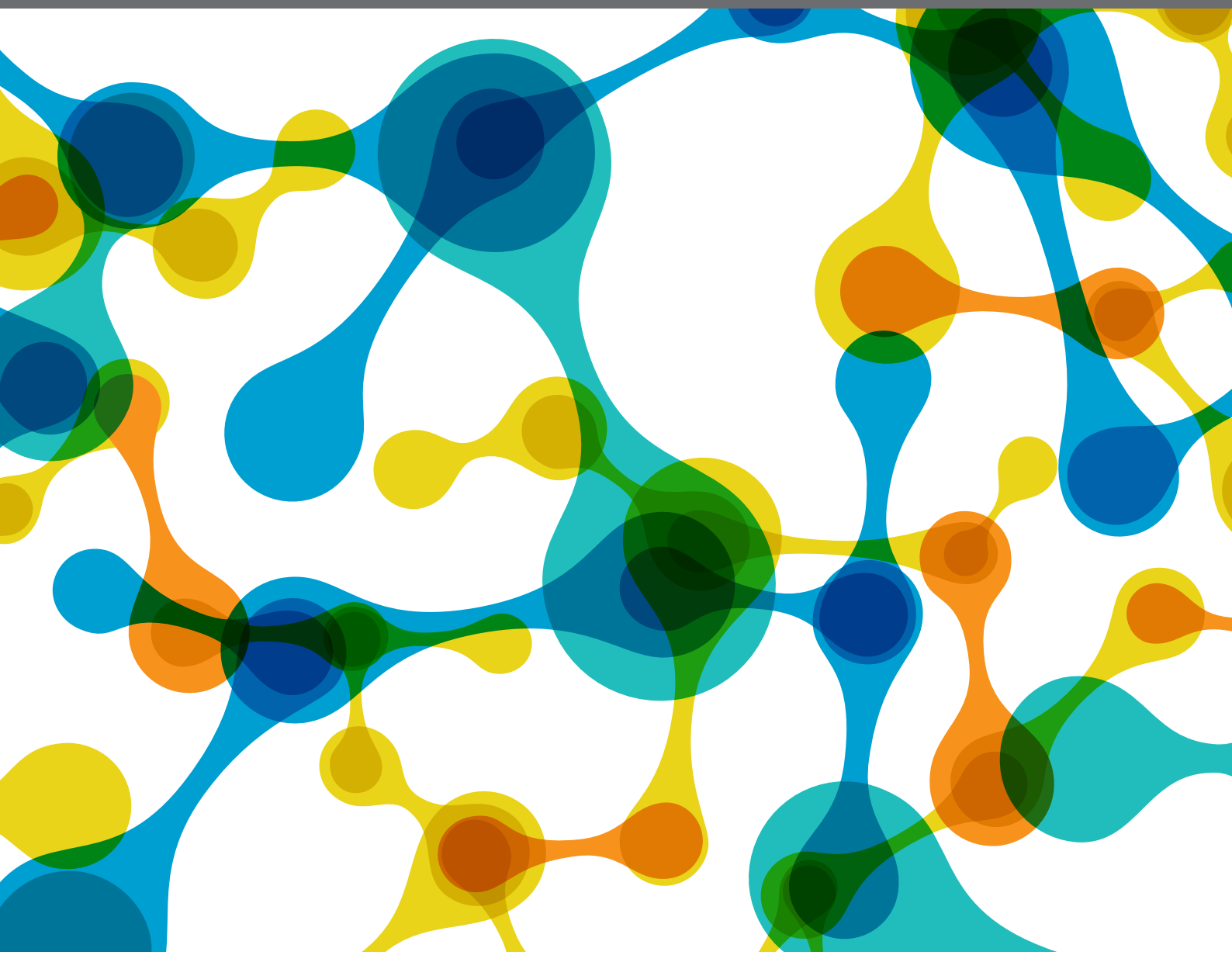
POST-TRANSLATIONAL MODIFICATIONS AND COMPARTMENTALIZED PROTEIN QUALITY CONTROL IN CARDIAC MUSCLE AND DISEASE

EDITED BY: Huabo Su, Aldrin V. Gomes and Mark J. Ranek

PUBLISHED IN: Frontiers in Physiology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88971-500-8
DOI 10.3389/978-2-88971-500-8

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

POST-TRANSLATIONAL MODIFICATIONS AND COMPARTMENTALIZED PROTEIN QUALITY CONTROL IN CARDIAC MUSCLE AND DISEASE

Topic Editors:
Huabo Su, Augusta University, United States
Aldrin V. Gomes, University of California, Davis, United States
Mark J. Ranek, Johns Hopkins University, United States

Citation: Su, H., Gomes, A. V., Ranek, M. J., eds. (2021). Post-Translational Modifications and Compartmentalized Protein Quality Control in Cardiac Muscle and Disease. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-500-8

Table of Contents

frontiers in Physiology

EDITORIAL
published: 30 August 2021
doi: 10.3389/fphys.2021.745887

Editorial: Post-translational Modifications and Compartmentalized Protein Quality Control in Cardiac Muscle and Disease

Mark J. Ranek[1], *Aldrin V. Gomes*[2] *and Huabo Su*[3]*

[1] *Department of Medicine, Division of Cardiology, The Johns Hopkins Medical Institutions, Baltimore, MD, United States,* [2] *Neurobiology, Physiology, and Behavior Department, University of California, Davis, Davis, CA, United States,* [3] *Department of Pharmacology and Toxicology, Vascular Biology Center, Augusta University, Augusta, GA, United States*

Keywords: post-translational modification, protein quality control, heart failure, mitochondria, endoplasmic reticulum

Editorial on the Research Topic

Post-translational Modifications and Compartmentalized Protein Quality Control in Cardiac Muscle and Disease

The proteome regulates the development, functioning, and adaptation of cells and organisms over time. Maintenance of a functional proteome is critical for the health of all cell types, especially to those with limited regeneration capacity such as cardiomyocytes. Cardiac development and maturation is accompanied by a drastic change in the composition of cardiac proteome due to increasing protein synthesis and a switch of fetal-to-adult protein expression (Rowton et al., 2021). Similarly, cardiac stress induces a dramatic alteration in the cardiac proteome that in turn has a significant impact on cardiac remodeling. Not surprisingly, derangement of the cardiac proteome is linked to various cardiomyopathy and heart failure (Lau et al., 2018). Thus, a better understanding of how cardiac structure and function is regulated at the protein level is crucial in delineating the molecular mechanisms underlying cardiac disease.

Cells maintain protein homeostasis (proteostasis) through an elegant protein quality control (PQC) system that involves targeted proteolysis and diverse protein post-translational modifications (PTMs). Proteolysis via the ubiquitin proteasome system (UPS) and autophagy offers the first line of PQC by removing unneeded normal proteins and misfolded/damaged proteins (Wang et al., 2008), which if not promptly removed will perturb other proteins, organelles, and cellular processes, causing cell malfunction and death. By directly controlling protein half-lives, targeted proteolysis is considered a coarse and bulk regulation of proteostasis. In contrast, a growing list over 300 different types of PTMs can serve as a fine-tuned PQC mechanism by regulating the stability, activity, subcellular localization and binding affinity to proteins/DNA of diverse protein substrates, thus having pleiotropic effects on various cellular processes. Partly due to these PTMs the complexity of the proteome is much larger than the genome. Targeted proteolysis and PTMs can occur at various subcellular compartments such as cytosol, mitochondria, and endoplasmic reticulum (ER) to preserve the normal function of these organelles. Inadequate targeted proteolysis and aberrant PTMs are known to contribute to impaired cardiac development and function, ultimately leading to failure (Wang et al., 2013; Willis and Patterson, 2013). Thus, improving cellular PQC has potential as a new therapeutic

OPEN ACCESS

Edited and reviewed by:
Paul M. L. Janssen,
The Ohio State University,
United States

***Correspondence:**
Huabo Su
hsu@augusta.edu

Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *22 July 2021*
Accepted: *06 August 2021*
Published: *30 August 2021*

Citation:
Ranek MJ, Gomes AV and Su H (2021) Editorial: Post-translational Modifications and Compartmentalized Protein Quality Control in Cardiac Muscle and Disease. Front. Physiol. 12:745887. doi: 10.3389/fphys.2021.745887

strategy for cardiac protection. Recent advances have identified new PTMs and compartmentalized proteolysis as novel avenues to protect the myocardium during development and disease. These exciting studies opened up new area of research. The present Research Topic, *Post-Translational Modifications and Compartmentalized Protein Quality Control in Cardiac Muscle and Disease*, highlights recent advances in our understanding of how various PTMs and compartmentalized proteolysis impact protein homeostasis and PQC pathways, along with discussing the promising roles of these discoveries in cardiac pathophysiology and therapeutic potential.

Cardiac development requires an intricately regulated process of protein expression and degradation to facilitate the genesis of the left ventricle/atrium from the first heart field and the right ventricle/atrium from the second heart field. We are beginning to understand the proteins involved in this complex process and even more recently, the PTMs influencing the proteins of this process. Chen et al. reviews the role of the Hippo pathway, including Yes-associated protein 1 (YAP1) and PDZ-binding motif (TAZ), during cardiac development, disease, and regeneration. While the Hippo pathway is conserved and has been shown to regulate organ size and the response of the heart to stress, only recently have PTMs such as phosphorylation, O-GlcNacylation, methylation, and ubiquitination been identified to regulate this pathway during development and disease (Chen et al.).

Cardiac pressure overload induces cardiomyocyte hypertrophy and proteotoxic stress, both of which can be attenuated through the activation of autophagy. Wu et al. demonstrate one of the mechanisms that autophagy protects the myocardium is by controlling nuclear factor erythroid factor 2-related factor (Nrf2). This exciting observation adds clarity to a field that was mystified as to whether Nrf2 was protective or detrimental. Multiples studies demonstrated the cardioprotection afforded by Nrf2; however other studies revealed Nrf2 is associated with the progression of cardiomyopathies. Here the authors propose that when autophagy is intact and can be stimulated, that activation of Nrf2 is protective; whereas when autophagy is inhibited Nrf2 is detrimental by driving the expression of angiotensinogen, which in turn promotes angiotensin II production (Wu et al.).

Compartmentalized regulation of organelle specific quality control mechanisms is a burgeoning area of research and has demonstrated therapeutic potential to attenuate cardiac disease. Mitochondria are tasked with producing enough energy for the heart to function, a challenge that is greater during disease. Indeed, mitochondrial dysfunction is a common hallmark of cardiovascular disease (Quiles and Gustafsson; Fan et al.). Cardiomyocytes possess quality control mechanisms to maintain mitochondrial quality control as reviewed by Quiles and Gustafsson and Fan et al. Mitochondrial health is a balance between mitochondrial biogenesis, fission, fusion, and mitophagy as the cardiomyocyte attempts to limit reactive oxygen species production, while meeting the energy demands of the heart. Mitochondrial quality control is achieved via the UPS, mitochondrial unfolded protein response, and mitophagy (Quiles and Gustafsson). PTMs regulate not only the stability of the proteins of the mitochondria but also the regulatory systems to increase mitochondrial health (Fan et al.). These manuscripts highlight targets for enhancing mitochondrial quality control mechanisms and the potential therapeutic benefit to treat cardiac disease. Endoplasmic reticulum (ER) proteostasis includes the unfolded protein response, which is initiated upon the accumulation of misfolded protein in ER lumen. Glembotski et al. reviews the role of one of the ER membrane proteins, ATF6, whose activation protects the myocardium by enhancing cardiomyocyte PQC. Seminal discoveries attributed a protective response of ATF6 activation to both inside and outside of the ER along with identifying novel small molecule activators of ATF6 to enhance proteostasis (Glembotski et al.).

Myofilament contraction is central to generate the force needed for cardiomyocytes to fulfill its mechanical function. The requirement of the heart to eject blood into the circulation places the sarcomeres of the cardiac cells under constant stress. Li et al. demonstrate Slit2, a secreted glycoprotein, protects the myocardium during ischemia reperfusion injury by inhibiting the inflammatory response to maintain myofilament contraction. The underlying mechanism for this protection was Slit2 expression inhibited the nuclear translocation of NFκB p65 along with decreasing IL-1β and IL-18 release (Li et al.).

The last few decades have seen many advances in our understanding of the mechanisms regulating cardiac PQC. Lewno et al. review the involvement of Cullin-RING ligases (CRLs) during cardiomyocyte necroptosis, focusing on the role of cullin neddylation (activates CRLs) and deneddylation by the COP9 signalosome. Downregulation of cullin deneddylation is associated with increased cardiac proteotoxicity and necroptosis during stress (Su et al., 2011). Wiley et al. explores the role of arginylation, mediated by arginyltransferase1 (ATE1), in the heart. They uncovered ATE1 interacts with select intracellular nodules important for cardiac physiology, namely translation/transcription regulation, biosynthesis, cell morphology, response to oxidative stress, and mitochondrial function (Wiley et al.). A major PTM, phosphorylation, is known to regulate many proteins associated with maintaining cardiomyocyte proteostasis. Mishra et al. review important cardiac proteins known to be phosphorylated, how this PTM regulates the proteins, the role of these modified proteins in cardiac patho-physiology, and potential therapeutic targets for cardiac disease.

This Research Topic illustrates exciting and significant advances the field of regulating cardiac PQC has made, and provides novel therapeutic targets that can be leveraged in the clinic for cardiac disease. Our understanding of these molecular mechanisms underlying cardiac proteostasis networks are continuously expanding and becoming better defined. Discovering key signaling nodes allows the field to identify and pursue new targets for therapeutic intervention against cardiac disease.

AUTHOR CONTRIBUTIONS

MR drafted and edited the editorial. AG reviewed and revised the editorial. HS planned, edited and finalized the editorial, and response to editors' report. All authors contributed to the article and approved the submitted version.

REFERENCES

Lau, E., Cao, Q., Lam, M. P. Y., Wang, J., Ng, D. C. M., Bleakley, B. J., et al. (2018). Integrated omics dissection of proteome dynamics during cardiac remodeling. *Nat. Commun.* 9:120. doi: 10.1038/s41467-017-02467-3

Rowton, M., Guzzetta, A., Rydeen, A. B., and Moskowitz, I. P. (2021). Control of cardiomyocyte differentiation timing by intercellular signaling pathways. *Semin. Cell. Dev. Biol.* 16:S1084-9521(21)00153-1. doi: 10.1016/j.semcdb.2021.06.002

Su, H., Li, J., Menon, S., Liu, J., Kumarapeli, A. R., Wei, N., et al. (2011). Perturbation of cullin deneddylation via conditional Csn8 ablation impairs the ubiquitin-proteasome system and causes cardiomyocyte necrosis and dilated cardiomyopathy in mice. *Circ. Res.* 108, 40–50. doi: 10.1161/CIRCRESAHA.110.230607

Wang, X., Pattison, J. S., and Su, H. (2013). Posttranslational modification and quality control. *Circ. Res.* 112, 367–381. doi: 10.1161/CIRCRESAHA.112.268706

Wang, X., Su, H., and Ranek, M. J. (2008). Protein quality control and degradation in cardiomyocytes. *J. Mol. Cell Cardiol.* 45, 11–27. doi: 10.1016/j.yjmcc.2008.03.025

Willis, M. S., and Patterson, C. (2013). Proteotoxicity and cardiac dysfunction–Alzheimer's disease of the heart? *N. Engl. J. Med.* 368, 455–464. doi: 10.1056/NEJMra1106180

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Physiology

REVIEW
published: 27 March 2020
doi: 10.3389/fphys.2020.00252

Mitochondrial Quality Control in Cardiomyocytes: A Critical Role in the Progression of Cardiovascular Diseases

Hualin Fan[1,2†], Zhengjie He[2†], Haofeng Huang[3], Haixia Zhuang[2], Hao Liu[2], Xiao Liu[2], Sijun Yang[4], Pengcheng He[5], Huan Yang[6] and Du Feng[2,7]**

[1] Guangdong Provincial People's Hospital, School of Medicine, South China University of Technology, Guangzhou, China, [2] Guangzhou Municipal and Guangdong Provincial Key Laboratory of Protein Modification and Degradation, State Key Laboratory of Respiratory Disease, School of Basic Medical Sciences, Guangzhou Medical University, Guangzhou, China, [3] Institute of Neurology, Affiliated Hospital of Guangdong Medical University, Zhanjiang, China, [4] ABSL-Laboratory at the Center for Animal Experiment and Institute of Animal Model for Human Disease, Wuhan University School of Medicine, Wuhan, China, [5] Department of Cardiology, Guangdong Cardiovascular Institute, Guangdong Provincial Key Laboratory of Coronary Heart Disease Prevention, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China, [6] Department of Pulmonary and Critical Care Medicine, Hunan Provincial People's Hospital, The First Affiliated Hospital of Hunan Normal University, Changsha, China, [7] The Sixth Affiliated Hospital of Guangzhou Medical University, Qingyuan People's Hospital, Guangzhou Medical University, Guangzhou, China

OPEN ACCESS

Edited by:
Huabo Su,
Augusta University Medical College of Georgia, United States

Reviewed by:
Jianqiu Zou,
Augusta University Medical College of Georgia, United States
Sebastiano Sciarretta,
Sapienza University of Rome, Italy
Xingguo Liu,
Guangzhou Institutes of Biomedicine and Health (CAS), China

***Correspondence:**
Du Feng
Fenglab@gzhmu.edu.cn;
feng_du@foxmail.com
Huan Yang
yanghuan_519@live.cn

[†] These authors share first authorship

Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: 23 December 2019
Accepted: 05 March 2020
Published: 27 March 2020

Citation:
Fan H, He Z, Huang H, Zhuang H, Liu H, Liu X, Yang S, He P, Yang H and Feng D (2020) Mitochondrial Quality Control in Cardiomyocytes: A Critical Role in the Progression of Cardiovascular Diseases. Front. Physiol. 11:252. doi: 10.3389/fphys.2020.00252

Mitochondria serve as an energy plant and participate in a variety of signaling pathways to regulate cellular metabolism, survival and immunity. Mitochondrial dysfunction, in particular in cardiomyocytes, is associated with the development and progression of cardiovascular disease, resulting in heart failure, cardiomyopathy, and cardiac ischemia/reperfusion injury. Therefore, mitochondrial quality control processes, including post-translational modifications of mitochondrial proteins, mitochondrial dynamics, mitophagy, and formation of mitochondrial-driven vesicles, play a critical role in maintenance of mitochondrial and even cellular homeostasis in physiological or pathological conditions. Accumulating evidence suggests that mitochondrial quality control in cardiomyocytes is able to improve cardiac function, rescue dying cardiomyocytes, and prevent the deterioration of cardiovascular disease upon external environmental stress. In this review, we discuss recent progress in understanding mitochondrial quality control in cardiomyocytes. We also evaluate potential targets to prevent or treat cardiovascular diseases, and highlight future research directions which will help uncover additional mechanisms underlying mitochondrial homeostasis in cardiomyocytes.

Keywords: mitochondrial quality control, mitophagy, post-translational modification, fission, fussion, cardiovascular disease, cardiomyocytes, cardiomyopathy

INTRODUCTION

Mitochondria are double-membraned organelles which play important roles in cellular homeostasis, including producing energy by oxidative phosphorylation, maintaining calcium homeostasis, and regulating the signaling that leads to programmed cell death (Giorgi et al., 2018; Sprenger and Langer, 2019). The mitochondrion is composed of the outer mitochondrial

membrane (OMM), the intermembrane space, the inner mitochondrial membrane (IMM) which is folded into projections called cristae, and the matrix. These substructures and compartments contain a number of proteins which maintain mitochondrial metabolism and homeostasis (Hammerling and Gustafsson, 2014). However, under stresses from environmental insults, mitochondrial shape, membrane potential and metabolism will be disturbed, and mitochondrial DNA, reactive oxidative species (ROS) and cytochrome c may be released into the cytoplasm. Moreover, mitochondrial proteins may even misfold under more severe conditions, causing a plethora of diseases such as cardiovascular disease (CVD), neurological diseases, aging and so on (Anzell et al., 2018; Tahrir et al., 2019).

Mitochondrial quality control (mitochondrial QC) pathways include post-translational modification (PTM) of mitochondrial proteins, mitochondrial dynamics (biogenesis, fission, and fusion) and mitochondrial autophagy (mitophagy). Together, these pathways repair or remove dysfunctional and damaged mitochondria to maintain mitochondrial morphology, quantity, and function, as well as to promote cell survival (Suliman and Piantadosi, 2015).

Cardiovascular disease is the one of most common causes of death globally, and its incidence and prevalence have increased over the years (Kyu et al., 2018). Remodeling of cardiomyocytes occurs in heart failure, ischemia/reperfusion (I/R) injury, and hypertrophic or dilated cardiomyopathy, which are characterized by mitochondrial dysfunction and perturbed cellular homeostasis (Tahrir et al., 2019). Given the high energy demands of cardiomyocytes as they pump blood around the body under normal physiological conditions, deregulated mitochondrial homeostasis causes cardiac dysfunction and remodeling, and will further induce CVD and complications. Through mitochondrial QC, the abnormal function and structure of heart will be improved (**Figure 1**).

In this review, we will discuss the progress in searching for evidence of the role of mitochondrial QC in cardiomyocytes, and for understanding the association between mitochondrial QC and development of CVD such as ischemia/reperfusion injury, cardiomyopathies, etc.

Abbreviations: 7,8-DHF, 7,8-dihydroxyflavone; AKT, RAC-alpha serine/threonine-protein kinase; AMPK, adenosine monophosphate-activated protein kinase; ANT, adenine nucleotide translocator; APC/CCdh1, Anaphase-promoting complex/cyclosome and its coactivator Cdh1; ATO, Arsenic trioxide; BDNF, brain-derived neurotrophic factor; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; BNIP3L/NIX, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like; CaMK1α, Ca^{2+}/calmodulin-dependent protein kinase-1α; CDK1, Cyclin-dependent kinase 1; CREB, cyclic AMP-responsive element-binding protein; CVD, cardiovascular disease; DRP1, dynamic relative-protein 1; DUSP1, Dual-specificity protein phosphatase1; E1, activating enzymes; E2, conjugating enzymes; E3, ligase enzymes; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FIS1, mitochondrial fission 1 protein; FOXO, Forkhead box protein O; FUNDC1, FUN14 domain-containing protein 1; GNAT, Guanine nucleotide-binding protein G(t) subunit alpha; GqPCR, Gq protein-coupled receptor; GSK, glycogen synthase kinase; H/R, Hypoxia/reoxygenation; HATs, histone acetyltransferases; HDACs, histone deacetylases; HO1, Heme oxygenase 1; I/R, Ischemia/reperfusion; IMM, inner mitochondrial membrane; IP_3R2, ER-resident inositol 1,4,5-trisphosphate type 2; KATs, lysine acetyltransferases; KDACs, lysine deacetylases; LAD, Left Anterior Descending Coronary Artery Ligation; LC3, Microtubule-associated proteins 1A/1B light chain 3; LIR, LC3 interaction region; MAMs, mitochondria-associated ER membranes; MAPL, mitochondrial-anchored protein ligase; MARCH, Membrane-associated RING-CH; MCL1, Induced myeloid leukemia cell differentiation protein Mcl-1 homolog; MFF, mitochondrial fission factor; MFN, Mitofusin; MiD49, Mitochondrial dynamics protein of 49 kDa homolog; MID51, Mitochondrial dynamics protein of 51 kDa homolog; Miro2, Mitochondrial Rho GTPase 2; MnSOD, manganese superoxide dismutase; mPTP, mitochondrial permeability transition pore; Mst1, mammalian Ste20-like kinase 1; mTOR, Mammalian target of rapamycin; NMNAT, nicotinamide mononucleotide adenylyltransferase; NR4A1, nuclear receptor subfamily 4 group A member 1; NRF1, Endoplasmic reticulum membrane sensor NFE2L1; NRF2, Nuclear factor erythroid 2-related factor 2; OGD, Oxygen/glucose deprivation; OMM, Outer mitochondrial membrane; OPA1, Optic atrophy protein 1 homolog; p300/CBP, CREB-binding protein; PARL, Presenilins-associated rhomboid-like protein, mitochondrial; PGC1, the transcriptional coregulator peroxisome proliferator-activated receptor γ coactivator 1; PI3K, Phosphoinositide 3-kinase; PINK, PTEN-induced putative kinase protein; PIP2, phosphatidylinositol (4,5) bisphosphate; PKA, Protein kinase A; PKD, Protein kinase D; PKδ, protein kinase C δ; PPARα, Peroxisome proliferator-activated receptor α; PTEN, Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase; PTM, post-translational modification; Q73, long polyglutamine repeat; QC, quality control; RIPK3, Receptor-interacting serine/threonine-protein kinase 3; ROCK1, Rho-associated coiled-coil-containing protein kinase 1; ROS, reactive oxidative species; S3, Diterpenoid derivative 15-oxospiramilactone; SENP, Sentrin/small ubiquitin-like modifier-specific protease; SIRT, NAD-dependent protein deacetylase sirtuin-1; SOD, Superoxide dismutase; SUMO, Small Ubiquitin-like Modifier; UCP1, Mitochondrial brown fat uncoupling protein 1; ULK1, Serine/threonine-protein kinase ULK1; UPS, Ubiquitination Proteasome System; VDAC1, Voltage-dependent anion-selective channel protein 1.

THE MOLECULAR MECHANISMS OF MITOCHONDRIAL QUALITY CONTROL (QC)

Post-translational Modification (PTM) of Mitochondrial Proteins

In physiological and pathological conditions, the activity of mitochondrial proteins is regulated by PTMs, including SUMOylation, ubiquitination, phosphorylation, acetylation and so on, to maintain mitochondrial metabolism or to trigger mitochondrial fission/fusion and mitophagy (Klimova et al., 2018).

SUMOylation

SUMOylation is a kind of PTM in which Small Ubiquitin-like Modifier (or SUMO) proteins are covalently attached to lysine residues of the substrate protein by SUMO E3 ligase. SUMO-1, SUMO-2, and SUMO-3 have been identified as the main members of the SUMO family (Hendriks and Vertegaal, 2016). SUMO is translated as an inactive precursor and subsequently cleaved by proteases such as the Sentrin/small ubiquitin-like modifier-specific protease (SENP) family to produce the mature form. SUMO undergoes an ATP-dependent interaction with SUMO E1 enzyme to form an E1-SUMO thioester conjugate through sequential adenylation and thioester bond formation at the carboxyl terminus of SUMO. Subsequently, SUMO is transferred to a cysteine residue of the SUMO E2 conjugating enzyme to form an E2-SUMO thioester bond, which then interacts with the E3 enzyme to facilitate transfer of SUMO to the substrate (Gareau and Lima, 2010; Wilkinson and Henley, 2010).

Several previous studies have reported that mitochondrial proteins are post-translationally modified via SUMOylation.

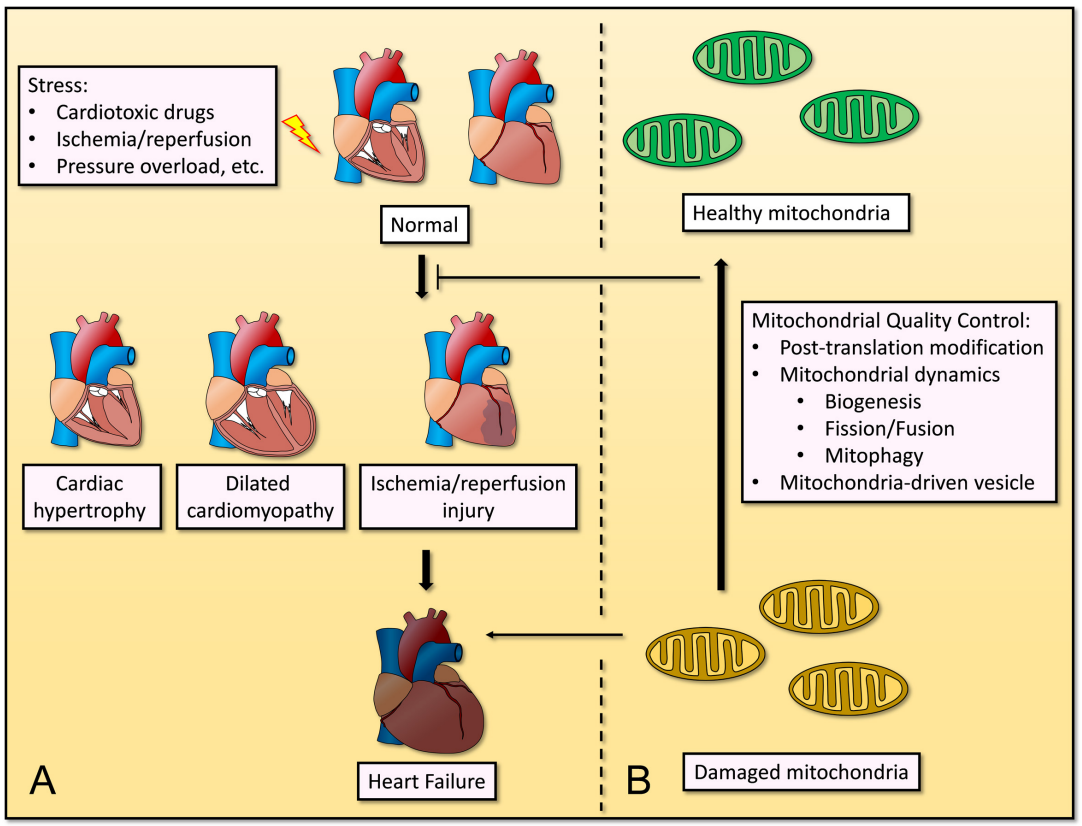


FIGURE 1 | Mitochondrial quality control in cardiovascular disease under stress. **(A)** Stresses such as cardiotoxic drugs, ischemia/reperfusion, pressure overload, etc., causes progression of several cardiovascular diseases, including cardiac hypertrophy, dilated cardiomyopathy, ischemia/reperfusion injury, and even heart failure. **(B)** Mitochondrial quality control including post-translation modification, mitochondrial dynamics (biogenesis, fission/fusion, and mitophagy) and mitochondria-driven vesicle, restores mitochondrial homeostasis and inhibits the progression of cardiovascular diseases.

Mitochondrial-anchored protein ligase (MAPL), the first known mitochondrial-anchored SUMO E3 ligase, can SUMOylate Dynamin relative-protein 1 (DRP1) and induce mitochondrial fission, but the mechanism was not clear (McBride et al., 2009). Further studies revealed that DRP1, is SUMOylated via MAPL-dependent activity of Bax/Bak during programmed cell death. SUMOylated DRP1 stabilizes an ER/mitochondrial signaling platform required for mitochondrial constriction, calcium flux, crista remodeling, and efficient cytochrome c release (Wasiak et al., 2007; Prudent et al., 2015). SENP3 is degraded during oxygen/glucose deprivation (OGD) and brain ischemia. This reduces deSUMOylation of DRP1 and leads to cytochrome c release and programmed cell death (Guo et al., 2013). Interestingly, as well as deSUMOylating DRP1, SENP3 also promotes the binding of DRP1 to mitochondrial fission factor (MFF), which leads to mitochondrial fragmentation and cytochrome c release (Guo et al., 2017).

Ubiquitination

Ubiquitination is a post-translational modification process that is critical for protein or organelle degradation. Ubiquitin is covalently attached to lysine residues on substrate proteins via Ub-activating enzymes (E1), Ub-conjugating enzymes (E2), and Ub-ligase enzymes (E3). Ub is activated by E1, and is subsequently transferred to E2 to form Ub-E2. The Ub-E2 conjugate then interacts with E3 and the substrate, which results in formation of an isopeptide bond between the C-terminus of Ub and a lysine residue in the substrate (Wang L. et al., 2019). Successive ubiquitination reactions between one ubiquitin to another lead to formation of a polyubiquitin chain, which binds to the proteasome and leads to degradation of the substrate (Ciechanover, 2005).

Several mitochondrial proteins are degraded via the Ubiquitination Proteasome System (UPS) pathway to maintain cellular homeostasis. The gene encoding Parkin, which serves as a Ub-ligase enzyme, is mutated in Parkinson's disease (Kitada et al., 1998). DRP1 interacts with Parkin and is subsequently ubiquitinated by Parkin for degradation. The depletion of Parkin reduces the ubiquitination and degradation of DRP1, causing increased mitochondrial fission (Wang et al., 2011; Buhlman et al., 2014). Ubiquitination of DRP1 is also catalyzed by APC/CCdh1 E3 ubiquitin ligase complex, leading to changes in mitochondrial dynamics when cellular mitosis is completed (Horn et al., 2011). MARCH-V, a ubiquitin ligase, promotes

the formation of elongated mitochondria by ubiquitinating DRP1 and interacting with mitofusin (MFN) 2 (Nakamura et al., 2006). Upon inhibition of USP30 by the diterpenoid derivative 15-oxospiramilactone (S3), non-degradative ubiquitination of MFN1/2 is induced to promote mitochondrial fusion (Yue et al., 2014). Additionally, ubiquitination also regulates mitophagy. For example, in cells treated with CCCP, which induces loss of mitochondrial membrane potential, MFN2 is ubiquitinated by the E3 ubiquitin ligase Parkin for degradation, which leads to impaired mitochondrial fusion and mitophagy (Gegg et al., 2010; Poole et al., 2010; Tanaka et al., 2010; Glauser et al., 2011). Also, MFN2 is ubiquitinated by the PINK/Parkin pathway to trigger p97-dependent disassembly of MFN2 complexes, which disrupts the contacts between mitochondria and ER and subsequent initiation of mitophagy (Tanaka et al., 2010; McLelland et al., 2018). Furthermore, the ubiquitination of other mitochondrial proteins such as MID49, MCL1 and FIS1 plays a role in mitochondrial dynamics and cellular homeostasis (Zhang et al., 2012; Cherok et al., 2017).

Phosphorylation

Phosphorylation is a typical form of PTM which occurs at several sites of target proteins such as serine, threonine, or tyrosine residues. DRP1 has been most widely studied to elucidate the mechanism of phosphorylation-mediated mitochondrial fragmentation. Phosphorylation of DRP1 occurs on several sites, such as Ser616 or Ser637 in human, and is catalyzed by several kinases, including PKA (Chang and Blackstone, 2007), CaMK1α (Cribbs and Strack, 2007; Han et al., 2008), CDK1, or PKδ (Zaja et al., 2014; Klimova et al., 2018). In addition, phosphorylation of DRP1 by c-Abl mediates oxidative stress-induced mitochondrial fragmentation and cell death (Chen and Dorn, 2013; Zhou H. et al., 2017). Under high-glucose conditions, a transient increase in Ca^{2+} levels and ROS production is induced, followed by activation of ERK1/2, which phosphorylates DRP1 and promotes mitochondrial fragmentation (Yu et al., 2011). In addition, other mitochondrial QC processes are mediated by phosphorylation of mitochondrial proteins. MFF is phosphorylated by adenosine monophosphate-activated protein kinase (AMPK) in response to energy stress, which is required for DRP1-mediated mitochondrial fission (Toyama et al., 2016). The pleiotropic ERK kinase phosphorylates MFN1 to reduce the mitochondrial fusion rate and to cause mitochondrial permeabilization (Pyakurel et al., 2015). By phosphorylating MFN2, PKA inhibits the proliferation of vascular smooth muscle cells, while PINK1 facilitates mitochondrial fragmentation and promotes mitophagy (Chen and Dorn, 2013; Zhou et al., 2010; Gong et al., 2015). FUNDC1, a mitophagy receptor under hypoxic conditions, is phosphorylated by ULK1 at Ser17. This enables FUNDC1 to bind to LC3, which leads to mitophagy for clearance of damaged mitochondria (Wu et al., 2014). Interestingly, under hypoxic conditions, Src kinase-mediated phosphorylation of FUNDC1 at Tyr18 in LIR motif is inhibited. This results in dephosphorylation of FUNDC1 and increased mitophagy (Liu et al., 2012).

Acetylation/Deacetylation

Acetylation is an evolutionarily conserved PTM, which plays an important role in gene expression, gene function and cellular metabolism via regulation of histones and non-histone proteins (Baeza et al., 2016). Recently, accumulating lines of studies have focused on lysine acetylation of non-histone proteins in mitochondria, which modulates mitochondrial homeostatic processes including mitochondrial dynamics and mitophagy. Initially, lysine acetylation was discovered as a PTM of histones which regulates the expression and function of nuclear genes and is mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Subsequently, lysine acetylation was identified to occur outside the nucleus. This form of lysine acetylation is mediated by lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) (Drazic et al., 2016). The exact number of KATs is unclear, but all identified KATs can be divided into three families: the GNAT family, the MYST family and the p300/CBP family. The human genome encodes 18 KDACs, which can be divided into two types: Zn^{2+}-dependent HDACs and NAD^{+}-dependent sirtuin deacetylases (Takeo et al., 2019). Multiple associations between acetylation or deacetylation and diseases have been revealed, including neuronal degeneration (Min et al., 2015; Li Y. et al., 2018), cancer (Audia and Campbell, 2016), disorders of glucose homeostasis (Mihaylova et al., 2011; Winkler et al., 2012), and cardiomyopathy(Vakhrusheva et al., 2008).

Mitochondrial Dynamics

Mitochondrial dynamics, including biogenesis, fusion, fission, and mitophagy, regulate mitochondrial morphology and maintain mitochondrial population health, which is critical for cellular responses to metabolic cues or environmental stresses. In physiological conditions, mitochondria continually and spontaneously produce reactive oxygen species, accompanied by the production of ATP, which impairs proteins, lipids and DNA within mitochondria. To eliminate these deleterious components and to maintain mitochondrial metabolism, fission helps the mitochondrion to segregate the deleterious components into a daughter mitochondrion which will be degraded by mitophagy. Mitochondrial fusion is necessary for maintaining the capacity of oxidative phosphorylation by healthy ones fused with damaged ones to compensate the deficient metabolism (Youle and van der Bliek, 2012).

Mitochondrial dynamics are mediated by several factors. In brief, the transcriptional coregulator peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC1α) is the core in mitochondrial biogenesis (Dorn et al., 2015); MFF, FIS1, MID49, and MID51 are required to recruit the fission factor DRP1 during mitochondrial fission, and OPA1 and MFN1/2 are required for mitochondrial fusion; characterized by PINK1/Parkin pathway, multiple pathways involve in mitophagy for the degradation of senescent and damaged mitochondria (Youle and van der Bliek, 2012).

Mitochondrial Biogenesis

PGC1 plays a key role in mitochondrial biogenesis through activating PPAR, increasing content of mitochondrial DNA

(mtDNA), and promoting expression of UCP1 and key mitochondrial enzymes of the respiratory chain in brown fat and skeletal muscle (Puigserver et al., 1998). PGC1 is regulated by several factors, such as cAMP, AMPK, and SIRT1, and plays its core role in mitochondrial biogenesis (Wu et al., 1999; Gerhart-Hines et al., 2007; Little et al., 2010). Through interacting with transcription factors at downstream, including NRF1/2, ERR and PPAR, PGC1 regulate the replication of mtDNA and the transcription of mitochondrial proteins genes for mitochondrial biogenesis (Dorn et al., 2015).

Mitochondrial Fission and Fusion

For mitochondrial fission, DRP1 is recruited from the cytosol to form spiral oligomers around the mitochondrion. DRP1 binds to 4 protein receptors, MFF, FIS1, MID49, and MID51, that constrict the mitochondrion and divide it into two daughters, in which mitochondrial components are asymmetrically distributed or segregated (Dorn and Kitsis, 2015; Ni et al., 2015). By fission, a daughter mitochondrion which may contain deleterious or damaged components can be removed via mitophagy (Youle and van der Bliek, 2012). The site where DRP1 constricts mitochondria is often the same place where mitochondria contact the ER (Friedman et al., 2011). In addition, fission is modulated via PTM of DRP1 as mentioned above. These PTMs, including SUMOylation and phosphorylation, are essential for the maintenance of mitochondrial homeostasis.

Mitochondrial fusion in mammals is mediated by MFN1 and MFN2, two membrane-anchored dynamin-related GTPases, which regulate fusion of the outer mitochondrial membrane, and by OPA1, also a dynamin-related GTPase, which regulates fusion of the IMM (Youle and van der Bliek, 2012; Ni et al., 2015).

Mitophagy

Autophagy is an evolutionarily conserved pathway in which double-membraned structures called autophagosomes encapsulate damaged organelles, protein aggregates, and invading microorganisms and fuse with lysosomes for degradation (Levine and Kroemer, 2008; Martinet and De Meyer, 2009; Gatica et al., 2015; Ikeda et al., 2015a). Macroautophagy is widely studied and is considered as the major cellular pathway for non-selective degradation of proteins and organelles. In addition to non-selective macroautophagy, selective autophagy, which acts via dedicated receptors on autophagic substrates, plays an important role in removal of specific organelles such as mitochondria (mitophagy), the ER (reticulophagy), ribosomes (ribophagy), lysosomes (lysophagy), etc. (Galluzzi et al., 2017).

Here, we focus on mitophagy, a critical mitochondrial QC process for maintenance of mitochondrial homeostasis, and we examine the link between mitophagy and physiological and pathological changes in cardiomyocytes.

Damaged mitochondria are removed by mitophagy via the PINK1-Parkin axis, which is the most widely studied mitophagy pathway. In normal conditions, after being imported into the IMM, PINK1 is constitutively cleaved by the IMM protease PARL, and is then released to the cytoplasm for further degradation. Upon loss of mitochondrial membrane potential, the import of PINK1 is blocked and therefore PINK1 cannot be accessed by PARL, resulting in its stabilization on the OMM. Subsequently, PINK1 phosphorylates Parkin and ubiquitin and further induces mitophagy to remove damaged mitochondria (Youle and van der Bliek, 2012; Lazarou et al., 2015; Nguyen et al., 2016; Pickles et al., 2018).

In addition to PINK1 and Parkin, other LC3-interacting proteins such as the mitophagy receptors FUNDC1, BNIP3, or BNIP3L/NIX are also involved in mitophagy (Liu et al., 2014).

Mitochondrial-Derived Vesicles (MDVs)

In addition to PTMs on mitochondrial proteins, changes in mitochondrial dynamics and mitophagy, increasing evidence has indicated that mitochondrial-derived vesicles (MDVs) are important for mitochondrial QC. In response to different stimuli, like starvation, small vesicles carrying specific components of mitochondria are generated and transported to lysosomes for degradation via the PINK1/Parkin pathway to maintain cellular homeostasis (Soubannier et al., 2012; McLelland et al., 2014; Suliman and Piantadosi, 2015). The size of MDVs ranges from 70 to 150 nm and the generation of MDVs is independent of DRP1 (Sugiura et al., 2014). Altogether, there are three distinct criteria to identify MDVs: independence from the core fission GTPase DRP1, incorporation of selected mitochondrial cargo, and a diameter of 70–150 nm as observed by electron microscopy (Cadete et al., 2016).

In summary, mitochondrial QC exerts its function to maintain mitochondrial and cellular homeostasis via multiple components, which is interacting with each other. PTMs serve as triggers to keep the balance of mitochondrial dynamics and MDVs. The deficiency or excess of PTMs is associated with abnormal mitochondrial dynamics and MDVs, which is involved in several pathological progressions in CVD.

THE MITOCHONDRIAL QUALITY CONTROL (QC) IN CARDIOVASCULAR DISEASE (CVD)

Post-translational Modification (PTM) of Mitochondrial Proteins in Cardiomyocytes

SUMOylation

DJ-1, also known as Parkinson disease protein 7, serves as a molecular chaperone and is activated in oxidative environments. Mutations in the gene encoding DJ-1 are associated with Parkinson's disease (Shendelman et al., 2004). Deficient DJ-1 enhanced accumulation of SUMO-1 modified proteins, reduced SUMO-2/3 modified proteins after I/R injury, which is associated with enhanced SUMOylation of DRP1 and excessive mitochondrial fission. The DJ-1 deletion heart displayed increased infarction size, deteriorated cardiac function *in vivo*. That study suggested that the activation of DJ-1 attenuates excessive mitochondrial fission and protects cardiomyocytes from I/R injury by decreasing the SUMOylation of DRP1 (Shimizu et al., 2016). In contrast, another study suggests that deSUMOylation of DRP1 is involved in the release or

cleavage of apoptotic factors, impaired mitochondrial fission, and development of cardiomyopathy induced by overexpression of SENP5 *in vivo* (Kim et al., 2014). Treatment with zinc protects cardiomyocytes from hypoxia/reoxygenation (H/R) injury *in vitro* and improves cardiac function against I/R injury *ex vivo* by inducing the SUMOylation of DRP1 and promoting mitophagy (Bian et al., 2019).

In summary, SUMOylation of DRP1 mediates mitochondrial dynamics and mitophagy, but the exact mechanism remains elusive. The existing research appears to suggest the levels of DRP1 SUMOylation should be controlled in a restrict range to maintain cardiac function. The association between SUMOylation of other mitochondrial proteins and cardiac protection remains unclear and needs further investigation.

Ubiquitination

In cardiomyocytes, beside mitophagy mediated by mitochondrial ubiquitination, ubiquitination regulates the cardiomyocytes homeostasis through specific function of protein substrates via activated UPS. Mitochondrial Rho GTPase 2 (Miro2) is an OMM protein which facilitates the formation of mitochondrial nanotubes along microtubules and mediates the transportation of mitochondria. The expression of Parkin increases during cardiac hypertrophy, which leads to ubiquitination of Miro2 and causes impairment of mitochondrial communication, whereas overexpressed Miro2 protects cardiomyocytes from transverse aortic constriction-induced cardiac dysfunction *in vivo* (Cao et al., 2019). On the contrary, VDAC1, a substrate of Parkin, is ubiquitinated when HL-1 mouse atrial cardiomyocytes are exposed to arsenic trioxide (ATO). This maintains mitochondrial homeostasis and protects cardiomyocytes during exposure to cardiotoxic agents *in vitro* (Watanabe et al., 2014).

In summary, previous studies have shown that ubiquitination of several mitochondrial proteins by PINK/Parkin or MARCH-V triggers changes of mitochondrial dynamics and mitophagy to maintain cellular homeostasis. However, the mechanism by which ubiquitination mediates mitochondrial QC as well as the different functions with specific ubiquitination of substrates in cardiomyocytes remains unclear and should be further investigated *in vivo* and *in vitro*.

Phosphorylation

Recently, several studies have focused on PTMs of mitochondrial proteins via phosphorylation. Fasudil has been reported to reduce mitochondrial fragmentation by inhibiting the effect of the RhoA/ROCK pathway, which catalyzes phosphorylation of DRP1, and subsequently induces mitophagy and protects cardiomyocytes from endotoxemia-induced impairment of heart function *in vivo* (Preau et al., 2016). Interestingly, in another study, RhoA activation was suggested to regulate the phosphorylation of DRP1 for cardioprotection *in vitro* (Brand et al., 2018). Furthermore, a recent study reported that protein kinase D (PKD), a downstream kinase of Gq protein-coupled receptor (GqPCR) signaling, phosphorylates DRP1 and promotes mitochondrial fragmentation in cardiomyocytes via α1-AR-PKD signaling *in vitro* and *in vivo* (Jhun et al., 2018). The protective effect of this pathway on stressed cardiac cells and the underlying mechanism should be further investigated. Also, MFN2 is a well-studied target protein phosphorylated by PINK1 for mitochondrial QC in cardiomyocytes (Chen and Dorn, 2013), which also plays a critical role in perinatal cardiac maturation (Gong et al., 2015). In addition, there is evidence that AMPK-α2 will be switched to AMPK-α1 increasingly in heart failure, which reduces the level of AMPK-α2 and promotes the development of cardiac dysfunction, whereas AMPK-α2 interacts with phosphorylated PINK1 to initiate PINK/Parkin-mediated mitophagy for protection against progression of heart failure under phenylephrine stimulation *in vivo* (Wang B. et al., 2018).

Like ubiquitination (discussed above), phosphorylation plays an important role in QC of mitochondria, and also triggers changes of mitochondrial dynamics and mitophagy. However, the association between the PTM and cardiomyocyte function in physiological and pathological processes is clearer for phosphorylation than for ubiquitination. Nevertheless, the functions of phosphorylation in heart cells still need to be further investigated.

Acetylation/Deacetylation

Several studies have investigated the link between the lysine acetylation/deacetylation balance and mitochondrial homeostasis in cardiomyocytes to understand the progress of CVDs. OPA1 is hyperacetylated under pathological stress and its GTPase activity is suppressed, whereas SIRT3, a NAD-dependent protein deacetylase (Onyango et al., 2002), can deacetylate OPA1 at Lys926 and Lys931 and restore its GTPase activity *in vivo* (Samant et al., 2014). These changes are accompanied by altered mitochondrial cAMP/PKA signaling (Signorile et al., 2017). Additionally, acetylation participates in regulation of mitophagy. SIRT3 depletion facilitates angiotensin II-induced PINK/Parkin acetylation and increases ROS generation, leading to impaired mitophagy, while the overexpression of SIRT3 enhances PINK/Parkin-dependent mitophagy, reduces ROS generation, restores vessel sprouting and tube formation, and improves cardiac function and microvascular network formation *in vivo* (Wei et al., 2017). Similarly, SIRT3 catalyzes the acetylation of Forkhead box protein O (FOXO) 3, which is upstream of Parkin-dependent mitophagy (Celestini et al., 2018), to maintain mitochondrial homeostasis during streptozotocin-induced diabetic cardiomyopathy *in vivo* (Das et al., 2014). In addition, deacetylation has been reported to regulate the levels of mitochondrial metabolism proteins that localize in the mitochondrial matrix, such as superoxide dismutase (SOD) 2 (Dikalova et al., 2017), nicotinamide mononucleotide adenylyltransferase (NMNAT) 3 (Yue et al., 2016), and glycogen synthase kinase (GSK) 3β (Sundaresan et al., 2015), to protect cardiomyocytes from hypertension, hypertrophy, and cardiac fibrosis *in vivo*. Furthermore, there is evidence that SIRT4 promotes development of hypertension by inhibiting the binding of manganese superoxide dismutase (MnSOD) to SIRT3. This results in decreased deacetylation of MnSOD by SIRT3 and reduced activity of MnSOD, and ROS accumulation during angiotensin II treatment *in vivo* (Luo et al., 2017).

Accumulating evidence reveals that the balance between acetylation and deacetylation has a crucial role in mitochondrial

dynamics, mitophagy and mitochondrial metabolism in cardiomyocytes. These mitochondrial QC processes prevent the progression of CVD. Further research is needed to find effective pharmacological agents that target the sites of acetylation, which may lead to the development of new therapies for CVD.

PTMs are crucial for mitochondrial homeostasis in cardiomyocytes, and dysregulation of PTMs is associated with development of CVD. Although different kinds of PTMs have been reported for mitochondrial proteins, further mechanistic studies in cardiomyocytes are needed (**Figure 2**).

MITOCHONDRIAL DYNAMICS IN CARDIOVASCULAR DISEASE (CVD)

For maintaining the pump function to meet the demand of organism, cardiomyocytes require health mitochondrial homeostasis to provide sufficient ATP for energy. Since mitochondria cannot be synthesized *de novo*, the dynamical renew cycle, including mitochondrial biogenesis, fission, fusion, and mitophagy, is required to maintain mitochondrial homeostasis health in cardiomyocytes (Suliman and Piantadosi, 2015). At baseline or in unstressed conditions, the expression of mitochondrial fusion-related proteins is higher than that of fission-related and mitophagy-related proteins in adult mouse heart (Song et al., 2015a). The high levels of fusion may contribute to maintaining the capacity of oxidative phosphorylation (Youle and van der Bliek, 2012), which meets the demand of cardiomyocytes.

Mitochondrial Biogenesis in Cardiomyocytes

Lehman et al. (2000) firstly reported the role of PGC1 in mitochondrial biogenesis in cardiomyocytes. The overexpression of PGC1 induced upregulated expression of mitochondrial proteins, accumulation of enlarged mitochondria *in vitro* and resulted in the loss of sarcomeric structure and a dilated cardiomyopathy *in vivo* (Lehman et al., 2000). Deficient PGC1 was not associated with abnormal mitochondrial and

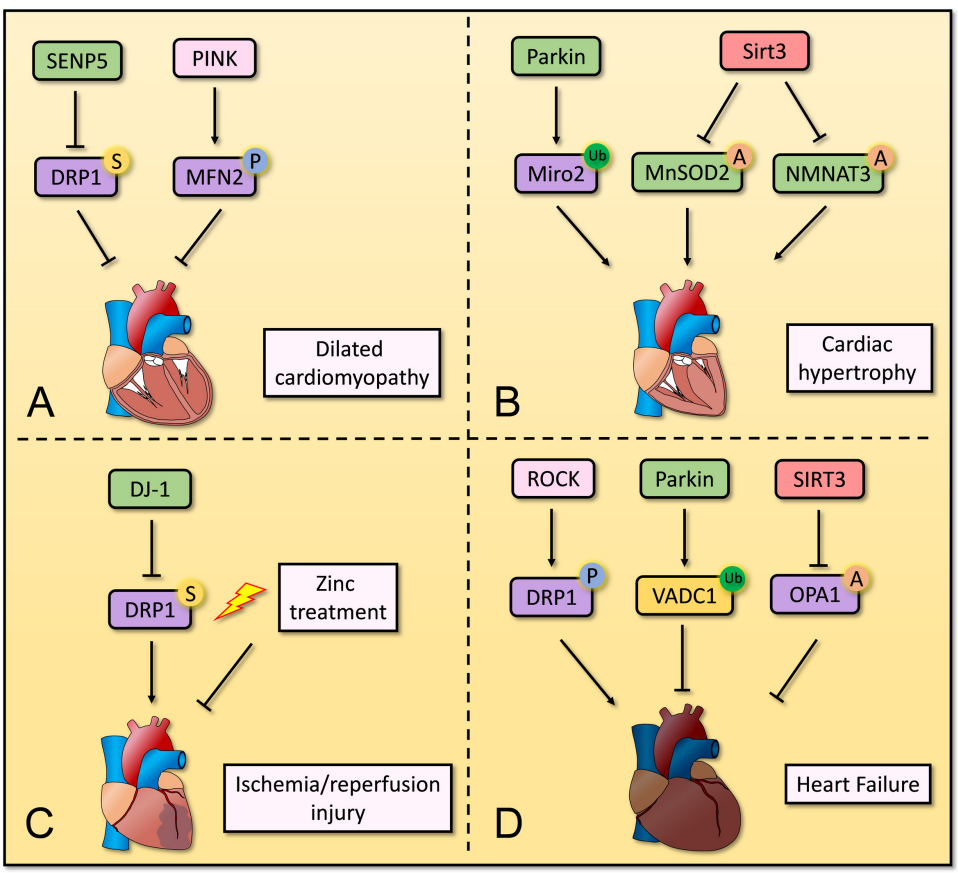


FIGURE 2 | Overview of post-translation modification in mitochondria in cardiomyocytes. Several post-translation modifications of mitochondrial proteins play important roles in mitochondrial homeostasis in cardiomyocytes. **(A)** Decreased SUMOylation of DRP1 and increased SUMOylation of MFN2 repress the progression of dilated cardiomyopathy. **(B)** Parkin induces ubiquitination of Miro2 and its degradation, leading to development of cardiac hypertrophy. SIRT3 deacetylates MnSOD2 and NMNAT3 to restores normal cardiac function and structure. **(C)** DJ-1 and Zinc treatment contribute to suppressing ischemia/reperfusion injury by regulating SUMOylation of DRP1. **(D)** Phosphorylation of DRP1 via RhoA/ROCK pathway induces heart failure, whereas ubiquitination of VADC1 and deacetylation of OPA1 repress development of heart failure.

cardiac structure, although it caused the deficient expression of mitochondrial proteins and cardiac contractile dysfunction in physiological conditions *in vivo* (Arany et al., 2005). The same group further revealed that when PGC1 deficiency mice were subjected to transverse aortic constriction, their heart appeared to enlarge and be heavier, consistent with hypertrophy *in vivo* (Arany et al., 2006).

In addition to PGC1, other factors also were reported to participate in the regulation of mitochondrial biogenesis. Heme oxygenase (HO) 1, a protective antioxidant enzyme that prevents cardiomyocyte apoptosis, promotes the endogenous generation of carbon monoxide (CO), which increases NRF1 levels via AKT-GSK3β-NRF2 pathways. Consequently, the overexpression of HO1 causes the accumulation of NRF1 to increase the mtDNA replication and promotes mitochondrial biogenesis, leading to protecting cardiomyocytes from cardiotoxic stress *in vivo* (Piantadosi et al., 2008).

These studies indicated that mitochondrial function is redundant to the demand for energy in cardiomyocytes, while mitochondrial biogenesis plays a crucial role when cardiomyocytes are subjected to external environmental stress, accompanied by increasing of mitophagy.

Mitochondrial Fission in Cardiomyocytes

In cardiomyocytes, exogenous inhibition of DRP1 improves impaired cardiac function, whereas endogenous DRP1 knockout promotes cardiac dysfunction. Reduced DRP1 expression levels induced by Mdivi-1, a mitochondrial fission inhibitor, reduced cell death, inhibited mitochondrial permeability transition pore (mPTP) opening, and protected cardiomyocytes from I/R injury *in vitro*. The pretreatment of Mdivi-1 followed by I/R injury improved LV developed pressure, reduced LV end-diastolic pressure and myocardial infarction size *in vivo* and *ex vivo* (Ong et al., 2010; Sharp et al., 2014). Besides Mdivi-1, the pretreatment of FK506 and therapeutic hypothermia (30°C) also preserved cardiac function via preventing Drp1-S637 dephosphorylation (Sharp et al., 2014).

Cardiac-specific DRP1 knockout mice have suppressed autophagy, dysfunctional mitochondria, and greater infarct size after ischemia/reperfusion. They also develop left ventricular dysfunction, and die after 13 weeks. *In vitro*, accumulated enlarged mitochondria induced by impaired mitochondrial fission fail to be degraded in lysosomes, although recruits abundant p62 and ubiquitin (Ikeda et al., 2015b). Interestingly, concomitant conditional deletion of Parkin suppresses the cardiac-specific DRP1 knockout-induced impaired ventricular ejection, adverse cardiac remodeling, and cardiac myocyte necrosis and fibrosis, but does not alter mitochondrial enlargement in cardiomyocytes (Song et al., 2015a). On the contrary, the whole body Parkin knockout mice fail to improve the function of heart with specific knockout of DRP1 (Kageyama et al., 2014). The underlying mechanism may be explained by the lower recruitment levels of p62 and ubiquitin on mitochondria in the former, which appears to block the degradation of mitochondria and maintain the cardiac function to some extent. The potential mechanisms should be also investigated in further study. These studies suggest that mitochondrial fission regulates cardiac function via mitophagy in physiological conditions.

Recently, it was reported that the interaction between DRP1 and FIS1 is enhanced under LPS treatment, and this is associated with increased ROS generation, mitochondrial fragmentation, and increased mortality *in vitro*. These effects are attenuated by treatment with P110 on LPS-treated mice, which inhibits the interaction between DRP1 and FIS1 (Haileselassie et al., 2019). Evidence also showed that cardiac dysfunction in Huntington's disease is related to DRP1/FIS1-mediated impairment of lysosomal function. Abnormal mitochondrial fragmentation and dysfunctional lysosomes were observed in H9C2 embryonic cardiomyocytes expressing mutant Huntingtin protein with a long polyglutamine repeat (Q73) and in human iPSC-derived cardiomyocytes transfected with Q77 (Joshi et al., 2019). In addition to DRP1, mitochondrial fission and fusion are also affected in phenylephrine (PE)-induced hypertrophy, where the mitochondrial permeability transition pore opens and the mitochondria are exposed to oxidative stress *in vivo* (Javadov et al., 2011).

Mitochondrial Fusion in Cardiomyocytes

Mitochondrial fusion has been reported to regulate the homeostasis of cardiomyocytes. Reduced expression of MFN2 and mitochondrial fission are observed in diabetic hearts from 12-week-old leptin receptor-deficient mice and in cardiomyocytes fed with high-glucose and high-fat medium. This is due to down-regulation of PPARα, which directly binds to the promoter of MFN2 and modulates its expression. The functional deficits in cardiomyocytes are attenuated by reconstitution of MFN2, which enhances mitochondrial fusion (Hu et al., 2019). The endogenous deficiency of MFN2 leads to hypertrophy and slight cardiac dysfunction, which is associated with a marked delay in mitochondrial permeability transition. Followed by the I/R procedure, MFN2 ablation results in improved cardiac performance and lower cell death ratio (Papanicolaou et al., 2011). In contrast, further research indicated that cardiac-specific MFN2 knockout mice caused a higher cell death ratio after the I/R procedure. MFN2 ablation in cardiomyocytes prevented the fusion between autophagosomes and lysosomes for mitochondrial degradation and caused the accumulation of autophagosomes (Zhao et al., 2012). These studies indicated the role of MFN2 in cardiomyocytes and cardiac function should be further investigated.

Optic atrophy 1 (OPA1) is also involved in mitochondrial homeostasis in cardiomyocytes. Reduction of OPA1 is associated with ischemia-induced heart failure, and with mitochondrial fragmentation and apoptosis in cardiomyocytes. Overexpression of OPA1 fails to prevent apoptosis, although it does increase the tubularity of mitochondria *in vitro* (Chen et al., 2009). However, further study *in vivo* suggested that overexpression of OPA1 refined cardiac injury accompanied by the upregulated long-OPA1 levels *in vivo*. The potential mechanism may be OPA1-dependent stabilization of mitochondrial cristae structure increases mitochondrial respiratory function, blocks cytochrome c release, ROS production, and apoptosis (Varanita et al., 2015).

In addition to MFN2 and OPA1, SIRT3 is another important factor that protects cardiomyocytes from post-infarction cardiac injury by normalizing the AMPK-DRP1 pathways. This has the effect of attenuating mitochondrial fission and preserving mitochondrial homeostasis, which improves cardiac fibrosis, maintains myocardial function, inhibits inflammatory responses, and reduces cellular death (Liu et al., 2019). Similar to SIRT3, NR4A1, which has no fission or fusion activity, promotes MFF-dependent mitochondrial fission and represses mitophagy, resulting in aggravation of cardiac microvascular I/R injury *in vivo* (Zhou H. et al., 2018). NR4A1 has also been reported to play an important role in protecting against vascular injury (Papac-Milicevic et al., 2012).

As mentioned above, Moshi Song and colleagues discovered that concomitant conditional Parkin deletion suppresses the effect of DRP1 ablation in heart (Ikeda et al., 2015b). To further investigate the association between mitochondrial dynamics and mitochondrial QC, the same group revealed that DRP1 conditional knockout in cardiomyocytes induces mitochondrial enlargement, dilated cardiomyopathy, and cardiomyocyte necrosis. Furthermore, MFN1/2 conditional knockout causes mitochondrial fragmentation and eccentric ventricular remodeling, namely eccentric hypertrophy without change in the ratio of left ventricular end-diastolic radius to wall thickness. Contrary to previous observations, DRP1 ablation increased mitophagy in this study, as evidenced by accumulation of LC3-II and increased engulfment of mitochondria into lysosomes (Song et al., 2015b).

Altogether, the accumulating evidence suggests that mitochondrial fission and fusion are both essential for mitochondrial QC, and deregulation of either process will result in cardiomyocyte dysfunction, cardiac remodeling and even cell death. Impaired mitochondrial fission causes dilated cardiomyopathy, while impaired mitochondrial fusion causes hypertrophy (Papanicolaou et al., 2011; Ikeda et al., 2015b; Song et al., 2015b; Hu et al., 2019). Nevertheless, the mechanisms underlying the associations between mitochondrial fission or fusion and pathological conditions are unclear. Additionally, there are some conflicting observations in mitophagy and mitochondrial dynamics, which should be further confirmed in future studies (**Figure 3**).

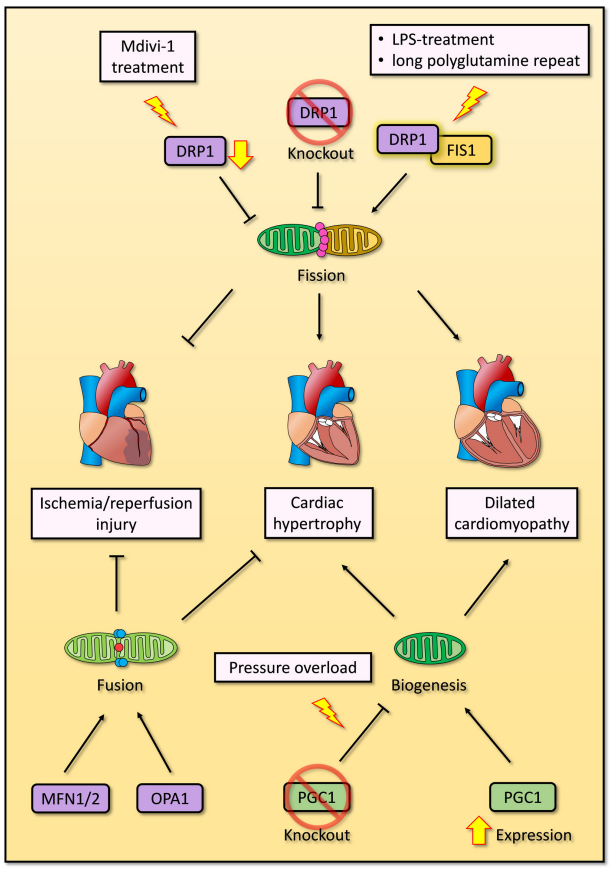


FIGURE 3 | Overview of mitochondrial biogenesis, fission and fusion in cardiomyocytes. Overexpression of PGC1 induces mitochondrial biogenesis and dilated cardiomyopathy, while deficient PGC1 results in cardiac hypertrophy under pressure overloading. Exogenous inhibition of DRP1 by Mdivi-1 improves impaired cardiac function after ischemia/reperfusion injury, whereas endogenous DRP1 knockout promotes cardiac hypertrophy. Enhanced interaction between DRP1 and FIS1 induced by LPS treatment or expression of long polyglutamine repeat causes excessive mitochondrial fission and dilated cardiomyopathy. Two crucial GTPase proteins of mitochondrial fusion, MFN1/2 and OPA1, are essential to repress cardiac hypertrophy and ischemia/reperfusion injury respectively.

MITOPHAGY IN CARDIOVASCULAR DISEASE (CVD)

PINK1/Parkin-Dependent Mitophagy

Parkin-dependent mitophagy has been reported to protect the heart from high-fat diet-induced cardiac hypertrophy, diastolic dysfunction, and lipid accumulation in mice (Tong et al., 2019). Evidence suggests that decreased PINK1/Parkin-dependent mitophagy is associated with development of dystrophic cardiomyopathy (Kang et al., 2018). Recently, it has been reported that PINK1/Parkin-dependent mitophagy is regulated by several factors (Sun et al., 2014; Tahrir et al., 2017; Yu et al., 2017; Li G. et al., 2018; Pickles et al., 2018; Thai et al., 2018; Xiong et al., 2018; Ren J. et al., 2019; Shang et al., 2019). PTEN is a PIP3 phosphatase that generates phosphatidylinositol (4,5) bisphosphate (PIP2), which is involved in multiple biological processes. PTEN was reported to bind Parkin via a form of the membrane binding helix in N-terminus and enhance the recruitment of Parkin onto mitochondria, leading to the removal of damaged mitochondria and maintenance of cardiomyocyte homeostasis (Li G. et al., 2018). In addition, mitophagy in cardiomyocytes is regulated by mammalian Ste20-like kinase 1 (Mst1), which rapidly increases in cardiomyocytes upon LPS treatment or diabetic stress. Mst1 depletion attenuates LPS-induced cardiomyocyte death or diabetic cardiomyopathy and improves cardiac function via promoting Parkin-dependent mitophagy (Shang et al., 2019). Further evidence reveals that the inhibition of mitophagy by Mst1 is associated with SIRT3, whereas depletion of SIRT3 restores cardiac function when

Mst1 is overexpressed in diabetic cardiomyopathy (Wang S. et al., 2019). However, depletion of SIRT3 aggravates impaired mitophagy in diabetes mellitus-associated cardiac dysfunction, accompanied by decreased deacetylation of FOXO3A and expression of Parkin (Yu et al., 2017).

A recent study revealed that adenine nucleotide translocator (ANT) complex drives mitophagy independent of its nucleotide translocase catalytic activity. ANT interacts with TIM23 and suppresses TIM23-mediated protein translocation, which results in the stabilization of PINK1 and subsequent mitophagy. In mouse heart, the lack of ANT causes the accumulation of mitochondria and abnormal mitochondrial morphology with swelling structure and aberrant cristae, which is associated with cardiac hypertrophy and reduced contractile function. Interestingly, this team also found a patient who suffered from homozygous loss of function mutations in ANT1, developed severe heart failure and the biopsy indicated this patient's cardiomyocytes had abnormal mitochondrial homeostasis (Hoshino et al., 2019).

Several lines of evidence suggested that normal PINK1/Parkin-dependent mitophagy is required for the maintenance of cardiac function (**Figure 4A**). However, a few proteins were known to regulate mitophagy in this manner and more definitive proofs of their clear mechanisms are needed.

PINK1/Parkin-Independent Mitophagy

It has been mentioned above that the activity of FUNDC1 in mitophagy is regulated by PTMs (Liu et al., 2012; Wu et al., 2014). FUNDC1, via binding to the ER-resident inositol 1,4,5-trisphosphate type 2 receptor (IP_3R2), modulates Ca^{2+} release from the ER at mitochondria-associated ER membranes (MAMs) to maintain mitochondrial function and homeostasis, which is essential for cardiac function, and to protect the heart from failure (Wu et al., 2017). Apart from regulating SIRT3, Mst1 represses FUNDC1 expression and FUNDC1-induced mitophagy in cardiac I/R injury via the ERK-CREB pathway. Depletion of Mst1 sustains mitochondrial homeostasis and protects cardiomyocytes (Yu W. et al., 2019). In contrast, Receptor-interacting serine/threonine-protein kinase 3 (RIPK3) induces mitochondrial apoptosis in cardiac I/R injury by inhibiting FUNDC1-mediated mitophagy (Quinsay et al., 2010).

The interaction between BNIP3 and LC3 is positively regulated by phosphorylation of Ser17 and Ser24 in BNIP3 (Liu et al., 2014). In cardiomyocytes isolated from rat heart, overexpression of BNIP3 induces extensive mitophagy, which is independent of cytosolic Ca^{2+} level, ROS generation and mPTP opening (Quinsay et al., 2010). The expression of BNIP3 increases in cardiomyocytes under PE or calcium stress *in vitro* or in response to pressure overload *in vivo*. By 3-methyladenine treatment and gene ablation assay, JNK signaling was proved to regulate BNIP3 expression via the transcription factor FOXO3a, thus protecting cardiomyocytes from cardiac remodeling in heart failure (Chaanine et al., 2012). JNK signaling is also regulated by Dual-specificity protein phosphatase1 (DUSP1) to alleviate cardiac I/R injury. This effect involves suppression of MFF-mediated mitochondrial fission and BNIP3-dependent mitophagy *in vivo* (Jin et al., 2018).

Structurally similar to BNIP3, BNIP3L, is also a receptor for mitophagy. BNIP3L interacts with LC3 or GABARAP by its WXXL motif in the N-terminal region to remove the excess or damaged mitochondria (Kanki, 2010; Moyzis and Gustafsson, 2019). However, serving as pro-apoptotic factors, BNIP3L is involved in cardiomyocyte apoptosis. Evidence indicates increased BNIP3L promotes cardiomyocyte apoptosis and causes cardiomyopathy with LV dilation and represses systolic function, whereas reduced BNIP3L protects against apoptotic cardiomyopathy (Lynch et al., 2002; Diwan et al., 2008). Although BNIP3L is widely studied in cardiomyocytes acting as a pro-apoptotic factor, definitive proofs linking cardiomyocytes to the role of BNIP3L in mitophagy are not available.

In summary, evidence from several studies suggests that mitophagy in cardiomyocytes may be a critical pathway for mitochondrial QC. Mitophagy receptors such as FUNDC1, BNIP3, BNIP3L, etc., play essential roles in maintenance of mitochondrial and cellular homeostasis (**Figure 4B**). For a number of these receptors, their mechanism of action remains unclear and needs to be further investigated. In addition, based on emerging knowledge about dedicated mitophagy receptors, some novel pharmacological agents have been proposed to prevent CVD. These agents include 6-Gingerol, berbamine and melatonin (Zhou L. et al., 2017; Ren Q. et al., 2019).

MITOCHONDRIAL-DERIVED VESICLES (MDVS) IN CARDIOVASCULAR DISEASE (CVD)

Although few researchers have studied the association between MDVs and cardiomyocytes, Cadete et al. (2016) revealed that MDVs maintain the homeostasis of mitochondria in cardiomyocytes in response to acute stress induced by doxorubicin. They also suggested that MDV formation serves as baseline housekeeping mechanism and a first line of defense against stress in the cardiac system (Cadete et al., 2016).

This preliminary evidence suggests that there may be a link between MDVs and cardiomyocyte function. However, much more work is needed to investigate the potential association between MDVs and CVD. The specific mechanisms underlying generation and maturation of MDVs also require further study. In particular, it will be important to identify the kinds of proteins that are involved in generating the selective asymmetry of MDVs.

PHARMACOLOGICAL AND THERAPEUTIC TARGETS BASED ON MITOCHONDRIAL QUALITY CONTROL SYSTEMS

Recently, many studies have focused on therapies targeted to mitochondrial QC including PTMs on mitochondrial proteins, mitochondrial dynamics, mitophagy, and MDVs. Melatonin treatment was reported to maintain myocardial function and cardiomyocyte viability in the context of I/R injury *in vivo*.

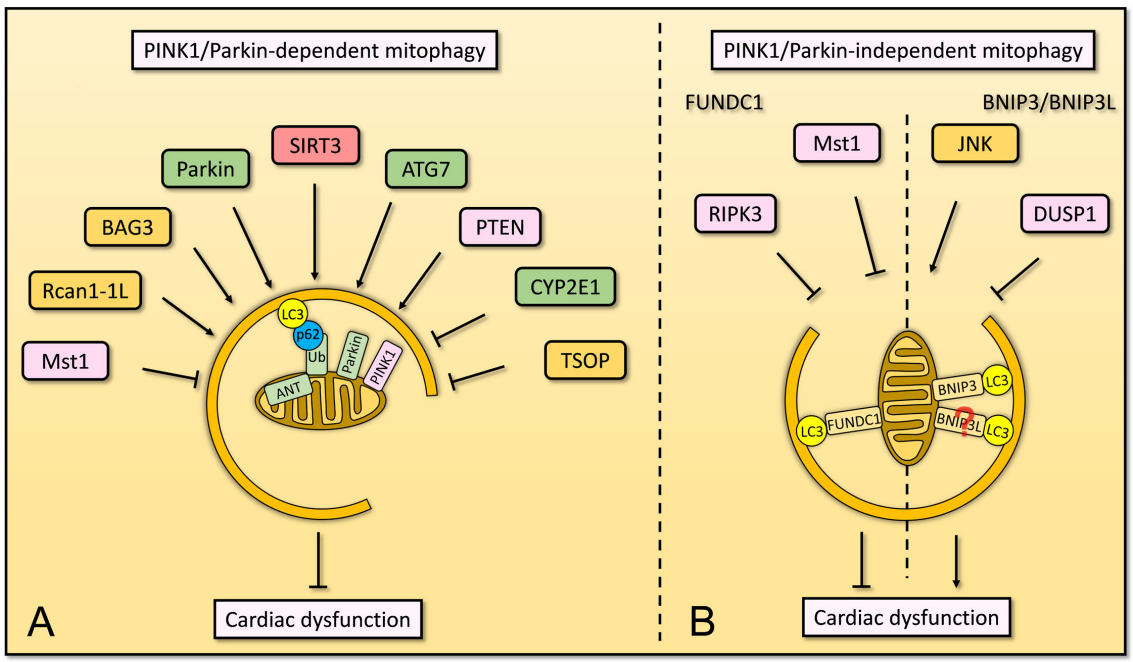


FIGURE 4 | Overview of mitophagy that involves in cardiac dysfunction. **(A)** In cardiomyocytes, PINK1/Parkin-dependent mitophagy is regulated by several factors. ANT localizes on the IMM, maintains the stabilization of PINK1 and subsequent mitophagy. SIRT3 plays an important role in mitophagy to suppress diabetes mellitus-associated cardiac dysfunction. Parkin and ATG7 are essential to mitophagy for homeostasis of cardiomyocytes. PTEN regulates mitophagy and maintains mitochondrial quality control as well as cardiac homeostasis. BAG3 and Rcan1-1L are involved in mitophagy for cardiomyocyte function, whereas CYP2E1 and TSOP negatively regulate mitophagy in cardiomyocytes. Mst1, serves as pro-apoptotic kinase, inhibits PINK1/Parkin-dependent mitophagy and promotes cardiac dysfunction. **(B)** Cardiac function is also regulated by PINK1/Parkin-independent mitophagy, including FUNDC1-dependent and BNIP3-dependent mitophagy. FUNDC1-dependent mitophagy restores cardiac function by Mst1 and RIPK3, whereas BNIP3-dependent mitophagy induces cardiac dysfunction which is promoted by JNK pathway but is inhibited by DUSP1. The knowledge linking BNIP3L to cardiomyocytes via mitophagy remains limited.

The effect was related to OPA1-dependent maintenance of mitochondrial fusion and mitophagy, and occurred through activation of AMPK by melatonin (Zhang et al., 2019). By inhibiting Mst1, melatonin also increases the expression of Parkin and thus restores Parkin-mediated mitophagy to protect the heart from diabetic cardiomyopathy (Wang S. et al., 2018). 7,8-dihydroxyflavone (7,8-DHF), a mimetic of Brain-derived neurotrophic factor (BDNF), also attenuates cardiomyocyte death and improves cardiac function in cardiac ischemic injury via repression of the OMA1-dependent proteolytic cleavage of L-OPA1 (Wang Z. et al., 2019). In addition to mitochondrial fusion, fission can also be targeted to improve cardiomyocyte function under pathological conditions. For example, during I/R injury, treatment with Mdivi-1, improves cardiac function, attenuates arrhythmia and reduces infarct size by maintaining mitochondrial function in cardiomyocytes, resulting in suppression of cellular death. Mdivi-1 is more efficient when administered prior to ischemia, but less effective at the onset of reperfusion (Cooper and Eguchi, 2018). Through reducing DRP1 expression levels, Mdivi-1, suppresses cell death, inhibited mPTP opening, and protected heart from I/R injury (Piantadosi et al., 2008; Ong et al., 2010). The pretreatment of FK506 and therapeutic hypothermia (30°C) also preserved cardiac function via preventing DRP1-S637 dephosphorylation (Ong et al., 2010). Also, treatment with P110, which inhibits the DRP1/FIS1 interaction, improves mitochondrial homeostasis in cardiomyocytes and promotes the removal of damaged mitochondria in Huntington's disease models (Joshi et al., 2019). A traditional Chinese medicine, Shenmai, induces mitophagy, regulates mitochondrial dynamics, improves intracellular Ca^{2+} homeostasis and mitochondrial potential to attenuate H/R injury *in vitro* and to prevent Left Anterior Descending Coronary Artery Ligation (LAD)-induced cardiac dysfunction *in vivo* (Yu J. et al., 2019).

As mentioned above, by targeting to autophagy receptors on mitochondria, or to regulators of mitochondrial function, several novel pharmacological therapies play a role in cardiovascular system. Through phosphorylating PI3K, AKT, mTOR and further suppressing BNIP3, 6-Gingerol attenuates hypoxia-induced cardiomyocyte injury (Ren Q. et al., 2019). Melatonin has also been identified to suppress platelet activation in cardiac I/R injury by restoring PPARγ and thus blocking FUNDC1-dependent mitophagy, which leads to reduction of platelet activity and improved cardiac outcome (Zhou L. et al., 2017). In addition to pharmacological therapies, several physical therapies are also efficient in preventing cardiac disease. For example, in aged mice after myocardial infarction, short-duration swimming exercise (15 min) was more effective than long-duration swimming (60 min) at regulating mitochondrial dynamics and mitophagy. Cardiac function was improved and the expression of SIRT3 was

promoted. However, depletion of SIRT3 in this study impairs mitochondrial dynamics, promotes ROS generation and release, and causes cell death (Zhao et al., 2018).

Thus, by targeting proteins involved in mitochondrial QC, both pharmacological and mechanical therapies can maintain mitochondrial homeostasis in cardiomyocytes. As described earlier in this review, there are several targets of mitochondrial QC in cardiomyocytes, and therefore more drugs or treatments are expected to be proposed and further developed for clinical use.

FUTURE PERSPECTIVES

Following different extracellular stresses, perturbations in mitochondrial homeostasis result in abnormal mitochondrial morphology, dysfunctional oxidative phosphorylation, leakage of components from the mitochondrial matrix, and activation of mitochondrion-dependent cellular signaling. Under physiological conditions, PTMs on mitochondrial proteins maintain the balance between mitochondrial fusion and fission as well as executing mitophagy to remove aged mitochondria for normal cellular homeostasis. Under pathological conditions, such as I/R injury or exposure to cardiotoxic agents, PTMs (SUMOylation, phosphorylation, ubiquitination) on critical mitochondrial proteins trigger changes in mitochondrial dynamics and mitophagy to rescue cellular homeostasis. Currently, many studies are focusing on the effect of mitochondrial QC in cardiomyocytes, including PTMs on mitochondrial proteins, mitochondrial dynamics, MDVs and mitophagy, trying to connect some known molecular mechanisms to cardiomyocytes and revealing the cardiac-specific mechanism of mitochondrial QC. The mechanisms by which mitochondrial QC protects cardiomyocytes against cardiac disease are becoming increasingly clear, and consequently more and more potential targets for clinical therapies are emerging. Furthermore, we should pay attention to that mitochondrial dynamics and network is highly dynamic and complex. Although accumulating tools or techniques are studied for real-time tracing of mitochondria *in vitro*, precise and effective methods remain in need of that *in vivo* (Guo et al., 2018).

Therefore, key goals for the future include: investigating more characterization of PTMs on mitochondrial proteins; developing more means to trace mitochondrial dynamics and network; identifying more solid and direct associations between protein targets of mitochondrial QC and specific CVDs; developing more efficient pharmacological or physical therapies for protecting the heart; and considering how to transfer basic research results into clinical practice.

AUTHOR CONTRIBUTIONS

HF and DF conceived and wrote the manuscript. All authors commented and revised the manuscript.

FUNDING

DF is supported by funding from the National Natural Science Foundation of China (NSFC Nos. 31771531 and 91754115), Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (GDUPS), Guangzhou Ling Nan Ying Ciai Project ((2019) 928), and the Science and Technology Planning Project of Guangdong Province (2017B090901051).

REFERENCES

Anzell, A. R., Maizy, R., Przyklenk, K., and Sanderson, T. H. (2018). Mitochondrial quality control and disease: insights into ischemia-reperfusion injury. *Mol. Neurobiol.* 55, 2547–2564. doi: 10.1007/s12035-017-0503-9

Arany, Z., He, H., Lin, J., Hoyer, K., Handschin, C., Toka, O., et al. (2005). Transcriptional coactivator PGC-1α controls the energy state and contractile function of cardiac muscle. *Cell Metab.* 1, 259–271. doi: 10.1016/j.cmet.2005.03.002

Arany, Z., Novikov, M., Chin, S., Ma, Y., Rosenzweig, A., and Spiegelman, B. M. (2006). Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-γ coactivator 1α. *Proc. Natl. Acad. Sci. U.S.A.* 103, 10086–10091. doi: 10.1073/pnas.0603615103

Audia, J. E., and Campbell, R. M. (2016). Histone modifications and cancer. *CSH Perspect. Biol.* 8:a19521.

Baeza, J., Smallegan, M. J., and Denu, J. M. (2016). Mechanisms and dynamics of protein acetylation in mitochondria. *Trends Biochem. Sci.* 41, 231–244. doi: 10.1016/j.tibs.2015.12.006

Bian, X., Xu, J., Zhao, H., Zheng, Q., Xiao, X., Ma, X., et al. (2019). Zinc-induced SUMOylation of dynamin-related protein 1 protects the heart against ischemia-reperfusion injury. *Oxid. Med. Cell Longev.* 2019:1232146. doi: 10.1155/2019/1232146

Brand, C. S., Tan, V. P., Brown, J. H., and Miyamoto, S. (2018). RhoA regulates Drp1 mediated mitochondrial fission through ROCK to protect cardiomyocytes. *Cell Signal* 50, 48–57. doi: 10.1016/j.cellsig.2018.06.012

Buhlman, L., Damiano, M., Bertolin, G., Ferrando-Miguel, R., Lombès, A., Brice, A., et al. (2014). Functional interplay between Parkin and Drp1 in mitochondrial fission and clearance. *Biochim. Biophys. Acta* 1843, 2012–2026. doi: 10.1016/j.bbamcr.2014.05.012

Cadete, V. J. J., Deschênes, S., Cuillerier, A., Brisebois, F., Sugiura, A., Vincent, A., et al. (2016). Formation of mitochondrial-derived vesicles is an active and physiologically relevant mitochondrial quality control process in the cardiac system. *J. Physiol.* 594, 5343–5362. doi: 10.1113/JP272703

Cao, Y., Xu, C., Ye, J., He, Q., Zhang, X., Jia, S., et al. (2019). Miro2 regulates inter-mitochondrial communication in the heart and protects against TAC-induced cardiac dysfunction. *Circ. Res.* 125, 728–743. doi: 10.1161/CIRCRESAHA.119.315432

Celestini, V., Tezil, T., Russo, L., Fasano, C., Sanese, P., Forte, G., et al. (2018). Uncoupling FoxO3A mitochondrial and nuclear functions in cancer cells undergoing metabolic stress and chemotherapy. *Cell Death Dis.* 9:231. doi: 10.1038/s41419-018-0336-0

Chaanine, A. H., Jeong, D., Liang, L., Chemaly, E. R., Fish, K., Gordon, R. E., et al. (2012). JNK modulates FOXO3a for the expression of the mitochondrial death and mitophagy marker BNIP3 in pathological hypertrophy and in heart failure. *Cell Death Dis.* 3:e265. doi: 10.1038/cddis.2012.5

Chang, C., and Blackstone, C. (2007). Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. *J. Biol. Chem.* 282, 21583–21587. doi: 10.1074/jbc.c700083200

Chen, L., Gong, Q., Stice, J. P., and Knowlton, A. A. (2009). Mitochondrial OPA1, apoptosis, and heart failure. *Cardiovasc. Res.* 84, 91–99. doi: 10.1093/cvr/cvp181

Chen, Y., and Dorn, G. W. (2013). PINK1-phosphorylated mitofusin 2 Is a parkin receptor for culling damaged mitochondria. *Science* 340, 471–475. doi: 10.1126/science.1231031

Cherok, E., Xu, S., Li, S., Das, S., Meltzer, W. A., Zalzman, M., et al. (2017). Novel regulatory roles of Mff and Drp1 in E3 ubiquitin ligase MARCH5-dependent degradation of MiD49 and Mcl1 and control of mitochondrial dynamics. *Mol. Biol. Cell* 28, 396–410. doi: 10.1091/mbc.E16-04-0208

Ciechanover, A. (2005). Intracellular protein degradation: from a vague idea, through the lysosome and the ubiquitin-proteasome system, and onto human diseases and drug targeting (Nobel Lecture). *Angew. Chem. Intern. Ed.* 44, 5944–5967. doi: 10.1002/anie.200501428

Cooper, H. A., and Eguchi, S. (2018). Inhibition of mitochondrial fission as a novel therapeutic strategy to reduce mortality upon myocardial infarction. *Clin. Sci.* 132, 2163–2167. doi: 10.1042/CS20180671

Cribbs, J. T., and Strack, S. (2007). Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. *EMBO Rep.* 8, 939–944. doi: 10.1038/sj.embor.7401062

Das, S., Mitrovsky, G., Vasanthi, H. R., and Das, D. K. (2014). Antiaging properties of a grape-derived antioxidant are regulated by mitochondrial balance of fusion and fission leading to mitophagy triggered by a signaling network of Sirt1-Sirt3-Foxo3-PINK1-PARKIN. *Oxid. Med. Cell Longev.* 2014, 1–13. doi: 10.1155/2014/345105

Dikalova, A. E., Itani, H. A., Nazarewicz, R. R., McMaster, W. G., Flynn, C. R., Uzhachenko, R., et al. (2017). Sirt3 impairment and SOD2 hyperacetylation in vascular oxidative stress and hypertension. *Circ. Res.* 121, 564–574. doi: 10.1161/CIRCRESAHA.117.310933

Diwan, A., Wansapura, J., Syed, F. M., Matkovich, S. J., Lorenz, J. N., and Dorn, G. W. (2008). Nix-Mediated apoptosis links myocardial fibrosis, cardiac remodeling, and hypertrophy decompensation. *Circulation* 117, 396–404. doi: 10.1161/CIRCULATIONAHA.107.727073

Dorn, G. W., and Kitsis, R. N. (2015). The mitochondrial dynamism-mitophagy-cell death interactome. *Circ. Res.* 116, 167–182. doi: 10.1161/CIRCRESAHA.116.303554

Dorn, G. W., Vega, R. B., and Kelly, D. P. (2015). Mitochondrial biogenesis and dynamics in the developing and diseased heart. *Gene Dev.* 29, 1981–1991. doi: 10.1101/gad.269894.115

Drazic, A., Myklebust, L. M., Ree, R., and Arnesen, T. (2016). The world of protein acetylation. *Biochim. Biophys. Acta* 1864, 1372–1401.

Friedman, J. R., Lackner, L. L., West, M., DiBenedetto, J. R., Nunnari, J., and Voeltz, G. K. E. R. (2011). Tubules mark sites of mitochondrial division. *Science* 334, 358–362. doi: 10.1126/science.1207385

Galluzzi, L., Baehrecke, E. H., Ballabio, A., Boya, P., Bravo-San, P. J., Cecconi, F., et al. (2017). Molecular definitions of autophagy and related processes. *EMBO J.* 36, 1811–1836. doi: 10.15252/embj.201796697

Gareau, J. R., and Lima, C. D. (2010). The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat. Rev. Mol. Cell Biol.* 11, 861–871. doi: 10.1038/nrm3011

Gatica, D., Chiong, M., Lavandero, S., and Klionsky, D. J. (2015). Molecular mechanisms of autophagy in the cardiovascular system. *Circ. Res.* 116, 456–467. doi: 10.1161/circresaha.114.303788

Gegg, M. E., Cooper, J. M., Chau, K., Rojo, M., Schapira, A. H. V., and Taanman, J. (2010). Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. *Hum. Mol. Genet.* 19, 4861–4870. doi: 10.1093/hmg/ddq419

Gerhart-Hines, Z., Rodgers, J. T., Bare, O., Lerin, C., Kim, S. H., Mostoslavsky, R., et al. (2007). Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. *EMBO J.* 26, 1913–1923. doi: 10.1038/sj.emboj.7601633

Giorgi, C., Marchi, S., and Pinton, P. (2018). The machineries, regulation and cellular functions of mitochondrial calcium. *Nat. Rev. Mol. Cell. Biol.* 19, 713–730. doi: 10.1038/s41580-018-0052-8

Glauser, L., Sonnay, S., Stafa, K., and Moore, D. J. (2011). Parkin promotes the ubiquitination and degradation of the mitochondrial fusion factor mitofusin 1. *J. Neurochem.* 118, 636–645. doi: 10.1111/j.1471-4159.2011.07318.x

Gong, G., Song, M., Csordas, G., Kelly, D. P., Matkovich, S. J., and Dorn, G. W. (2015). Parkin-mediated mitophagy directs perinatal cardiac metabolic maturation in mice. *Science* 350:d2459. doi: 10.1126/science.aad2459

Guo, C., Hildick, K. L., Luo, J., Dearden, L., Wilkinson, K. A., and Henley, J. M. (2013). SENP3-mediated deSUMOylation of dynamin-related protein 1 promotes cell death following ischaemia. *EMBO J.* 32, 1514–1528. doi: 10.1038/emboj.2013.65

Guo, C., Wilkinson, K. A., Evans, A. J., Rubin, P. P., and Henley, J. M. (2017). SENP3-mediated deSUMOylation of Drp1 facilitates interaction with Mff to promote cell death. *Sci. Rep.* 7:43811. doi: 10.1038/srep43811

Guo, Y., Li, D., Zhang, S., Yang, Y., Liu, J., Wang, X., et al. (2018). Visualizing intracellular organelle and cytoskeletal interactions at nanoscale resolution on millisecond timescales. *Cell* 175, 1430–1442. doi: 10.1016/j.cell.2018.09.057

Haileselassie, B., Mukherjee, R., Joshi, A. U., Napier, B. A., Massis, L. M., Ostberg, N. P., et al. (2019). Drp1/Fis1 interaction mediates mitochondrial dysfunction in septic cardiomyopathy. *J. Mol. Cell Cardiol.* 130, 160–169. doi: 10.1016/j.yjmcc.2019.04.006

Hammerling, B. C., and Gustafsson, ÅB. (2014). Mitochondrial quality control in the myocardium: cooperation between protein degradation and mitophagy. *J. Mol. Cell Cardiol.* 75, 122–130. doi: 10.1016/j.yjmcc.2014.07.013

Han, X., Lu, Y., Li, S., Kaitsuka, T., Sato, Y., Tomizawa, K., et al. (2008). CaM kinase Iα–induced phosphorylation of Drp1 regulates mitochondrial morphology. *J. Cell Biol.* 182, 573–585. doi: 10.1083/jcb.200802164

Hendriks, I. A., and Vertegaal, A. C. (2016). A comprehensive compilation of SUMO proteomics. *Nat. Rev. Mol. Cell Biol.* 17, 581–595. doi: 10.1038/nrm.2016.81

Horn, S. R., Thomenius, M. J., Johnson, E. S., Freel, C. D., Wu, J. Q., Coloff, J. L., et al. (2011). Regulation of mitochondrial morphology by APC/CCdh1-mediated control of Drp1 stability. *Mol. Biol. Cell* 22, 1207–1216. doi: 10.1091/mbc.E10-07-0567

Hoshino, A., Wang, W., Wada, S., McDermott-Roe, C., Evans, C. S., Gosis, B., et al. (2019). The ADP/ATP translocase drives mitophagy independent of nucleotide exchange. *Nature* 575, 375–379. doi: 10.1038/s41586-019-1667-4

Hu, L., Ding, M., Tang, D., Gao, E., Li, C., Wang, K., et al. (2019). Targeting mitochondrial dynamics by regulating Mfn2 for therapeutic intervention in diabetic cardiomyopathy. *Theranostics* 9, 3687–3706. doi: 10.7150/thno.33684

Ikeda, Y., Shirakabe, A., Brady, C., Zablocki, D., Ohishi, M., and Sadoshima, J. (2015a). Molecular mechanisms mediating mitochondrial dynamics and mitophagy and their functional roles in the cardiovascular system. *J. Mol. Cell Cardiol.* 78, 116–122. doi: 10.1016/j.yjmcc.2014.09.019

Ikeda, Y., Shirakabe, A., Maejima, Y., Zhai, P., Sciarretta, S., Toli, J., et al. (2015b). Endogenous Drp1 mediates mitochondrial autophagy and protects the heart against energy stress. *Circ. Res.* 116, 264–278. doi: 10.1161/CIRCRESAHA.116.303356

Javadov, S., Rajapurohitam, V., Kilić, A., Hunter, J. C., Zeidan, A., Said Faruq, N., et al. (2011). Expression of mitochondrial fusion–fission proteins during post-infarction remodeling: the effect of NHE-1 inhibition. *Basic Res. Cardiol.* 106, 99–109. doi: 10.1007/s00395-010-0122-3

Jhun, B. S., O-Uchi, J., Adaniya, S. M., Mancini, T. J., Cao, J. L., King, M. E., et al. (2018). Protein kinase D activation induces mitochondrial fragmentation and dysfunction in cardiomyocytes. *J. Physiol.* 596, 827–855. doi: 10.1113/JP275418

Jin, Q., Li, R., Hu, N., Xin, T., Zhu, P., Hu, S., et al. (2018). DUSP1 alleviates cardiac ischemia/reperfusion injury by suppressing the Mff-required mitochondrial fission and Bnip3-related mitophagy via the JNK pathways. *Redox Biol.* 14, 576–587. doi: 10.1016/j.redox.2017.11.004

Joshi, A. U., Ebert, A. E., Haileselassie, B., and Mochly-Rosen, D. (2019). Drp1/Fis1-mediated mitochondrial fragmentation leads to lysosomal dysfunction in cardiac models of Huntington's disease. *J. Mol. Cell Cardiol.* 127, 125–133. doi: 10.1016/j.yjmcc.2018.12.004

Kageyama, Y., Hoshijima, M., Seo, K., Bedja, D., Sysa-Shah, P., Andrabi, S. A., et al. (2014). Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain. *EMBO J.* 33, 2798–2813. doi: 10.15252/embj.201488658

Kang, C., Badr, M. A., Kyrychenko, V., Eskelinen, E., and Shirokova, N. (2018). Deficit in PINK1/PARKIN-mediated mitochondrial autophagy at late stages of dystrophic cardiomyopathy. *Cardiovasc. Res.* 114, 90–102. doi: 10.1093/cvr/cvx201

Kanki, T. (2010). Nix: A receptor protein for mitophagy in mammals. *Autophagy* 6, 433–435. doi: 10.4161/auto.6.3.11420

Kim, E. Y., Zhang, Y., Beketaev, I., Segura, A. M., Yu, W., Xi, Y., et al. (2014). SENP5, a SUMO isopeptidase, induces apoptosis and cardiomyopathy. *J. Mol. Cell Cardiol.* 78, 154–164. doi: 10.1016/j.yjmcc.2014.08.003

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., et al. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392, 605–608. doi: 10.1038/33416

Klimova, N., Long, A., and Kristian, T. (2018). Significance of mitochondrial protein post-translational modifications in pathophysiology of brain injury. *Transl. Stroke Res.* 9, 223–237. doi: 10.1007/s12975-017-0569-8

Kyu, H. H., Abate, D., Abate, K. H., Abay, S. M., Abbafati, C., Abbasi, N., et al. (2018). Global, regional, and national disability-adjusted life-years (DALYs) for 359 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990–2017: a systematic analysis for the global burden of disease study 2017. *Lancet* 392, 1859–1922.

Lazarou, M., Sliter, D. A., Kane, L. A., Sarraf, S. A., Wang, C., Burman, J. L., et al. (2015). The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* 524, 309–314. doi: 10.1038/nature14893

Lehman, J. J., Barger, P. M., Kovacs, A., Saffitz, J. E., Medeiros, D. M., and Kelly, D. P. (2000). Peroxisome proliferator–activated receptor γ coactivator-1 promotes cardiac mitochondrial biogenesis. *J. Clin. Invest.* 106, 847–856. doi: 10.1172/jci10268

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell* 132, 27–42. doi: 10.1016/j.cell.2007.12.018

Li, G., Yang, J., Yang, C., Zhu, M., Jin, Y., McNutt, M. A., et al. (2018). PTENα regulates mitophagy and maintains mitochondrial quality control. *Autophagy* 14, 1742–1760. doi: 10.1080/15548627.2018.1489477

Li, Y., Stockton, M. E., Eisinger, B. E., Zhao, Y., Miller, J. L., Bhuiyan, I., et al. (2018). Reducing histone acetylation rescues cognitive deficits in a mouse model of Fragile X syndrome. *Nat. Commun.* 9:2494. doi: 10.1038/s41467-018-04869-3

Little, J. P., Safdar, A., Cermak, N., Tarnopolsky, M. A., and Gibala, M. J. (2010). Acute endurance exercise increases the nuclear abundance of PGC-1α in trained human skeletal muscle. *Am. J. Physiol Regul. Integ. Compar. Physiol.* 298, R912–R917. doi: 10.1152/ajpregu.00409.2009

Liu, J., Yan, W., Zhao, X., Jia, Q., Wang, J., Zhang, H., et al. (2019). Sirt3 attenuates post-infarction cardiac injury via inhibiting mitochondrial fission and normalization of AMPK-Drp1 pathways. *Cell Signal.* 53, 1–13. doi: 10.1016/j.cellsig.2018.09.009

Liu, L., Feng, D., Chen, G., Chen, M., Zheng, Q., Song, P., et al. (2012). Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells. *Nat. Cell Biol.* 14, 177–185. doi: 10.1038/ncb2422

Liu, L., Sakakibara, K., Chen, Q., and Okamoto, K. (2014). Receptor-mediated mitophagy in yeast and mammalian systems. *Cell Res.* 24, 787–795. doi: 10.1038/cr.2014.75

Luo, Y. X., Tang, X., An, X. Z., Xie, X. M., Chen, X. F., Zhao, X., et al. (2017). SIRT4 accelerates Ang II-induced pathological cardiac hypertrophy by inhibiting manganese superoxide dismutase activity. *Eur. Heart J.* 38, 1389–1398. doi: 10.1093/eurheartj/ehw138

Lynch, R. A., Aronow, B. J., Odley, A., Lorenz, J. N., Toyokawa, T., Wu, G., et al. (2002). Mitochondrial death protein Nix is induced in cardiac hypertrophy and triggers apoptotic cardiomyopathy. *Nat. Med.* 8, 725–730. doi: 10.1038/nm719

Martinet, W., and De Meyer, G. R. Y. (2009). Autophagy in atherosclerosis. *Circ. Res.* 104, 304–317. doi: 10.1161/CIRCRESAHA.108.188318

McBride, H. M., Braschi, E., and Zunino, R. (2009). MAPL is a new mitochondrial SUMO E3 ligase that regulates mitochondrial fission. *Embo. Rep.* 10, 748–754. doi: 10.1038/embor.2009.86

McLelland, G. L., Soubannier, V., Chen, C. X., McBride, H. M., and Fon, E. A. (2014). Parkin and PINK1 function in a vesicular trafficking pathway regulating mitochondrial quality control. *EMBO J.* 33, 282–295. doi: 10.1002/embj.201385902

McLelland, G., Goiran, T., Yi, W., Dorval, G., Chen, C. X., Lauinger, N. D., et al. (2018). Mfn2 ubiquitination by PINK1/parkin gates the p97-dependent release of ER from mitochondria to drive mitophagy. *eLife* 7:e32866. doi: 10.7554/eLife.32866

Mihaylova, M. M., Vasquez, D. S., Ravnskjaer, K., Denechaud, P., Yu, R. T., Alvarez, J. G., et al. (2011). Class iia histone deacetylases are hormone-activated regulators of FOXO and mammalian glucose homeostasis. *Cell* 145, 607–621. doi: 10.1016/j.cell.2011.03.043

Min, S., Chen, X., Tracy, T. E., Li, Y., Zhou, Y., Wang, C., et al. (2015). Critical role of acetylation in tau-mediated neurodegeneration and cognitive deficits. *Nat. Med.* 21, 1154–1162. doi: 10.1038/nm.3951

Moyzis, A., and Gustafsson, ÅB. (2019). Multiple recycling routes: canonical vs. non-canonical mitophagy in the heart. *Biochim. Biophys. Acta* 1865, 797–809. doi: 10.1016/j.bbadis.2018.09.034

Nakamura, N., Kimura, Y., Tokuda, M., Honda, S., and Hirose, S. (2006). MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology. *EMBO Rep.* 7, 1019–1022. doi: 10.1038/sj.embor.7400790

Nguyen, T. N., Padman, B. S., and Lazarou, M. (2016). Deciphering the molecular signals of PINK1/Parkin mitophagy. *Trends Cell Biol.* 26, 733–744. doi: 10.1016/j.tcb.2016.05.008

Ni, H., Williams, J. A., and Ding, W. (2015). Mitochondrial dynamics and mitochondrial quality control. *Redox Biol.* 4, 6–13. doi: 10.1016/j.redox.2014.11.006

Ong, S., Subrayan, S., Lim, S. Y., Yellon, D. M., Davidson, S. M., and Hausenloy, D. J. (2010). Inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury. *Circulation* 121, 2012–2022. doi: 10.1161/CIRCULATIONAHA.109.906610

Onyango, P., Celic, I., McCaffery, J. M., Boeke, J. D., and Feinberg, A. P. (2002). SIRT3, a human SIR2 homologue, is an NAD-dependent deacetylase localized to mitochondria. *Proc. Natl. Acad. Sci. U.S.A.* 99, 13653–13658. doi: 10.1073/pnas.222538099

Papac-Milicevic, N., Breuss, J. M., Zaujec, J., Ryban, L., Plyushch, T., Wagner, G. A., et al. (2012). The interferon stimulated gene 12 inactivates vasculoprotective functions of NR4A nuclear receptors. *Circ. Res.* 110, e50–e63. doi: 10.1161/CIRCRESAHA.111.258814

Papanicolaou, K. N., Khairallah, R. J., Ngoh, G. A., Chikando, A., Luptak, I., O'Shea, K. M., et al. (2011). Mitofusin-2 maintains mitochondrial structure and contributes to stress-induced permeability transition in cardiac myocytes. *Mol. Cell Biol.* 31, 1309–1328. doi: 10.1128/MCB.00911-10

Piantadosi, C. A., Carraway, M. S., Babiker, A., and Suliman, H. B. (2008). Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via nrf2-mediated transcriptional control of nuclear respiratory factor-1. *Circ. Res.* 103, 1232–1240. doi: 10.1161/01.RES.0000338597.71702.ad

Pickles, S., Vigié, P., and Youle, R. J. (2018). Mitophagy and quality control mechanisms in mitochondrial maintenance. *Curr. Biol.* 28, R170–R185. doi: 10.1016/j.cub.2018.01.004

Poole, A. C., Thomas, R. E., Yu, S., Vincow, E. S., and Pallanck, L. (2010). The mitochondrial fusion-promoting factor mitofusin is a substrate of the PINK1/parkin pathway. *PLoS One* 5:e10054. doi: 10.1371/journal.pone.0010054

Preau, S., Delguste, F., Yu, Y., Remy-Jouet, I., Richard, V., Saulnier, F., et al. (2016). Endotoxemia engages the RhoA Kinase pathway to impair cardiac function by altering cytoskeleton, mitochondrial fission, and autophagy. *Antioxid. Redox Sign.* 24, 529–542. doi: 10.1089/ars.2015.6421

Prudent, J., Zunino, R., Sugiura, A., Mattie, S., Shore, G. C., and McBride, H. M. M. A. P. L. (2015). SUMOylation of Drp1 stabilizes an ER/mitochondrial platform required for cell death. *Mol. Cell* 59, 941–955. doi: 10.1016/j.molcel.2015.08.001

Puigserver, P., Wu, Z., Park, C. W., Graves, R., Wright, M., and Spiegelman, B. M. A. (1998). Cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92, 829–839. doi: 10.1016/s0092-8674(00)81410-5

Pyakurel, A., Savoia, C., Hess, D., and Scorrano, L. (2015). Extracellular regulated kinase phosphorylates mitofusin 1 to control mitochondrial morphology and apoptosis. *Mol. Cell* 58, 244–254. doi: 10.1016/j.molcel.2015.02.021

Quinsay, M. N., Thomas, R. L., Lee, Y., and Gustafsson, ÅB. (2010). Bnip3-mediated mitochondrial autophagy is independent of the mitochondrial permeability transition pore. *Autophagy* 6, 855–862. doi: 10.4161/auto.6.7.13005

Ren, J., Pei, Z., Chen, X., Berg, M. J., Matrougui, K., Zhang, Q., et al. (2019). Inhibition of CYP2E1 attenuates myocardial dysfunction in a murine model of insulin resistance through NLRP3-mediated regulation of mitophagy. *Biochim. Biophys. Acta* 1865, 206–217. doi: 10.1016/j.bbadis.2018.08.017

Ren, Q., Zhao, S., and Ren, C. (2019). 6-Gingerol protects cardiocytes H9c2 against hypoxia-induced injury by suppressing BNIP3 expression. *Artif. Cells Nanomed. Biotechnol.* 47, 2016–2023. doi: 10.1080/21691401.2019.1610415

Samant, S. A., Zhang, H. J., Hong, Z., Pillai, V. B., Sundaresan, N. R., Wolfgeher, D., et al. (2014). SIRT3 deacetylates and activates OPA1 to regulate mitochondrial

dynamics during stress. *Mol. Cell Biol.* 34, 807–819. doi: 10.1128/MCB.01483-13

Shang, X., Lin, K., Zhang, Y., Li, M., Xu, J., Chen, K., et al. (2019). Mst1 deletion reduces septic cardiomyopathy via activating Parkin-related mitophagy. *J. Cell Physiol.* 235, 317–327. doi: 10.1002/jcp.28971

Sharp, W. W., Fang, Y. H., Han, M., Zhang, H. J., Hong, Z., Banathy, A., et al. (2014). Dynamin-related protein 1 (Drp1)-mediated diastolic dysfunction in myocardial ischemia-reperfusion injury: therapeutic benefits of Drp1 inhibition to reduce mitochondrial fission. *FASEB J.* 28, 316–326. doi: 10.1096/fj.12-226225

Shendelman, S., Jonason, A., Martinat, C., Leete, T., and Abeliovich, A. (2004). DJ-1 is a redox-dependent molecular chaperone that inhibits α-synuclein aggregate formation. *PLoS Biol.* 2:e362. doi: 10.1371/journal.pbio.0020362

Shimizu, Y., Lambert, J. P., Nicholson, C. K., Kim, J. J., Wolfson, D. W., Cho, H. C., et al. (2016). DJ-1 protects the heart against ischemia–reperfusion injury by regulating mitochondrial fission. *J. Mol. Cell Cardiol.* 97, 56–66. doi: 10.1016/j.yjmcc.2016.04.008

Signorile, A., Santeramo, A., Tamma, G., Pellegrino, T., D'Oria, S., Lattanzio, P., et al. (2017). Mitochondrial cAMP prevents apoptosis modulating Sirt3 protein level and OPA1 processing in cardiac myoblast cells. *Biochim. Biophys Acta* 1864, 355–366. doi: 10.1016/j.bbamcr.2016.11.022

Song, M., Gong, G., Burelle, Y., Gustafsson, ÅB., Kitsis, R. N., Matkovich, S. J., et al. (2015a). Interdependence of parkin-mediated mitophagy and mitochondrial fission in adult mouse hearts. *Circ. Res.* 117, 346–351. doi: 10.1161/CIRCRESAHA.117.306859

Song, M., Mihara, K., Chen, Y., Scorrano, L., and Dorn, G. W. (2015b). Mitochondrial fission and fusion factors reciprocally orchestrate mitophagic culling in mouse hearts and cultured fibroblasts. *Cell Metab.* 21, 273–286. doi: 10.1016/j.cmet.2014.12.011

Soubannier, V., McLelland, G., Zunino, R., Braschi, E., Rippstein, P., Fon, E. A., et al. (2012). A vesicular transport pathway shuttles cargo from mitochondria to lysosomes. *Curr. Biol.* 22, 135–141. doi: 10.1016/j.cub.2011.11.057

Sprenger, H., and Langer, T. (2019). The good and the bad of mitochondrial breakups. *Trends Cell Biol.* 29, 888–900. doi: 10.1016/j.tcb.2019.08.003

Sugiura, A., McLelland, G. L., Fon, E. A., and McBride, H. M. (2014). A new pathway for mitochondrial quality control: mitochondrial-derived vesicles. *EMBO J.* 33, 2142–2156. doi: 10.15252/embj.201488104

Suliman, H. B., and Piantadosi, C. A. (2015). Mitochondrial quality control as a therapeutic target. *Pharmacol. Rev.* 68, 20–48. doi: 10.1124/pr.115.011502

Sun, L., Hao, Y., An, R., Li, H., Xi, C., and Shen, G. (2014). Overexpression of Rcan1-1L inhibits hypoxia-induced cell apoptosis through induction of mitophagy. *Mol. Cells* 37, 785–794. doi: 10.14348/molcells.2014.0103

Sundaresan, N. R., Bindu, S., Pillai, V. B., Samant, S., Pan, Y., Huang, J. Y., et al. (2015). SIRT3 blocks aging-associated tissue fibrosis in mice by deacetylating and activating glycogen synthase kinase 3beta. *Mol. Cell Biol.* 36, 678–692. doi: 10.1128/MCB.00586-15

Tahrir, F. G., Knezevic, T., Gupta, M. K., Gordon, J., Cheung, J. Y., Feldman, A. M., et al. (2017). Evidence for the role of BAG3 in mitochondrial quality control in cardiomyocytes. *J. Cell Physiol.* 232, 797–805. doi: 10.1002/jcp.25476

Tahrir, F. G., Langford, D., Amini, S., Mohseni, A. T., and Khalili, K. (2019). Mitochondrial quality control in cardiac cells: mechanisms and role in cardiac cell injury and disease. *J. Cell Physiol.* 234, 8122–8133. doi: 10.1002/jcp.27597

Takeo, N., Brian, T. W., and Choudhary, C. (2019). Functions and mechanisms of non-histone protein acetylation. *Nat. Rev. Mol. Cell Biol.* 20, 156–174. doi: 10.1038/s41580-018-0081-3

Tanaka, A., Cleland, M. M., Xu, S., Narendra, D. P., Suen, D., Karbowski, M., et al. (2010). Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin. *J. Cell Biol.* 191, 1367–1380. doi: 10.1083/jcb.201007013

Thai, P. N., Daugherty, D. J., Frederich, B. J., Lu, X., Deng, W., Bers, D. M., et al. (2018). Cardiac-specific conditional knockout of the 18-kDa mitochondrial translocator protein protects from pressure overload induced heart failure. *Sci. Rep.* 8:16213. doi: 10.1038/s41598-018-34451-2

Tong, M., Saito, T., Zhai, P., Oka, S., Mizushima, W., Nakamura, M., et al. (2019). Mitophagy is essential for maintaining cardiac function during high fat diet-induced diabetic cardiomyopathy. *Circ. Res.* 124, 1360–1371. doi: 10.1161/CIRCRESAHA.118.314607

Toyama, E. Q., Herzig, S., Courchet, J., Lewis, T. J., Loson, O. C., Hellberg, K., et al. (2016). Metabolism. AMP-activated protein kinase mediates mitochondrial fission in response to energy stress. *Science* 351, 275–281. doi: 10.1126/science.aab4138

Vakhrusheva, O., Smolka, C., Gajawada, P., Kostin, S., Boettger, T., Kubin, T., et al. (2008). Sirt7 increases stress resistance of cardiomyocytes and prevents apoptosis and inflammatory cardiomyopathy in mice. *Circ. Res.* 102, 703–710. doi: 10.1161/CIRCRESAHA.107.164558

Varanita, T., Soriano, M. E., Romanello, V., Zaglia, T., Quintana-Cabrera, R., Semenzato, M., et al. (2015). The opa1-dependent mitochondrial cristae remodeling pathway controls atrophic, apoptotic, and ischemic tissue damage. *Cell Metab.* 21, 834–844. doi: 10.1016/j.cmet.2015.05.007

Wang, B., Nie, J., Wu, L., Hu, Y., Wen, Z., Dong, L., et al. (2018). AMPKα2 protects against the development of heart failure by enhancing mitophagy via PINK1 phosphorylation. *Circ. Res.* 122, 712–729. doi: 10.1161/CIRCRESAHA.117.312317

Wang, H., Song, P., Du, L., Tian, W., Yue, W., Liu, M., et al. (2011). Parkin Ubiquitinates Drp1 for proteasome-dependent degradation. *J. Biol. Chem.* 286, 11649–11658. doi: 10.1074/jbc.M110.144238

Wang, L., Qi, H., Tang, Y., and Shen, H. (2019). Post-translational modifications of key machinery in the control of mitophagy. *Trends Biochem. Sci.* 45, 58–75. doi: 10.1016/j.tibs.2019.08.002

Wang, S., Zhao, Z., Fan, Y., Zhang, M., Feng, X., Lin, J., et al. (2019). Mst1 inhibits Sirt3 expression and contributes to diabetic cardiomyopathy through inhibiting Parkin-dependent mitophagy. *Biochim. Biophys. Acta* 1865, 1905–1914. doi: 10.1016/j.bbadis.2018.04.009

Wang, S., Zhao, Z., Feng, X., Cheng, Z., Xiong, Z., Wang, T., et al. (2018). Melatonin activates parkin translocation and rescues the impaired mitophagy activity of diabetic cardiomyopathy through Mst1 inhibition. *J. Cell Mol. Med.* 22, 5132–5144. doi: 10.1111/jcmm.13802

Wang, Z., Wang, S., Shao, Q., Li, P., Sun, Y., Luo, L., et al. (2019). Brain-derived neurotrophic factor mimetic, 7,8-dihydroxyflavone, protects against myocardial ischemia by rebalancing optic atrophy 1 processing. *Free Radical Biol. Med.* 145, 187–197. doi: 10.1016/j.freeradbiomed.2019.09.033

Wasiak, S., Zunino, R., and McBride, H. M. (2007). Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death. *J. Cell Biol.* 177, 439–450. doi: 10.1083/jcb.200610042

Watanabe, M., Funakoshi, T., Unuma, K., Aki, T., and Uemura, K. (2014). Activation of the ubiquitin–proteasome system against arsenic trioxide cardiotoxicity involves ubiquitin ligase Parkin for mitochondrial homeostasis. *Toxicology* 322, 43–50. doi: 10.1016/j.tox.2014.04.008

Wei, T., Huang, G., Gao, J., Huang, C., Sun, M., Wu, J., et al. (2017). Sirtuin 3 deficiency accelerates hypertensive cardiac remodeling by impairing angiogenesis. *J. Am. Heart Assoc.* 6:e006114. doi: 10.1161/JAHA.117.006114

Wilkinson, K. A., and Henley, J. M. (2010). Mechanisms, regulation and consequences of protein SUMOylation. *Biochem. J.* 428, 133–145. doi: 10.1042/BJ20100158

Winkler, R., Benz, V., Clemenz, M., Bloch, M., Foryst-Ludwig, A., Wardat, S., et al. (2012). Histone deacetylase 6 (HDAC6) is an essential modifier of glucocorticoid-induced hepatic gluconeogenesis. *Diabetes* 61, 513–523. doi: 10.2337/db11-0313

Wu, S., Lu, Q., Wang, Q., Ding, Y., Ma, Z., Mao, X., et al. (2017). Binding of FUN14 domain containing 1 with inositol 1,4,5-trisphosphate receptor in mitochondria-associated endoplasmic reticulum membranes maintains mitochondrial dynamics and function in hearts in vivo. *Circulation* 136, 2248–2266. doi: 10.1161/CIRCULATIONAHA.117.030235

Wu, W., Tian, W., Hu, Z., Chen, G., Huang, L., Li, W., et al. (2014). ULK1 translocates to mitochondria and phosphorylates FUNDC1 to regulate mitophagy. *EMBO Rep.* 15, 566–575. doi: 10.1002/embr.201438501

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., et al. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* 98, 115–124. doi: 10.1016/s0092-8674(00)80611-x

Xiong, W., Hua, J., Liu, Z., Cai, W., Bai, Y., Zhan, Q., et al. (2018). PTEN induced putative kinase 1 (PINK1) alleviates angiotensin II-induced cardiac injury by

ameliorating mitochondrial dysfunction. *Int. J. Cardiol.* 266, 198–205. doi: 10.1016/j.ijcard.2018.03.054

Youle, R. J., and van der Bliek, A. M. (2012). Mitochondrial fission, fusion, and stress. *Science* 337, 1062–1065. doi: 10.1126/science.1219855

Yu, J., Li, Y., Liu, X., Ma, Z., Michael, S., Orgah, J. O., et al. (2019). Mitochondrial dynamics modulation as a critical contribution for shenmai injection in attenuating hypoxia/reoxygenation injury. *J. Ethnopharmacol.* 237, 9–19. doi: 10.1016/j.jep.2019.03.033

Yu, T., Jhun, B. S., and Yoon, Y. (2011). High-glucose stimulation increases reactive oxygen species production through the calcium and mitogen-activated protein kinase-mediated activation of mitochondrial fission. *Antioxid. Redox Signal.* 14, 425–437. doi: 10.1089/ars.2010.3284

Yu, W., Gao, B., Li, N., Wang, J., Qiu, C., Zhang, G., et al. (2017). Sirt3 deficiency exacerbates diabetic cardiac dysfunction: role of Foxo3A-Parkin-mediated mitophagy. *Biochim. Biophys. Acta* 1863, 1973–1983. doi: 10.1016/j.bbadis.2016.10.021

Yu, W., Xu, M., Zhang, T., Zhang, Q., and Zou, C. (2019). Mst1 promotes cardiac ischemia–reperfusion injury by inhibiting the ERK-CREB pathway and repressing FUNDC1-mediated mitophagy. *J. Physiol. Sci.* 69, 113–127. doi: 10.1007/s12576-018-0627-3

Yue, W., Chen, Z., Liu, H., Yan, C., Chen, M., Feng, D., et al. (2014). A small natural molecule promotes mitochondrial fusion through inhibition of the deubiquitinase USP30. *Cell Res.* 24, 482–496. doi: 10.1038/cr.2014.20

Yue, Z., Ma, Y., You, J., Li, Z., Ding, Y., He, P., et al. (2016). NMNAT3 is involved in the protective effect of SIRT3 in Ang II-induced cardiac hypertrophy. *Exp. Cell Res.* 347, 261–273. doi: 10.1016/j.yexcr.2016.07.006

Zaja, I., Bai, X., Liu, Y., Kikuchi, C., Dosenovic, S., Yan, Y., et al. (2014). Cdk1, PKCδ and calcineurin-mediated Drp1 pathway contributes to mitochondrial fission-induced cardiomyocyte death. *Biochem. Biophys. Res.* 453, 710–721. doi: 10.1016/j.bbrc.2014.09.144

Zhang, Q., Wu, J., Wu, R., Ma, J., Du, G., Jiao, R., et al. (2012). DJ-1 promotes the proteasomal degradation of Fis1: implications of DJ-1 in neuronal protection. *Biochem. J.* 447, 261–269. doi: 10.1042/bj20120598

Zhang, Y., Wang, Y., Xu, J., Tian, F., Hu, S., Chen, Y., et al. (2019). Melatonin attenuates myocardial ischemia-reperfusion injury via improving mitochondrial fusion/mitophagy and activating the AMPK-OPA1 signaling pathways. *J. Pineal. Res.* 66:e12542. doi: 10.1111/jpi.12542

Zhao, D., Sun, Y., Tan, Y., Zhang, Z., Hou, Z., Gao, C., et al. (2018). Short-duration swimming exercise after myocardial infarction attenuates cardiac dysfunction and regulates mitochondrial quality control in aged mice. *Oxid. Med. Cell Longev.* 2018, 1–16. doi: 10.1155/2018/4079041

Zhao, T., Huang, X., Han, L., Wang, X., Cheng, H., Zhao, Y., et al. (2012). Central role of mitofusin 2 in autophagosome-lysosome fusion in cardiomyocytes. *J. Biol. Chem.* 287, 23615–23625. doi: 10.1074/jbc.M112.379164

Zhou, H., Li, D., Zhu, P., Hu, S., Hu, N., Ma, S., et al. (2017). Melatonin suppresses platelet activation and function against cardiac ischemia/reperfusion injury via PPARγ/FUNDC1/mitophagy pathways. *J. Pineal. Res.* 63:e12438. doi: 10.1111/jpi.12438

Zhou, H., Wang, J., Zhu, P., Zhu, H., Toan, S., Hu, S., et al. (2018). NR4A1 aggravates the cardiac microvascular ischemia reperfusion injury through suppressing FUNDC1-mediated mitophagy and promoting Mff-required mitochondrial fission by CK2α. *Basic Res. Cardiol.* 113:23. doi: 10.1007/s00395-018-0682-1

Zhou, L., Zhang, Q., Zhang, P., Sun, L., Peng, C., Yuan, Z., et al. (2017). c-Abl-mediated Drp1 phosphorylation promotes oxidative stress-induced mitochondrial fragmentation and neuronal cell death. *Cell Death Dis.* 8:e3117. doi: 10.1038/cddis.2017.524

Zhou, W., Chen, K., Cao, W., Zeng, J., Liao, H., Zhao, L., et al. (2010). Mutation of the protein kinase A phosphorylation site influences the anti-proliferative activity of mitofusin 2. *Atherosclerosis* 211, 216–223. doi: 10.1016/j.atherosclerosis.2010.02.012

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 27 March 2020
doi: 10.3389/fphys.2020.00228

Slit2 Protects Hearts Against Ischemia-Reperfusion Injury by Inhibiting Inflammatory Responses and Maintaining Myofilament Contractile Properties

Xiang Li[1†], Shuang Zheng[1,2†], Weijiang Tan[1†], Hongqi Chen[1†], Xiaohui Li[3], Jian Wu[4], Ting Luo[1], Xuecong Ren[1], W. Glen Pyle[5], Lijing Wang[2], Peter H. Backx[6,7], Ren Huang[1]* and Feng Hua Yang[1,3]**

[1] Guangdong Province Key Laboratory of Laboratory Animals, Cardiovascular Model Research Center, Guangdong Laboratory Animals Monitoring Institute, Guangzhou, China, [2] School of Basic Medicine, Vascular Biology Institute, Guangdong Pharmaceutical University, Guanghzou, China, [3] Department of Cardiovascular Surgery, The First Affiliated Hospital, Jinan University, Guangzhou, China, [4] Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital and Institutes of Biomedical Sciences, Fudan University, Shanghai, China, [5] Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada, [6] Department of Biology, York University, Toronto, ON, Canada, [7] Division of Cardiology and the Peter Munk Cardiac Centre, University Health Network, Toronto, ON, Canada

Background: The secreted glycoprotein Slit2, previously known as an axon guidance cue, has recently been found to protect tissues in pathological conditions; however, it is unknown whether Slit2 functions in cardiac ischemia–reperfusion (IR) injury.

Methods: Langendorff-perfused isolated hearts from Slit2-overexpressing (Slit2-Tg) mice and C57BL/6J mice (background strain) were subjected to 20 min of global ischemia followed by 40 min of reperfusion. We compared Slit2-Tg with C57BL/6J mice in terms of left ventricular function and infarct size of post-IR hearts along with tissue histological and biochemical assessments (mRNA and protein expression, phosphorylation status, and myofilament contractile properties).

Results: Slit2 played cardioprotective roles in maintaining contractile function and reducing infarct size in post-IR hearts. IR increased the expression of the Slit2 receptor Robo4 and the membrane receptor Slamf7, but these increases were suppressed by Slit2 overexpression post IR. This suppression was associated with inhibition of the nuclear translocation of NFκB p65 and reductions in IL-1β and IL-18 release into perfusates. Furthermore, Slit2 overexpression attenuated the increases in myofilament-associated PKCs and phosphorylation of cTnI at Ser43 in the post-IR myocardium. The myofilament calcium sensitivity and actomyosin MgATPase activity were preserved in the post-IR Slit2 myocardium.

Conclusion: Our work demonstrates that Slit2 inhibits inflammatory responses and maintains myofilament contractile properties, thus contributing, at least in part, to the prevention of structural and functional damage during IR.

Keywords: ischemia-reperfusion injury, contractile function, RNA sequencing, myofilament phosphorylation, inflammatory response, cTnI, Slit2, protein kinase C

OPEN ACCESS

Edited by:
Mark J. Ranek, Johns Hopkins University, United States

Reviewed by:
James William McNamara, University of Cincinnati, United States
Margaret Westfall, University of Michigan, United States
Brian Leei Lin, Johns Hopkins University, United States

***Correspondence:**
Feng Hua Yang
fenghua.yang@gdlami.com
Peter H. Backx
pbackx@yorku.ca
Ren Huang
1649405216@qq.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *26 June 2019*
Accepted: *27 February 2020*
Published: *27 March 2020*

Citation:
Li X, Zheng S, Tan W, Chen H, Li X, Wu J, Luo T, Ren X, Pyle WG, Wang L, Backx PH, Huang R and Yang FH (2020) Slit2 Protects Hearts Against Ischemia-Reperfusion Injury by Inhibiting Inflammatory Responses and Maintaining Myofilament Contractile Properties. Front. Physiol. 11:228. doi: 10.3389/fphys.2020.00228

INTRODUCTION

WHO statistics indicate that the annual deaths from ischemic heart disease were ∼8.9 million worldwide in 2017 (GBD 2017; Causes of Death Collaborators, 2018), despite the availability of various drugs and other therapeutic approaches that have been developed to tackle this epidemic condition (Hausenloy and Yellon, 2013). Reestablishment of blood flow to previously ischemic tissues during treatment results in tissue and functional damage called ischemia–reperfusion (IR) injury (Murphy and Steenbergen, 2008; Prasad et al., 2009). Inflammatory response is the primary mechanism of IR injury, in which reperfusion triggers leukocyte recruitment and reactive oxygen species (ROS) production in endothelial cells. Subsequently, transcriptional factors such as NFκB activate the expression of proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-18 (Barnes and Karin, 1997; Kawaguchi et al., 2011; Burma et al., 2014; Lu et al., 2016). Blockade of leukocytes' adherence to endothelial cells significantly reduces the infarct size in the IR myocardium of cats (Ma et al., 1992), and inhibition of NFκB reduces neutrophil activation and cell death in IR rabbit hearts (Yeh et al., 2005).

IR depresses the contractile function of the heart. Cardiac contractile function is executed by myofilaments, including thin filaments [actin, tropomyosin, and the troponin complex of cardiac troponin C (cTnC), cardiac troponin I (cTnI), and cardiac troponin T (cTnT)] and thick filaments [myosin heavy and light chains, myosin binding protein C (MyBP-C), and titin] (van der Velden and Stienen, 2019) and regulated by the balance of myofilament phosphorylation (Metzger and Westfall, 2004; Verduyn et al., 2007; Kooij et al., 2010). Among the phosphorylation sites of myofilaments, Ser23/24 and Ser43/45 of cTnI are the most investigated sites. Ser23/24 and Ser43/45 are preferentially phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) isoforms, respectively (Jideama et al., 1996). Phosphorylation of Ser23/24 accelerates the relaxation rate (Takimoto et al., 2004), and phosphorylation of Ser43/45 reduces maximum ATPase activity (Noland et al., 1995). Protein kinase signaling has been explored as a therapeutic target for improvement of cardiac function in heart disease (Singh et al., 2017).

Slit2 is one of three Slit family members that are secreted from the extracellular matrix and expressed in many tissues, including the hearts (Dickson and Gilestro, 2006). Slit2 can exist in a full-length or in a cleaved form of an NH2-terminal fragment or a COOH-terminal fragment (Nguyen Ba-Charvet et al., 2001). Slit2 is a ligand of the Robo family of immunoglobulin receptors (Robo1–4). The Slit2 isoform, similar to its family members, guides heart tube formation during development (Wong et al., 2002), but the roles of Slit2 in adult hearts are unknown. Evidence has shown that Slit2 protects several organs and tissues through anti-inflammatory mechanisms. For example, Ye et al. (2010) reported that Slit2 suppresses chemotaxis of neutrophils to inhibit lung inflammation in mice. In addition, Zhao et al. (2014) showed that Robo4, a ligand of Slit2, inhibits lipopolysaccharide-induced expression of the cytokine and cell adhesion molecule ICAM-1 in human microvascular endothelial cells. Furthermore, Slit2 inhibits neutrophil and macrophage infiltration in response to IR and reduces renal tubular necrosis in mice (Chaturvedi et al., 2013). As the anti-inflammatory mechanisms of Slit2 that occur during other organ stresses, particularly renal IR, are similar to those that occur during cardiac IR, we hypothesized that Slit2 suppresses inflammatory responses in the IR myocardium.

Slit2 regulates intracellular PKC signaling and plays key roles in development and physiological function. PKC is recruited to colocalize with Robo3 in murine brain regions that govern learning, memory, and reward (Samelson et al., 2015). Increases in Slit2/Robo1 expression caused by prostaglandin F2 are PKC-dependent during murine luteolysis (Zhang et al., 2013). In addition, PKC-dependent substrate phosphorylation is required for F-actin function in murine commissural growth cones in response to Slit2 (Shen et al., 2002). However, under pathological conditions, Slit2-associated PKC activation might exert detrimental effects. For example, treatment with phospholipase C (PLC), the upstream protein of PKC (Essen et al., 1997), impairs Slit2-induced angiogenesis in cultured endothelial cells (Dunaway et al., 2011). In the myocardium, PKC includes more than 10 isoenzymes that might play beneficial or detrimental roles upon activation (Steinberg, 2012). For example, PKCδ contributes to ATP depletion, ROS accumulation, apoptosis, and infarction during cardiac IR (Inagaki et al., 2003a; Murriel et al., 2004), while treatment with a PKCδ inhibitor reduces infarct size and improves the functions of reperfused swine hearts (Inagaki et al., 2003a). Additionally, PKCε, another intensively investigated isoform, shows either protective or undetectable effects during the reperfusion period (Inagaki et al., 2003b). These results suggest that Slit2 might regulate PKC-dependent contractile function during IR.

In this study, we aimed to explore the effects of Slit2 and its regulatory mechanisms regarding anti-inflammatory responses and myofilament phosphorylation in the post-IR myocardium. Understanding the regulatory mechanisms of Slit2 will provide a novel therapeutic strategy for combating IR injury.

MATERIALS AND METHODS

Animals

C57BL/6 mice overexpressing the Slit2 gene (Slit2-Tg) were originally generated by the Geng Laboratory (University of Michigan, United States) (Yang et al., 2010). Briefly, full-length human Slit2 cDNA was cloned into a pCEP4F vector containing the CMV promoter, and then injected into the pronuclei of fertilized C57 × CBA F1 oocytes to construct transgenic mice. The genotypes were confirmed by Southern blotting and PCR analysis. PCR screening of Slit2 heterozygotes was performed using a pair of primers specific for Slit2 cDNA (forward: 5′-CCCTCCGGATC CTTTACCTGTCAAGGTCCT-3′; reverse: 5′-TGGAGAGAGCTCACAGAACAAGCCACTGTA-3′). Slit2-Tg mice and C57BL/6J (background strain) mice were bred and maintained in a specific pathogen-free (SPF), AAALAC-accredited facility of the Guangdong Laboratory Animals Monitoring Institute, Guangzhou, China. The temperature and humidity were 24 ± 2°C and 40–60%, respectively, and the

light cycle included 12 h of light and 12 h of dark. Three- to five-month-old male mice were used in this study. All animal experiments were approved by the Institutional Animal Care and Use Committee (No. IACUC2017002).

Langendorff Heart Preparation and Perfusion Protocol

To determine the effects of Slit2 on myocardial IR injury, a Langendorff heart perfusion system for rodents (Radnoti LLC, United States) equipped with a pressure transducer was used (Yang and Pyle, 2012). Briefly, mice were euthanized with isoflurane, and their hearts were excised. The aortas were then cannulated and the hearts were perfused using a modified Krebs-Henseleit (KH) buffer solution (37°C) containing (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 0.05 EDTA, 0.5 sodium pyruvate, and 11 glucose. The KH buffer was continuously gassed with 95% O_2/5% CO_2, and the perfusion pressure was maintained at 70 mmHg. A water-filled balloon connected to the pressure transducer was inserted into the left ventricle, and left ventricular pressures were recorded with a PowerLab 16/35 system (AD Instruments Inc., United States). Hemodynamic parameters, including the left ventricular developed pressure (LVDP), heart rate (HR), left ventricular end diastolic pressure (LVEDP), rate of pressure product (RPP), and minimum and maximum rates of left ventricular pressure development [dP/dt(min) and dP/dt(max), respectively] were analyzed with Chart 7 Software (ADInstruments Inc., United States).

For the IR protocol, hearts were subjected to 20 min of stable perfusion, followed by 20 min of no-flow global ischemia and 40 min of reperfusion. Control hearts were continuously perfused for 80 min (**Figure 1A**). At the end of the experiment, some heart tissues were fixed for hematoxylin–eosin (HE) staining, transmission electron microscopy (TEM), and immunofluorescence staining, and some were flash frozen in liquid nitrogen for subsequent RNA sequencing analysis, RT-qPCR, Western blotting, Pro-Q staining, and actomyosin MgATPase activity assay.

Myocardial Infarct Size

To determine post-IR myocardial infarct size, hearts were subjected to TTC staining. Briefly, the frozen tissues were sliced into 2 mm thick sections and incubated in a 1% TTC (Sigma-Aldrich, United States) solution at 37°C for 15 min and in 10% neutral formalin for 1 h. TTC stained the viable areas red, while the unstained areas (white) were infarcted tissue. The infarct size in the myocardial tissue was measured using ImageJ software (NIH, United States), and the infarct size (%) was calculated as a percentage of the total section area. Different technicians performed the sectioning and observations, and the technicians did not know the individual identities of the samples.

HE Staining

Hearts were fixed in 10% neutral formalin and subjected to HE staining, which was used to detect necrosis of heart tissues. First, the hearts were embedded in paraffin, sliced into 3 μm sections, and dewaxed with dimethylbenzene and dehydrated with gradient ethanol solutions. Next, the sections were stained with hematoxylin for 10 min and soaked in 1% hydrochloric acid–ethanol for 2 s. The sections were then stained with alcohol-soluble eosin for 25 s. Finally, the sections were sealed with neutral gum and observed under an optical microscope (Leica DM 2000, Germany). During evaluation of the histological changes, different technicians performed the sectioning and observations, and the technicians did not know the sample IDs.

Transmission Electron Microscopy

TEM was further used to determine the subcellular changes associated with Slit2 in the post-IR myocardium. Left ventricular tissues were cut into small blocks (about 1 mm^3), fixed with 2.5% glutaraldehyde, fixed with 1% OsO_4, dehydrated in ethanol, and embedded in Araldite. The tissue blocks were cut into slices at a thickness of 60 nm using a Leica cryostat system (EM UC7/FC7, Germany) and collected on copper grids. The ultrathin sections were double stained with 3% uranyl acetate and lead citrate. The subcellular structure was observed with a Tecnai G2 Spirit transmission electron microscope (FEI Company, United States). During evaluation of the subcellular differences between the groups, different technicians performed the slice preparation and observations, and the technicians did not know the sample IDs.

RNA Sequencing and Raw Data Processing

Total RNA was isolated using TRIzol® Reagent (Thermo Fisher Scientific, United States), and RNA concentrations were determined using a Qubit® 2.0 Fluorometer and an assay kit (Life Technologies, United States). RNA integrity was assessed using an RNA Nano 6000 Assay Kit with a Bioanalyzer 2100 system (Agilent Technologies, United States). Sequencing libraries were generated using an NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, United States). After adding index codes, the samples were clustered with a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina, United States). An Illumina HiSeq platform (Illumina, United States) was employed to sequence the libraries, generating 125 bp/150 bp paired-end reads. Raw data with proper paired-end clean reads were mapped for further analysis. The original reads of the genes for the samples in the four groups (the C57-non-IR, C57-IR, Slit2-non-IR, and Slit2-IR groups) were compared in pairs, and the fold changes in the expressed genes between the groups were log2 transformed. A P value < 0.05 was the cutoff for identification of differentially expressed genes (DEGs). Furthermore, DEGs with log2 (fold change) values > 0 were considered upregulated, while those with log2 (fold change) values < 0 were considered downregulated. Thus, we defined eight datasets of DEGs: an upregulated dataset and a downregulated dataset each for the C57-IR vs. C57-non-IR, Slit2-IR vs. Slit2-non-IR, Slit2-non-IR vs. C57-non-IR, and Slit2-IR vs. C57-IR comparisons. The gene expression profiles are presented using volcano plots (**Supplementary Figure S3**).

The raw RNA-seq data have been deposited in NCBI's Gene Expression Omnibus (GEO) (#GSE133796)[1].

Target Gene Prediction and Gene Set Enrichment Analysis (GSEA)

First, to identify genes regulated by Slit2 in post-IR hearts, five datasets of DEGs (the downregulated DEGs in the C57-IR vs. C57-non-IR comparison, the upregulated DEGs in the C57-IR vs. C57-non-IR upregulated, the downregulated DEGs in the Slit2-IR vs. C57-IR comparison, the upregulated DEGs in the Slit2-IR vs. C57-IR comparison, and the up-/downregulated DEGs in the Slit2-non-IR vs. C57-non-IR comparison) were input into the TBtools program[2] to generate a Venn diagram. By analyzing this diagram, two final intersections were obtained: (1) the intersection of the upregulated DEGs in the C57-non-IR vs. C57-IR comparison and the downregulated DEGs in the Slit2-IR vs. C57-IR comparison, and (2) the intersection of the downregulated DEGs in the C57-IR vs. C57-non-IR comparison and the upregulated DEGs in the Slit2-IR vs. C57-IR comparison. These two final intersections further excluded the up-/downregulated DEGs in theSlit2-non-IR vs. C57-non-IR comparison. We therefore identified two new sets of DEGs: one set was upregulated by IR in C57BL/6J hearts but downregulated by Slit2 overexpression in post-IR hearts, and another was downregulated by IR in C57BL/6J hearts but upregulated by Slit2 overexpression in post-IR hearts. The Venn diagram results are presented in **Figures 4B,C**.

Second, to predict the effects of Slit2 on cardiac function, the GSEA tool Metascape (Zhou et al., 2019) was used to construct protein interaction networks of gene sets. Briefly, the enrichment gene sets (Biological Processes, KEGG pathways, or Reactome pathways) with similar functions were clustered, and the identification algorithm "MCODE" revealed that these enrichment clusters formed interaction networks. The interaction network of the Slit2-non-IR vs. C57-non-IR suggested the effects of Slit2 on the molecular function of the heart (**Figures 3A,B**), and the interaction network of the Slit2-IR vs. C57-IR suggested the effects of Slit2 on molecular function in post-IR hearts (**Figures 3C,D**).

RT-qPCR

RT-qPCR was used to verify the expression of genes identified by the Venn diagram analysis and to determine the expression of Robo receptors. Total RNA extracted from the left ventricle was subjected to reverse transcription and quantitative PCR. The primers are listed in **Supplementary Table S1**). TB Green® Premix Ex Taq™ II (Takara Bio Inc., Japan) was used. The program was as follows: 95°C for 30 s (1 cycle); 95°C for 5 s, 60°C for 34 s (40 cycles); and 72°C for 10 min (1 cycle). Gene expression levels were normalized to GAPDH levels, and the results were expressed as the ratio of the expression in each experimental group to that in the C57-non-IR group.

[1]https://wwwn.cbi.nlm.nih.gov/geo/

[2]https://omictools.com/tbtools-tool

Immunofluorescence Staining

Immunofluorescence detection of Slamf7 and nuclear translocation of NFκB were used to assess inflammatory responses. Heart tissues were fixed in 10% neutral formalin, cryoprotected in 30% sucrose for 24 h, embedded in optimal cutting temperature (OCT) compound, and sectioned with a freezing microtome at a thickness of 5 μm (Leica CM1950, Germany). The heart sections were processed using a standard immunostaining protocol. After routine hydration, the sections were subjected to permeabilization with PBS containing 0.05% Triton X-100 followed by incubation with a primary antibody against Slamf7 (A5782, ABclonal, China) or NFκB p65 (8242S, CST, United States) at 4°C overnight. The primary antibodies were visualized using Alexa Fluor 594- and Alexa Fluor 488-conjugated secondary antibodies (Invitrogen, CA). The sections were photographed under a confocal microscope (Leica TCS SP5, Germany). The technicians performing immunofluorescent staining and observations were different and did not know the sample ID.

Enzyme-Linked Immunosorbent Assay (ELISA)

Heart perfusates were collected at the perfusion baseline and 0, 5, 10, and 40 min after reperfusion. ELISA kits (Cusabio Biotech Co., China) were used to test the levels of the proinflammatory cytokines IL-1β and IL-18 in the perfusates according to the manufacturer's instructions. Briefly, 100 μl of the standard or sample was added to each well, and the plates were incubated for 2 h at 37°C. After removing the liquid, 100 μl/well of anti-biotin antibody was added, and the plates were incubated for 1 h at 37°C before being incubated with 100 μl of HRP-avidin and 90 μl of TMB substrate for 25 min at 37°C in the dark. The optical density was measured within 5 min using a microplate reader at 450 nm.

Myofilament Isolation for Western Blotting, Myofilament Phosphorylation Analysis, and Actomyosin MgATPase Activity Assay

Myofilaments were extracted according to a modified protocol from Yang et al. (2008) and Yang and Pyle (2011, 2012). Briefly, hearts were homogenized in ice-cold lysis buffer composed of (in mM) 60 KCl, 30 imidazole (pH 7.0), 2 $MgCl_2$, 0.01 leupeptin, 0.1 phenylmethylsulfonyl fluoride (PMSF), and 0.2 benzamidine and then centrifuged at 12,000 × *g* and 4°C for 15 min. The pellets were extracted with ice-cold lysis buffer containing 1% Triton X-100 and centrifuged at 1,100 × *g* to obtain myofilaments. The protein concentrations of the myofilaments were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, United States).

Western Blotting

First, cytosolic fractions extracted from cardiac tissues were used to detect the expression of protein-encoding genes selected from RNA-seq analyses, Slit2 and the Slit2 fragment. Briefly, hearts were homogenized with a cell lysis buffer (#9803, CST,

United States) containing protease inhibitors and 1% Triton X-100. The samples were centrifuged at 12,000 × *g* and 4°C for 15 min, and the supernatants were retained for use. Primary antibodies against Slamf7 (A5782, ABclonal, China), RasGRP1 (sc-365358, Santa Cruz, United States), Slit2 (ab7665, Abcam, United States), and Slit2-C (BM5312, Bostern, China) were used. Second, myofilament fractions extracted from cardiac tissues were used to determine myofilament-associated PKCs, PKA, protein phosphatases (PPs), and phosphorylation sites of cardiac myofilaments. Primary antibodies against PKCδ (2058S, CST, United States), PKCε (2683S, CST, United States), PKA (4782S, CST, United States), PP1α (2582S, CST, United States), PP type 2A (PP2A; 2038S, CST, United States), p-cTnISer43 (PA5-35412, Invitrogen, CA), p-cTnISer23/24 (4004S, CST, United States), Slit2 (ab7665, Abcam, United States), Slit2-C (BM5312, Bostern, China), GAPDH (2118S, CST, United States), and actin (MAB1501R, Millipore, United States) were used.

Cytosolic (40 μg) or myofilament (60 μg) proteins were separated using 10% SDS-PAGE and transferred to PVDF blotting membranes (Millipore, United States). After blocking with 5% skim milk, the membranes were incubated with primary antibodies (as listed above) overnight at 4°C before being incubated with species-appropriate secondary antibodies (CST, United States). The bands were detected with Immobilon Western chemiluminescent HRP substrate (Millipore, United States). The densities of the bands were analyzed using ImageJ software (NIH, United States).

Myofilament Phosphorylation

The phosphorylation levels of myofilament proteins (MyBP-C, desmin, cTnT, and cTnI) were measured using the Pro-Q Diamond phosphorylation gel staining method, as previously described (Yang and Pyle, 2011, 2012). Briefly, myofilaments (60 μg) were separated using 12% SDS-PAGE. After fixation with 50% methanol/10% acetic acid for 30 min, the gels were incubated with Pro-Q Diamond phosphorylation gel staining solution (Molecular Probes, United States) for 90 min. The phosphorylated bands were visualized using a Gel Doc^{TM} XR + gel documentation system (Bio-Rad Laboratories Ltd., United States), and the band densities were quantified using ImageJ (NIH, United States). Total protein was stained with Coomassie solution, and actin bands were used as the loading controls.

Actomyosin MgATPase Activity

An actomyosin MgATPase activity assay was performed as described previously (Yang et al., 2008; Yang and Pyle, 2011, 2012) to assess myofilament function. Briefly, myofilaments (50 μg) were incubated in reaction solutions containing various concentrations of free calcium. The free calcium concentration in each solution was calculated using a Patton assay program (Patton et al., 2004). The myofilaments were incubated in the reaction solutions for 5 min at 32°C. After the reactions were stopped with 10% trichloroacetic acid, the amount of inorganic phosphate produced was measured after adding 0.5% $FeSO_4$ and 0.5% ammonium molybdate in 0.5 M H_2SO_4 at 630 nm using a spectrophotometer (Biochrom, United Kingdom).

Statistical Analysis

All experimental data are presented as the mean ± SEM. Statistical differences between two groups were analyzed with unpaired Student's *t*-tests, and differences among multiple groups of data were analyzed by two-way ANOVA followed by Tukey's multiple comparisons test (GraphPad Prism 7, United States). $P < 0.05$ were considered to indicate statistical significance.

RESULTS

Slit2 Ameliorates Myocardial Decline and Prevents Cardiac Damage in the Post-IR Myocardium

First, we examined cardiac function in all groups. There were no significant differences in cardiac function (HR, LVDP, and RPP) between C57BL/6J and Slit2-Tg mice at baseline (0–20 min, **Figures 1C–E**). However, following 20 min of ischemia, HR, LVDP, RPP, and dP/dt(max) were significantly higher and dP/dt(min) was significantly lower in Slit2-Tg mice than in C57BL/6J mice at the time points of 60, 65, 70, 75, and 80 min (**Figures 1C–H**). These improvements in contractility were associated with lower LVEDP values at the above time points in post-IR Slit2-Tg hearts compared to control hearts (**Figure 1H**, **Table 1**, and **Supplementary Figure S2**) as well as with markedly reduced infarct size in post-IR Slit2-Tg hearts (**Figures 1I,J**). These results establish that Slit2 protein overexpression significantly improves cardiac systolic and diastolic function following IR and reduces infarct area. The functional parameters are presented in **Table 1**. Next, we explored the effects of Slit2 on cardiac cellular damage post IR. Under a microscope, we found focal necrosis, some vacuoles, and swollen and dissolved myofibers in cardiac tissues of post-IR C57BL/6J mice (**Figure 2A**). However, Slit2 overexpression reduced the IR-induced myofiber swelling and vacuole formation between fibers seen in C57BL/6J hearts (**Figure 2A**). We further employed TEM technology to examine the subcellular structure in post-IR hearts. We found that reperfused C57BL/6J hearts displayed edematous mitochondria, fractured internal crests of mitochondria, ruptured myofibrils, and ruptured Z-discs in cardiomyocytes (**Figure 2B**). Compared with C57BL/6J hearts, Slit2-Tg hearts had less mitochondrial edema and better myofilament maintenance and Z-line morphology (**Figure 2B**) after reperfusion. The results from HE staining and TEM examination demonstrate that Slit2 reduces tissue and cellular damage in post-IR hearts.

Slit2 Shifts Gene Expression Profiles to Boost the Regulation of Cardiac Contraction and Reduce Inflammation in Normal and Post-IR Slit2 Hearts

Slit2 mRNA and protein levels were significantly higher in Slit2-Tg hearts than in C57BL/6J (background strain) hearts, but there were no functional or histological differences (**Supplementary Figure S1** and **Supplementary Table S2**). The overall gene expression profiles are displayed in

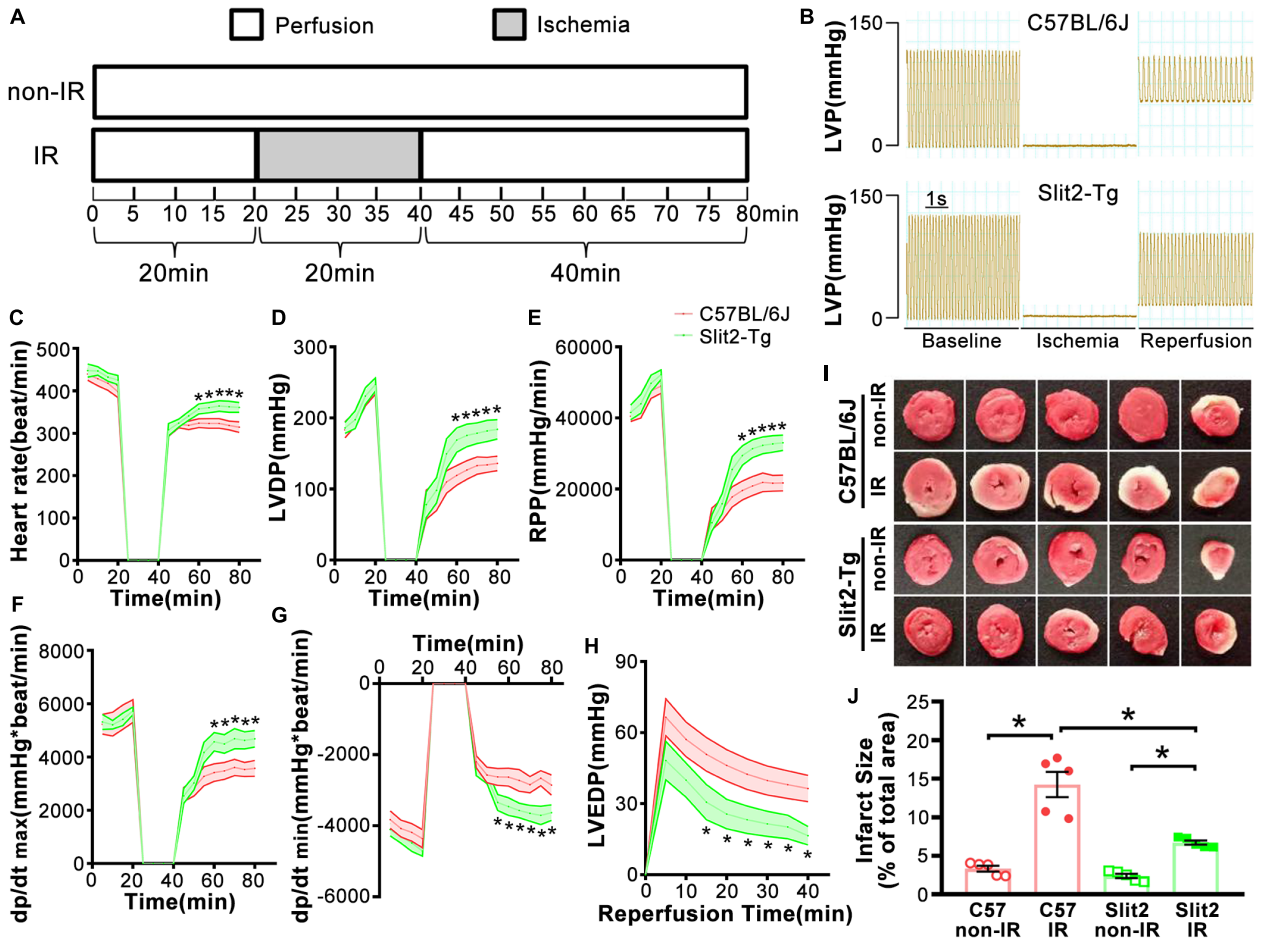


FIGURE 1 | Effects of Slit2 on post-ischemia–reperfusion (IR) cardiac function. **(A)** Perfusion protocol. All hearts were perfused for 20 min for equilibration. The IR groups were subjected to 20 min of global ischemia and 40 min of reperfusion, while the non-IR groups were perfused continuously for 80 min without global ischemia. **(B)** Representative traces of left ventricular pressure (LVP) for C57BL/6J (top) and Slit2-Tg (bottom) hearts during IR. Functional measurements of C57BL/6J and Slit2-Tg hearts were taken. **(C–H)** Heart rate, left ventricular developed pressure (LVDP), rate pressure product (RPP), dP/dt(max), dP/dt(min), and left ventricular end-diastolic pressure (LVEDP) values. $n = 7$ mice per group. The values are the means ± SEMs; $^{*}P < 0.05$ vs. C57BL/6J hearts (unpaired Student's *t*-test). **(I)** Representative images of serial TTC-stained sections of C57BL/6J and Slit2-Tg hearts subjected or not subjected to IR. **(J)** Quantitative analysis of the infarct area in each group. $n = 5$ mice per group. All data are presented as the mean ± SEM; $^{*}P < 0.05$ between the groups (two-way ANOVA, Tukey's multiple comparisons test).

TABLE 1 | Hemodynamic analysis of Slit2-Tg and C57BL/6J mouse hearts after IR.

	HR (beats/min)	LVDP (mmHg)	dP/dt(max) (mmHg/s)	RPP (mmHg*beats/min)	LVEDP (mmHg)
C57-non-IR	336.4 ± 13.7	122.7 ± 11.87	5, 821 ± 326	40, 447 ± 3, 004	0.610 ± 0.60
Slit2-non-IR	360.2 ± 11.1	119.0 ± 4.20	5, 490 ± 303	41, 518 ± 2, 067	1.184 ± 0.86
C57-IR	314.3 ± 12.4	68.02 ± 5.08*	3, 574 ± 195*	21, 680 ± 2, 235*	36.42 ± 5.57*
Slit2-IR	361.1 ± 11.7#	92.01 ± 6.88*#	4, 663 ± 305*#	32, 885 ± 2, 115*#	16.50 ± 3.91*#

*n = 7. All data are presented as the mean ± SEM. *P < 0.05 vs. non-IR and #P < 0.05 vs. C57-IR (two-way ANOVA, Tukey's multiple comparisons test). HR, heart rate; LVDP, left ventricular developed pressure; dp/dt(max), maximum increase rate of LV pressure; RPP, rate pressure product; LVEDP, left ventricular end-diastolic pressure.*

Supplementary Figure S3 and **Supplementary Table S4**. Furthermore, the RNA sequencing data revealed that multiple molecular functions were altered in Slit2-Tg hearts (**Figure 3**). Slit2 overexpression boosted regulation of cell adhesion, protein kinase B (Akt) signaling, the response to hypoxia, organic acid catabolic processes, and the response to oxidative stress (**Figure 3A** and **Supplementary Table S3**). Slit2 reduced positive regulation of glycogen metabolic processes, the PPAR signaling pathway, regulation of the inflammatory response, and regulation of the release of cytochrome C from mitochondria (**Figure 3B** and **Supplementary Table S3**). These results suggest that hearts overexpressing Slit2 are sensitive to external

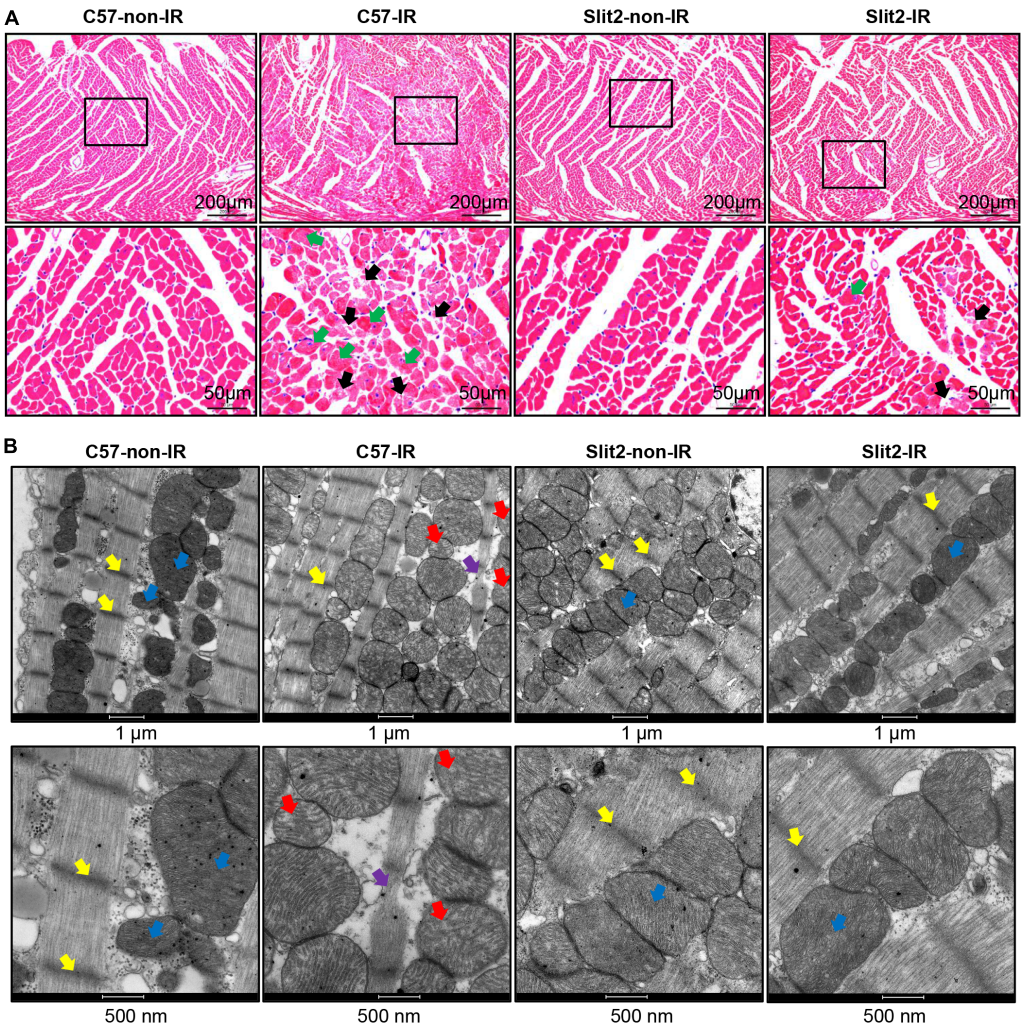


FIGURE 2 | Post-ischemia–reperfusion (IR) injury in C57BL/6J and Slit2-Tg hearts. **(A)** Representative images of hematoxylin–eosin (HE) staining in C57BL/6J and Slit2-Tg hearts. Scale bar = 200 μm (top) or 50 μm (bottom). The bottom panels show the magnified views of the black framed areas of the corresponding upper panels. The black framed area of the C57-IR heart section displays necrosis. The black arrows indicate ruptured cardiomyocytes; the green arrows indicate swollen cardiomyocytes. **(B)** Subcellular structures of cardiomyocytes as examined by TEM in C57BL/6J and Slit2-Tg hearts. Scale bar = 1 μm (top) or 500 nm (bottom). C57-non-IR, C57BL/6J hearts not subjected to IR; Slit2-non-IR, Slit2-Tg hearts not subjected to IR; C57-IR, C57BL/6J hearts subjected to IR; Slit2-IR, Slit2-Tg hearts subjected to IR. Arrows: yellow, Z-discs; blue, normal mitochondria; red, post-IR mitochondria with disappearing crista; purple, ruptured Z-discs.

stimuli, while they resist inflammatory insults and might exhibit improved cell survival.

In hearts with post-IR injury, Slit2 enhanced collagen chain trimerization, regulation of ion transport, regulation of system processes, responses to mechanical stimuli, potassium ion transport, and ion homeostasis (**Figure 3C** and **Supplementary Table S3**). Moreover, Slit2 specifically protected cardiac contractile function in the post-IR myocardium, as observed from the enrichment of the "regulation of system process" term: a cluster of gene sets upregulated in post-IR Slit2 hearts was enriched for muscle contraction, regulation of HR, regulation of heart contraction, heart contraction, actin-mediated cell contraction, regulation of blood circulation, and adrenergic signaling in cardiomyocytes (**Figure 3C** and **Supplementary Table S3**). In addition, Slit2 reduced regulation of immune effector processes, the inflammatory response, the response to interferon-gamma, monocyte chemotactic protein-1 production, and regulation of leukocyte activation in the post-IR myocardium (**Figure 3D** and **Supplementary Table S3**). These results suggest that Slit2 maintains cardiac contractile function and extracellular structure and prevents inflammatory responses during IR.

Slit2 Regulates Robo1, Robo4, and Slamf7 and Inhibits Inflammatory Responses in the Post-IR Myocardium

In exploring the intracellular targets of Slit2 in the protected myocardium, we identified 385, 646, 112, 183, and 431 DEGs from among the downregulated DEGs in the C57-IR vs. C57-non-IR comparison, the upregulated DEGs in the C57-IR vs.

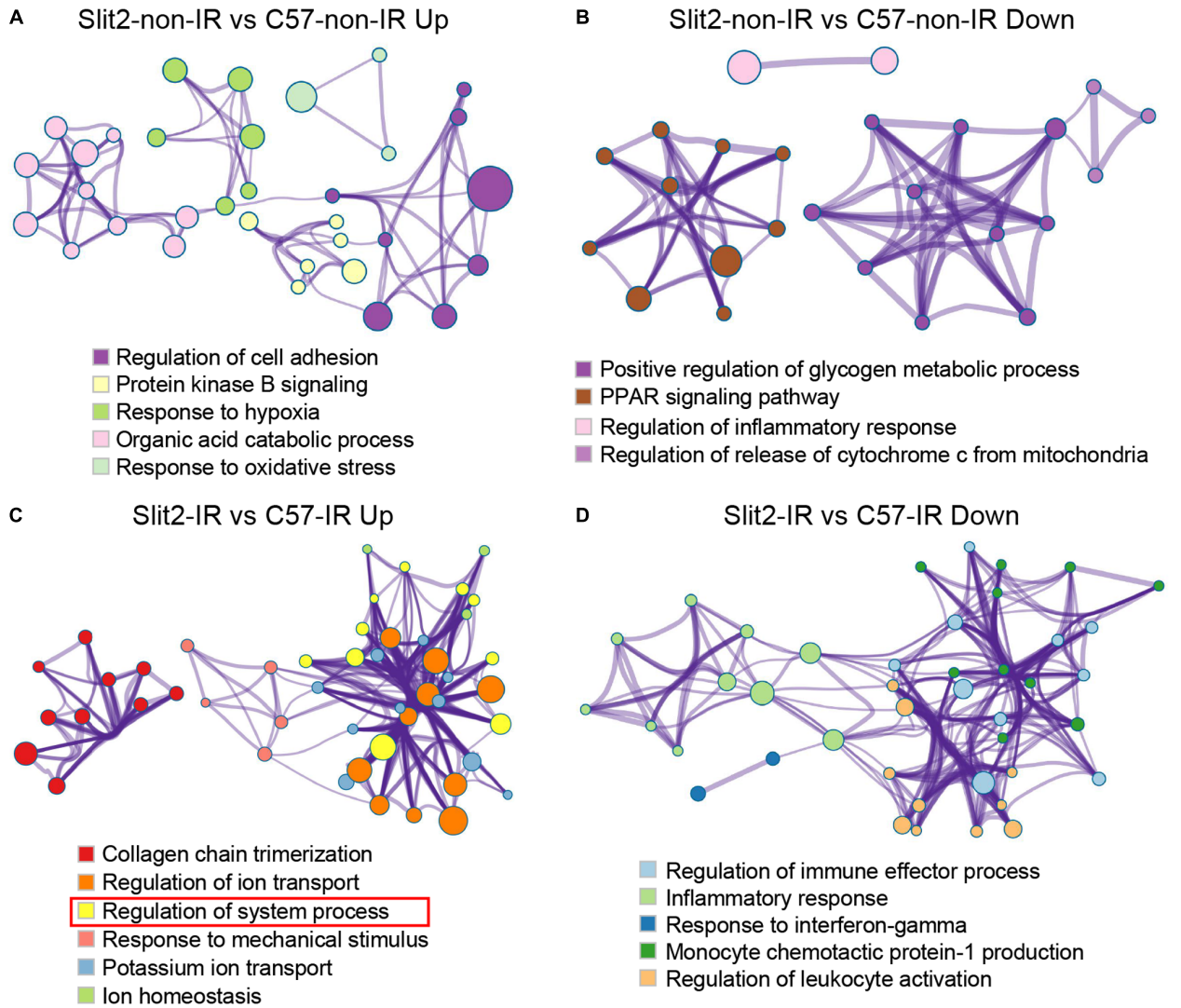


FIGURE 3 | Gene set enrichment analysis of differentially expressed genes (DEGs). Shown are the results for the upregulated DEGs in the Slit2-non-IR vs. C57-non-IR comparison **(A)**, the downregulated DEGs in the Slit2-non-IR vs. C57-non-IR comparison **(B)**, the upregulated DEGs in the Slit2-IR vs. C57-IR comparison (the rectangles in red indicate gene sets enriched for muscle contraction, regulation of heart rate, regulation of heart contraction, heart contraction, actin-mediated cell contraction, regulation of blood circulation, and adrenergic signaling in cardiomyocytes) **(C)**, and the downregulated DEGs in the Slit2-IR vs. C57-IR comparison **(D)**. In the Metascape protein interaction network, two or more gene sets enriched for biological processes/KEGG pathways/Reactome pathways with similar functions were clustered. Within each cluster, the gene set with the highest enrichment score (log *P*-value) was used to annotate the cluster. The links between the clusters are shown. The cluster annotations are color-coded. The size of a node (representing an enriched gene set) is proportional to the number of DEGs in the set, and the width of an edge is proportional to the number of genes shared between the two enriched sets.

C57-non-IR comparison, the downregulated DEGs in the Slit2-IR vs. C57-IR comparison, the upregulated DEGs in the Slit2-IR vs. C57-IR comparison, and the up-/downregulated DEGs in the Slit2-non-IR vs. C57-non-IR comparison, respectively (**Figures 4A,B** and **Supplementary Table S4**). By using Venn diagram analysis (**Figures 4A,B**), we identified 18 genes (6 upregulated and 12 downregulated) that might be regulated by Slit2 to protect the post-IR myocardium. The genes are shown in **Figure 4C**.

The expression of the two genes most affected by Slit2 overexpression post IR (Slamf7 and RasGRP1) was verified using RT-qPCR and Western blotting. The gene expression and protein expression of Slamf7 were both upregulated by IR in C57BL/6J hearts, but Slit2 overexpression blunted this upregulation in post-IR conditions (**Figures 5A,F,G**). No differences in RasGRP1 were detected between the groups (**Figures 5B,F,H**). In addition, RT-qPCR and Western blotting were used to determine the gene and/or protein expression of the Slit2-C fragment and Robo receptors. The protein expression of Slit2-C did not differ between the groups (**Figure 5I**). Robo1 expression did not change in C57BL/6J hearts after IR but was activated in post-IR Slit2-Tg hearts (**Figure 5C**). Robo2 was not different between any groups

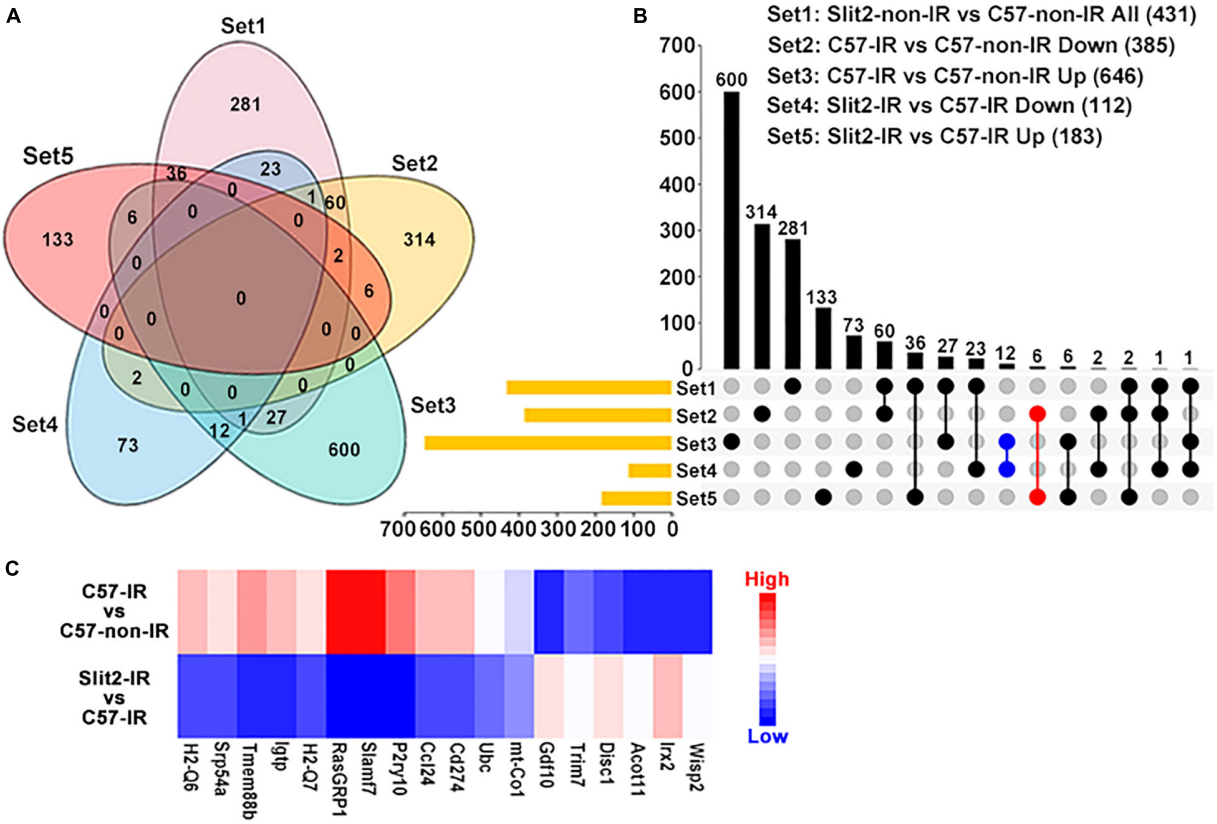


FIGURE 4 | Gene expression profiles of C57BL/6J and Slit2-Tg hearts subjected or not subjected to ischemia–reperfusion (IR) (n = 3 per group), as determined by RNA sequencing analysis. **(A)** Venn diagram of the differentially expressed genes (DEGs) in all comparisons, including all DEGs in the Slit2-non-IR vs. C57-non-IR comparison (pink), the upregulated DEGs in the C57-IR vs. C57-non-IR comparison (green), the downregulated DEGs in the C57-IR vs. C57-non-IR comparison (orange), the upregulated DEGs in the Slit2-IR vs. C57-IR comparison (red), and the downregulated DEGs in the Slit2-IR vs. C57-IR comparison (blue). **(B)** UpSet plot displaying the number of DEGs between groups for each intersection. The blue dots represent the genes (12) at the intersection between the downregulated DEGs in the Slit2-IR vs. C57-IR comparison and the upregulated DEGs in the C57-IR vs. C57-non-IR comparison, and the red dots represent the genes (6) at the intersection between the upregulated DEGs in the Slit2-IR vs. C57-IR comparison and the downregulated DEGs in the C57-IR vs. C57-non-IR comparison; both exclude overlap with all DEGs in the Slit2-non-IR vs. C57-non-IR comparison. **(C)** Heatmap displaying the fold changes in 18 genes obtained from the Venn diagram analysis. DEGs with log2 (fold change) values > 0 are indicated in red, and those with log2 (fold change) values < 0 are indicated in blue; a darker color indicates a higher P-value.

(**Figure 5D**). Robo4 was upregulated by IR in C57BL/6J hearts, but the IR-induced increases were blunted in Slit2-Tg hearts (**Figure 5E**). Therefore, we identified Robo1, Robo4, and Slamf7 to be regulated by Slit2 in post-IR hearts.

A previous study has shown that Slit-Robo signaling plays anti-inflammatory roles, and our RNA sequencing data indicated that Slit2 overexpression reduced inflammatory responses in post-IR conditions (**Figure 3D** and **Supplementary Table S3**). In addition, RNA sequencing and expression verification confirmed that Slamf7 was an effector of Slit2 overexpression. Slamf7 is known to be expressed on immune cells (Malaer and Mathew, 2017). Thus, we further investigated the inflammatory responses within cells. First, we found that Slamf7 was diffusely distributed in cardiomyocytes in C57BL/6J hearts. The expression of Slamf7 increased significantly when the myocardium was subjected to IR, but this activation was suppressed by Slit2 overexpression (**Figures 6A,C**). These results were consistent with the Western blot and RT-qPCR findings (**Figures 5A,F,G**). Moreover, we found that Slit2 overexpression inhibited the IR-induced nuclear translocation of NFκB p65 in cells (mainly fibroblasts) (**Figures 6B,D**).

The levels of the cytokines IL-1β and IL-18 did not differ between C57BL/6J and Slit2-Tg heart perfusates at baseline. At the beginning of reperfusion, the release of IL-1β sharply increased before exhibiting a U-shaped change in C57BL/6J hearts, but Slit2-Tg hearts showed smaller increases, and the levels rapidly returned to baseline levels during reperfusion (**Figure 6E**). Elevations in IL-18 release were seen in C57BL/6J hearts but not in Slit2-Tg hearts at the beginning of reperfusion (**Figure 6F**). The above results establish that Slit2 plays an anti-inflammatory role during IR.

Slit2 Regulates Myofilament-Associated PKC in Post-IR Hearts

In previous experiments, we found that Slit2 improved contractile function, protected cardiac tissues (**Figures 1**, **2**), and shifted gene expression profiles to enhance the regulation of

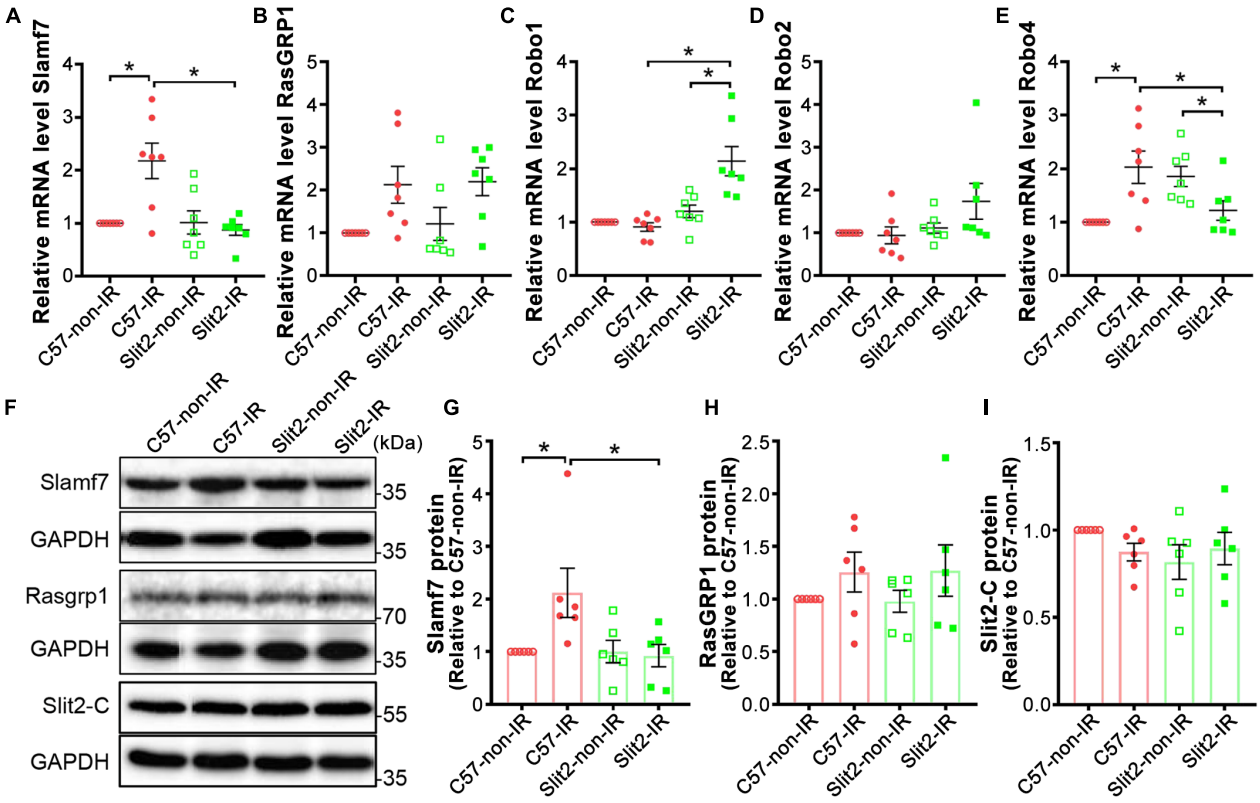


FIGURE 5 | Expression of Slamf7, RasGRP1, Robos, and the Slit2-C fragment in C57BL/6J and Slit2-Tg hearts. **(A–E)** Gene expression of Slamf7 **(A)**, RasGRP1 **(B)**, Robo1 **(C)**, Robo2 **(D)**, and Robo4 **(E)**. $n = 7$ mice per group. **(F)** Representative Western blots of Slamf7, RasGRP1, Slit2-C, and GAPDH (the loading control). **(G–I)** Quantitative results for Slamf7 **(G)**, RasGRP1 **(H)**, and the Slit2-C fragment **(I)** normalized to GAPDH. The values are relative to those in the C57-non-IR group. $n = 6$ mice per group. All data are presented as the mean ± SEM; $^*P < 0.05$ between the groups (two-way ANOVA, Tukey's multiple comparisons test).

cardiac contraction (**Figure 3C**). Consequently, we investigated myofilament-associated PKA and PKC and their counterparts PP type 1 (PP1) and PP2A, which are known to be activated during IR and to regulate cardiac contraction (Lochner et al., 1999; Armstrong, 2004; Nicolaou et al., 2009). Here, we found that myofilament-associated PKCδ was significantly upregulated in C57BL/6J hearts after IR, but the increases in PKCδ were blocked by Slit2 post IR (**Figure 7B**). Slit2 did not fully block the IR-induced upregulation of PKCε in C57BL/6J mice (**Figure 7A**). The IR-induced increases in myofilament-associated PKA and PP2A were not altered by Slit2 overexpression post IR (**Figures 7C,E**). The expression of myofilament-associated PP1α was not different between any groups (**Figure 7D**). The above results establish that Slit2 targets myofilament-associated PKC phosphorylation signaling in the post-IR myocardium.

Slit2 Suppresses cTnI Ser43 Phosphorylation and Maintains Myofilament Contractile Function

Several cardiac myofilament proteins, including cTnI, cTnT, MyBP-C, and desmin, can be targeted by protein kinases (Mohamed et al., 1998; Solaro, 2008; Streng et al., 2013). Following identification of myofilament-associated PKC regulation by Slit2, we explored myofilament phosphorylation levels and specific sites affected by Slit2 in the post-IR myocardium. We found that cTnI phosphorylation was increased in both post-IR C57BL/6J and Slit2-Tg hearts, but the increase was relatively suppressed in post-IR Slit2-Tg hearts (**Figures 8A,B**). MyBP-C phosphorylation was decreased in C57BL/6J and Slit2-Tg hearts after IR, and the decreases did not differ between the groups (**Figures 8A,B**). Desmin and cTnT did not show any differences between the groups (**Figures 8A,B**). Moreover, we found that the phosphorylation levels of cTnI at Ser43 were higher than baseline levels in post-IR C57BL/6J hearts, but Slit2 significantly decreased the IR-induced phosphorylation at this site (**Figures 8C,E**). These changes were consistent with the findings for cTnI phosphorylation. cTnI phosphorylation at Ser23/24 did not differ between any groups (**Figures 8C,D**). Thus, we identified that cTnI Ser43 was targeted by Slit2 during IR, and this site is known for phosphorylation by PKCs. Given the findings regarding the activation of myofilament-associated PKC, we conclude that Slit2 regulates myofilament activation at least in part by targeting PKC-dependent cTnI phosphorylation.

Myofilament contractile properties (maximum actomyosin MgATPase activity and calcium sensitivity) were examined to further explain the post-IR protective effects of Slit2. We found

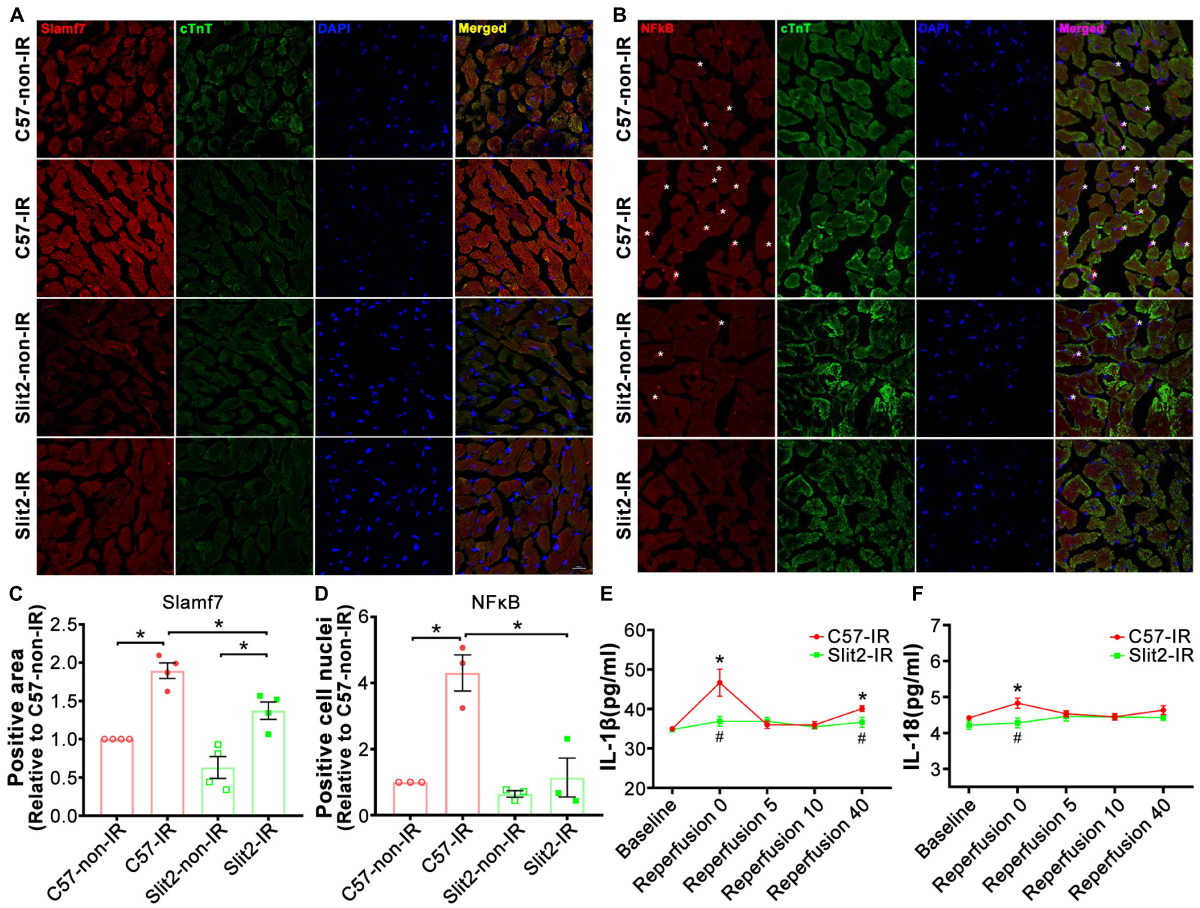


FIGURE 6 | Immunofluorescence staining of Slamf7 and NFκB in the myocardium. **(A–D)** Representative confocal images of Slamf7 (red) **(A)** and NFκB p65 (red) **(B)** in the left ventricular myocardium in C57BL/6J and Slit2-Tg mice. Images of cTnT staining (green), nuclear staining (blue), and merged staining are displayed. Scale bar = 100 nm. The relative levels of Slamf7 were calculated by determining the Slamf7 + area as a percentage of the cTnT + area **(C)**, and the relative levels of NFκB p65 were calculated by determining the number of NFκB p65 + DAPI + cell nuclei (asterisk) as a percentage of the number of DAPI + cell nuclei **(D)**. n = 3–4 mice per group. All data are presented as the mean ± SEM; $^{*}P < 0.05$ between the groups (two-way ANOVA, Tukey's multiple comparisons test). **(E,F)** Release of the inflammatory cytokines IL-1β **(E)** and IL-18 **(F)** at different time points of reperfusion. n = 5 mice per group. All data are presented as the mean ± SEM; $^{*}P < 0.05$ vs. baseline; $^{\#}P < 0.05$ vs. reperfusion at 0 min (unpaired Student's t-test).

that while maximum actomyosin MgATPase activity did not differ among non-ischemic hearts (**Figures 8F,H**), Slit2-Tg hearts had higher calcium sensitivity than C57BL/6J hearts (**Figure 8G**). Maximum actomyosin MgATPase activity decreased significantly in both C57BL/6J and Slit2-Tg hearts post IR, but Slit2-Tg hearts maintained relatively higher contractility than C57BL/6J hearts (**Figures 8F,H**). The calcium sensitivity of C57BL/6J myofilaments was significantly decreased after IR, but it was not altered in post-IR Slit2 myofilaments (**Figure 8G**). In summary, Slit2 protects myofilament contractile function in post-IR hearts, and this effect is associated with Slit2-mediated depression of cTnI phosphorylation.

DISCUSSION

Our current study demonstrates that Slit2 overexpression protects hearts against IR injury by maintaining postischemic contractile function and reducing tissue damage. The beneficial effects of Slit2 post IR were confirmed by evaluating cardiac contractile function, cellular structure, anti-inflammatory responses of the myocardium, and myofilament phosphorylation status and contractile properties. The proposed mechanisms for the cardioprotective effects of Slit2 in the post-IR myocardium are shown in **Figure 9**. Briefly, Slit2 overexpression inhibits the expression of the membrane receptors Robo4 and Slamf7 but activates Robo1, which in turn blocks the nuclear translocation of NFκB p65 and impedes the release of IL-1β and IL-18 from cells in post-IR hearts. Next, Slit2 regulates intracellular signaling pathways to attenuate the increases in myofilament-associated PKC levels and cTnI Ser43 phosphorylation in the post-IR myocardium, maintaining myofilament contractility and calcium sensitivity. Our study establishes, for the first time, that Slit2 protects hearts against IR injury and regulates myofilament phosphorylation post IR.

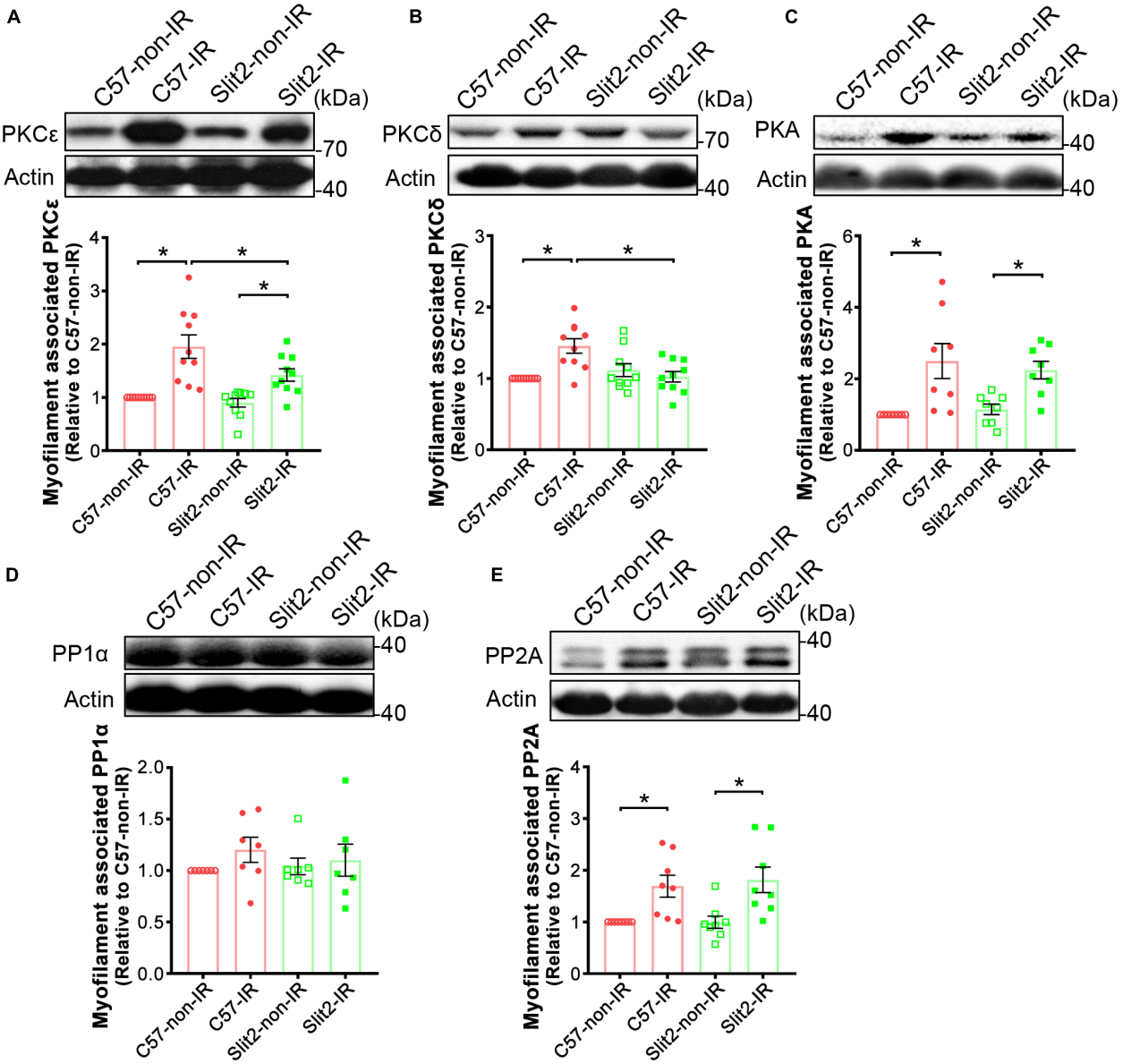


FIGURE 7 | Expression of myofilament-associated PKCs, PKA, and PPs. Cardiac myofilaments were probed for **(A)** PKCε, **(B)** PKCδ, **(C)** PKA, **(D)** PP1α, and **(E)** PP2A in C57BL/6J and Slit2-Tg hearts subjected or not subjected to IR. The protein expression levels were normalized to those in non-ischemic C57BL/6J hearts. n = 7–10 mice per group. All data are presented as the mean ± SEM; $^*P < 0.05$ between the groups (two-way ANOVA, Tukey's multiple comparisons test).

The Slit2-Robo signaling pathway has also been implicated in the response to IR injury in other organ systems. For example, Slit2 levels in the brain increase following ischemic injury (Park et al., 2016), and Slit2 is a crucial element in glial scar formation and brain remodeling in response to ischemic stroke (Jin et al., 2016). Slit2 has been shown to antagonize the actions of neutrophils in the kidney and protect against IR (Chaturvedi et al., 2013). The mechanisms of renal IR injury are similar to those of cardiac IR injury: ATP depletion, ROS generation, ionic dysregulation, and increased inflammatory cytokine and chemokine production. Thus, Slit2 might offer similar protection against IR injury in the heart through similar mechanisms. Moreira et al. (2014) found that changes in Slit2 levels associated with peptide treatment were correlated with cardiac recovery following sepsis, but the authors did not test whether increases in Slit2 were sufficient to mediate cardioprotection. Our current study is the first to show that increased expression of Slit2 protects against cardiac IR injury and supports the therapeutic potential of Slit2 treatment for a variety of ischemic injuries. One potential limitation for the translation of Slit2 treatment might be the inhibitory effects of Slit2 on the immune system. Interestingly, the effects of Slit2 on neutrophils in the kidney in response to IR do not negatively impact innate immune neutrophil functions, showing that Slit2-focused treatments do

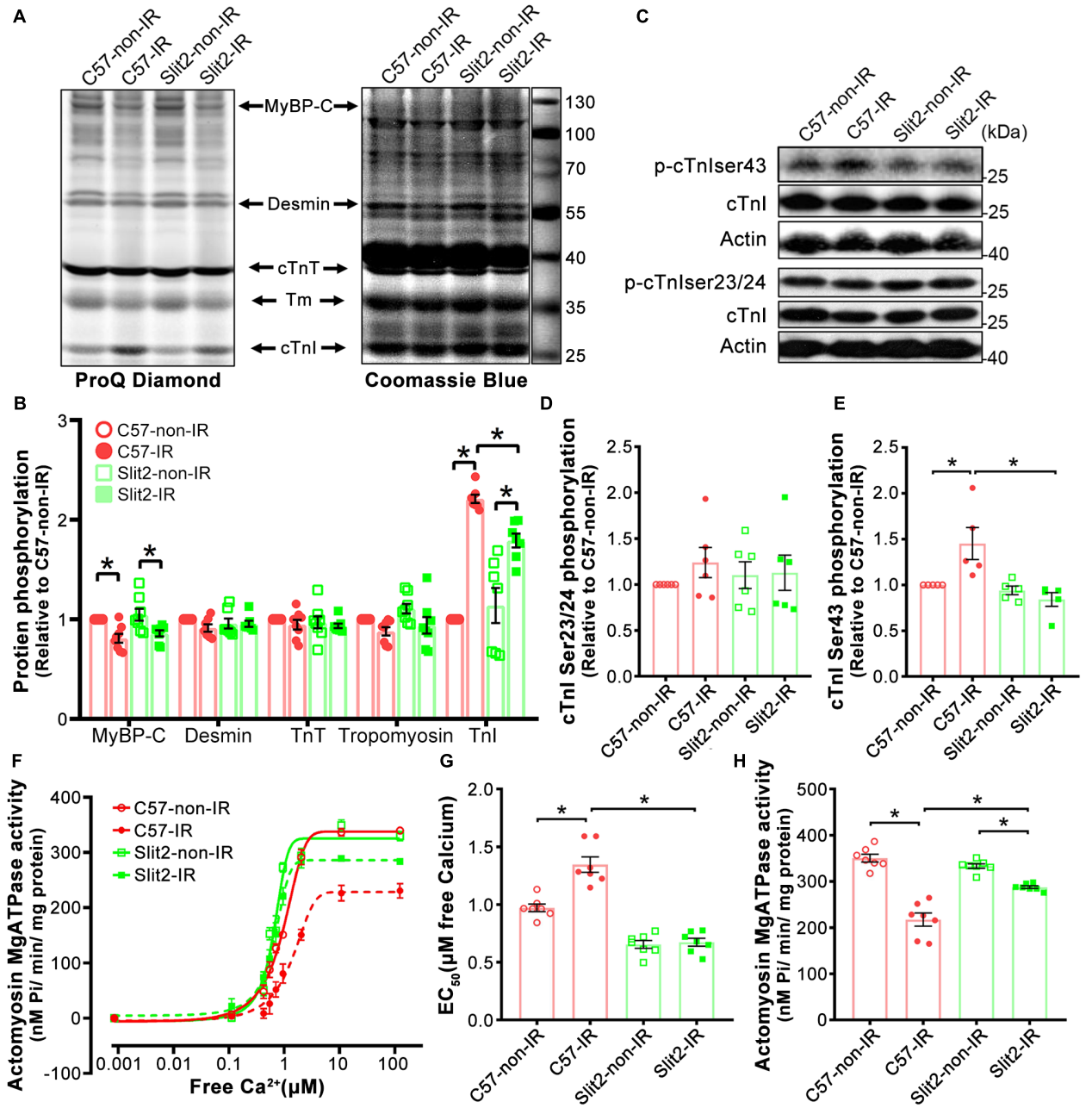


FIGURE 8 | Myofilament phosphorylation and contractile properties. **(A)** ProQ phosphorylation analysis and Coomassie staining of cardiac myofilament proteins after IR. **(B)** Phosphorylation levels of MyBP-C, desmin, cTnT, and cTnI. n = 7–8 mice per group. **(C–E)** Representative Western blots of cTnI phosphorylation **(C)** and quantification of cTnI phosphorylation at Ser23/24 **(D)** and Ser43 **(E)**. n = 5–6 mice per group. **(F)** Illustrations of actomyosin MgATPase activity in each group. **(G)** Myofilament EC_{50} values (indicators of myofilament calcium sensitivity). **(H)** Maximum actomyosin MgATPase activity. n = 7 mice per group. All data are presented as the mean ± SEM; $^*P < 0.05$ between the groups (two-way ANOVA, Tukey's multiple comparisons test).

not increase the risk of opportunistic infections (Chaturvedi et al., 2013). Furthermore, acute application of Slit2 agonists around the time of reperfusion may be a therapeutic approach that avoids any immune complications that could limit the potential of chronic Slit2 treatment.

Investigation into the inflammatory response machinery supported our above conclusions about an antagonistic role for Slit2 in postischemic immune activation. RNA sequencing analysis showed that hearts overexpressing Slit2 were sensitive to external stimuli, resisted inflammatory insults, and exhibited improved cell survival. Among the 12 genes that were upregulated during IR, 7 were inflammatory genes (Ubc, Cd274, Ccl24, Slamf7, H2Q7, Igtp, and H2Q6) (**Figure 4C**). In this study, we also found that Slit2 blocked the nuclear translocation of

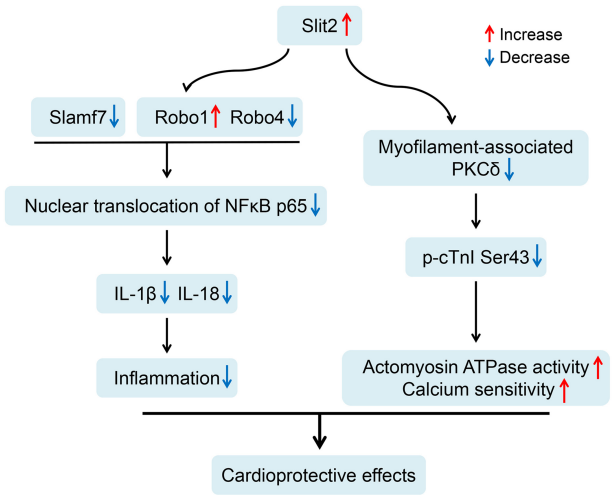


FIGURE 9 | Proposed cardioprotective mechanisms of Slit2 in the post-ischemia–reperfusion (IR) myocardium. Slit2 overexpression inhibits the expression of the membrane receptors Robo4 and Slamf7 but activates Robo1, which in turn blocks the nuclear translocation of NFκB p65 and impedes the release of IL-1β and IL-18 from the cells in post-IR hearts. Next, Slit2 regulates intracellular signaling pathways to attenuate the increases in myofilament-associated PKC levels and cTnI Ser43 phosphorylation in the post-IR myocardium, maintaining myofilament contractility and calcium sensitivity.

NFκB p65 in both cardiomyocytes and fibroblasts, indicating that multiple cell types might be involved in Slit2-induced cardioprotective effects. These findings are consistent with those of Pilling et al. (2014), who reported that Slit2 has direct inhibitory effects on fibrosis. Furthermore, we found that Slamf7, a receptor expressed on natural killer (NK) cells, B cells, T cells, NK T cells, dendritic cells, and monocytes, is involved in functional regulation in the post-IR myocardium. This is the first study to show that Slamf7 is present in cardiomyocytes and is linked to IR injury and Slit2-regulated cardioprotection. It has previously been shown that cleaved Slamf7 promotes the growth of multiple myeloma (Kikuchi et al., 2020) and that inhibition of SLAMF7 blocks adhesion of multiple myeloma cells to bone marrow stromal cells (Bouchon et al., 2001; Hsi et al., 2008). Slamf7 has been found to regulate PLCγ and PI_3K in human NK cells (Tassi and Colonna, 2005) and to be upregulated in cultured neonatal cardiomyocytes following acute cathepsin G exposure (Shukla et al., 2018). These findings suggest that Slamf7 can be present in cardiomyocytes and that it responds to various stresses. The mechanisms associated with the interaction of Slamf7 with intracellular signaling pathways and the cardiac expression/translocation of Slamf7 need to be further explored.

Roman et al. (2004) found that inhibition of PKC-mediated phosphorylation of cTnI improves cardiac performance. We observed that the levels of myofilament-associated PKCs were significantly increased by IR, but such increases were suppressed by Slit2 overexpression; consistent with these findings, Slit2 attenuated the upregulated phosphorylation of cTnI Ser43, a PKC phosphorylation site in the post-IR myocardium. Thus, our results suggest that the suppression of Slit2-regulated PKCs plays crucial roles in cardioprotection post IR. Additionally, PKC can directly interact with cytokines, as its activation is essential for the synthesis of cytokines such as TNF-α, IL-1β, and IL-6 in human monocytes (Kontny et al., 1999), suggesting that other mechanisms beyond those investigated in our study might be involved in Slit2-regulated PKC signaling. Furthermore, the levels of myofilament-associated PKA and PP2A were increased in the post-IR myocardium in C57BL/6J mice, but these changes were not modified by Slit2. This finding suggests that PKA and PP2A might not be crucial effectors of Slit2 in the postischemic heart.

Myofilament phosphorylation status is critical for normal contractile function (Verduyn et al., 2007; Kooij et al., 2010). In the present study, we found that the increases in cTnI phosphorylation post IR could be attenuated by Slit2, which supports claims that cTnI phosphorylation status is the key player in determining cardiac contractile function (McDonough and Van Eyk, 2004; Heijman et al., 2013). Furthermore, we identified the Ser43 site of cTnI as a downstream target of Slit2 and determined cTnI phosphorylation levels in post-IR Slit2-Tg hearts. Phosphorylation of cTnI Ser43/45 sites increases force in response to phenylephrine (Montgomery et al., 2002) and reduces ATPase activity (Jideama et al., 1996). Here, we found that altered cTnI phosphorylation was associated with preserved actomyosin MgATPase activity and calcium sensitivity in post-IR Slit2-Tg hearts. Thus, we clearly show that Slit2 targets myofilament phosphorylation, which plays crucial roles in maintaining myofilament contractile properties after IR injury.

In conclusion, we demonstrate that Slit2 protects cardiac function and reduces IR injury and that these effects are regulated by Slit2 interaction with membrane receptors that inhibit inflammatory responses and maintain myofilament contractile properties. This is the first study to show that Slit2 can protect hearts against IR injury and to identify that myofilament phosphorylation is regulated by Slit2.

LIMITATIONS OF THE STUDY

The Slit2-Tg mice were constructed using a CMV promoter. Niwa et al. (1991) developed the CMV vector system, which has been widely used to stably express foreign genes in mammalian models and normal cells. However, as a non-tissue-specific promoter, CMV might drive Slit2 expression in multiple organs and multiple cell types within an organ. In this study, Slit2 overexpression was driven by the CMV promoter, suggesting that Slit2 might play roles in all types of cells residing in the heart, such as cardiomyocytes, fibroblasts, and endothelial cells. When interpreting the results of this study, we should consider the overall effects of Slit2 on cardiac tissues, not limiting our conclusions to cardiomyocytes. In addition, an isolated perfused heart model that represents an acute ischemic condition was used in this study to understand the cardioprotective mechanisms of Slit2. The effects of Slit2 during chronic ischemia need to be further investigated. It is anticipated that an *in vivo* model will help to reveal the long-term effects of Slit2.

In this study, although the contractile phenotype is consistent with phosphorylation of Ser43/45 on cardiac troponin I (cTnI), some regulation mechanisms regarding Slit2-induced myofilament phosphorylation were not clear. For example, cTnI Thr144 is phosphorylated by PKC under acidosis, a condition induced by ischemia (Engel et al., 2009). Although it exhibits functions different from those of cTnI Ser43/45 after phosphorylation, this site might contribute to the functional adaptation regulated by Slit2 post IR. Besides cTnI, other myofilaments including MyBP-C (Mohamed et al., 1998) and cTnT (Jideama et al., 1996) were targeted by PKC. Although we did not detect the overall changes in the phosphorylation levels of these myofilaments in the post-IR myocardium, PKC might collaborate with other protein kinases and phosphatases to exert the effects. Another unclear mechanism is the roles of MyBP-C dephosphorylation in the IR context. We found that MyBP-C was dephosphorylated in post-IR Slit2-Tg hearts, which contrasts with previous reports showing that MyBP-C dephosphorylation is associated with heart dysfunction (Sadayappan et al., 2006). Thus, further study is needed for evaluating the roles of the site-specific and/or PKC-dependent myofilament phosphorylation in the Slit2-regulated post-IR cardiac function.

DATA AVAILABILITY STATEMENT

The RNA sequencing data has been deposited in the Gene Expression Omnibus (accession: GSE133796).

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (No. IACUC2017002).

AUTHOR CONTRIBUTIONS

FY, RH, PB, and XL designed and initiated the project. XL, SZ, WT, HC, XHL, JW, TL, and XR were responsible for the laboratory experiments, data analysis, and animal care. WP and LW provided critical comments during manuscript preparation. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (31672376, FY), the Guangzhou Science and Technology Program (201804010206, FY), the Guangdong Science and Technology Program (2018B030317001 and 2019A030317014), and the Guangdong Key Laboratory of Laboratory Animals (2017B030314171).

ACKNOWLEDGMENTS

We wish to thank the Center for Genetic Engineering Mouse Research of the Guangdong Laboratory Animals Monitoring Institute for maintaining the genetically modified mice used in this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00228/full#supplementary-material

REFERENCES

Armstrong, S. C. (2004). Protein kinase activation and myocardial ischemia/reperfusion injury. *Cardiovasc. Res.* 61, 427–436. doi: 10.1016/j.cardiores.2003.09.031

Barnes, P. J., and Karin, M. (1997). Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* 336, 1066–1071. doi: 10.1056/nejm199704103361506

Bouchon, A., Cella, M., Grierson, H. L., Cohen, J. I., and Colonna, M. (2001). Activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family. *J. Immunol.* 167, 5517–5521. doi: 10.4049/jimmunol.167.10.5517

Burma, O., Onat, E., Uysal, A., Ilhan, N., Erol, D., Ozcan, M., et al. (2014). Effects of rosuvastatin on ADMA, rhokinase, NADPH oxidase, caveolin-1, hsp 90 and NFkB levels in a rat model of myocardial ischaemia-reperfusion. *Cardiovasc J Afr* 25, 212–216. doi: 10.5830/CVJA-2014-038

Chaturvedi, S., Yuen, D. A., Bajwa, A., Huang, Y. W., Sokollik, C., Huang, L., et al. (2013). Slit2 prevents neutrophil recruitment and renal ischemia-reperfusion injury. *J. Am. Soc. Nephrol.* 24, 1274–1287. doi: 10.1681/ASN.2012090890

Dickson, B. J., and Gilestro, G. F. (2006). Regulation of commissural axon pathfinding by slit and its Robo receptors. *Annu. Rev. Cell Dev. Biol.* 22, 651–675. doi: 10.1146/annurev.cellbio.21.090704.151234

Dunaway, C. M., Hwang, Y., Lindsley, C. W., Cook, R. S., Wu, J. Y., Boothby, M., et al. (2011). Cooperative signaling between Slit2 and Ephrin-A1 regulates a balance between angiogenesis and angiostasis. *Mol. Cell. Biol.* 31, 404–416. doi: 10.1128/MCB.00667-10

Engel, P. L., Hinken, A., and Solaro, R. J. (2009). Differential effects of phosphorylation of regions of troponin I in modifying cooperative activation of cardiac thin filaments. *J. Mol. Cell Cardiol.* 47, 359–364. doi: 10.1016/j.yjmcc.2009.04.016

Essen, L. O., Perisic, O., Katan, M., Wu, Y., Roberts, M. F., and Williams, R. L. (1997). Structural mapping of the catalytic mechanism for a mammalian phosphoinositide-specific phospholipase C. *Biochemistry* 36, 1704–1718. doi: 10.1021/bi962512p

GBD 2017 Causes of Death Collaborators (2018). Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet* 392, 1736–1788. doi: 10.1016/S0140-6736(18)32203-7

Hausenloy, D. J., and Yellon, D. M. (2013). Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *J. Clin. Invest.* 123, 92–100. doi: 10.1172/JCI62874

Heijman, J., Dewenter, M., El-Armouche, A., and Dobrev, D. (2013). Function and regulation of serine/threonine phosphatases in the healthy and diseased heart. *J. Mol. Cell Cardiol.* 64, 90–98. doi: 10.1016/j.yjmcc.2013.09.006

Hsi, E. D., Steinle, R., Balasa, B., Szmania, S., Draksharapu, A., Shum, B. P., et al. (2008). CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma. *Clin. Cancer Res.* 14, 2775–2784. doi: 10.1158/1078-0432.CCR-07-4246

Inagaki, K., Chen, L., Ikeno, F., Lee, F. H., Imahashi, K., Bouley, D. M., et al. (2003a). Inhibition of delta-protein kinase C protects against reperfusion injury of the ischemic heart *in vivo*. *Circulation* 108, 2304–2307. doi: 10.1161/01.cir.0000101682.24138.36

Inagaki, K., Hahn, H. S., Dorn, G. W. II, and Mochly-Rosen, D. (2003b). Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. *Circulation* 108, 869–875. doi: 10.1161/01.cir.0000081943.93653.73

Jideama, N. M., Noland, T. A. Jr., Raynor, R. L., Blobe, G. C., Fabbro, D., Kazanietz, M. G., et al. (1996). Phosphorylation specificities of protein kinase C isozymes for bovine cardiac troponin I and troponin T and sites within these proteins and regulation of myofilament properties. *J. Biol. Chem.* 271, 23277–23283. doi: 10.1074/jbc.271.38.23277

Jin, X., Shin, Y. J., Riew, T. R., Choi, J. H., and Lee, M. Y. (2016). Increased expression of Slit2 and its robo receptors during astroglial scar formation after transient focal cerebral ischemia in rats. *Neurochem. Res.* 41, 3373–3385. doi: 10.1007/s11064-016-2072-0

Kawaguchi, M., Takahashi, M., Hata, T., Kashima, Y., Usui, F., Morimoto, H., et al. (2011). Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. *Circulation* 123, 594–604. doi: 10.1161/CIRCULATIONAHA.110.982777

Kikuchi, J., Hori, M., Iha, H., Toyama-Sorimachi, N., Hagiwara, S., Kuroda, Y., et al. (2020). Soluble SLAMF7 promotes the growth of myeloma cells via homophilic interaction with surface SLAMF7. *Leukemia* 34, 180–195. doi: 10.1038/s41375-019-0525-6

Kontny, E., Ziolkowska, M., Ryzewska, A., and Maslinski, W. (1999). Protein kinase c-dependent pathway is critical for the production of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6). *Cytokine* 11, 839–848. doi: 10.1006/cyto.1998.0496

Kooij, V., Boontje, N., Zaremba, R., Jaquet, K., Dos Remedios, C., Stienen, G. J., et al. (2010). Protein kinase C alpha and epsilon phosphorylation of troponin and myosin binding protein C reduce Ca2+ sensitivity in human myocardium. *Basic Res. Cardiol.* 105, 289–300. doi: 10.1007/s00395-009-0053-z

Lochner, A., Genade, S., Tromp, E., Podzuweit, T., and Moolman, J. A. (1999). Ischemic preconditioning and the beta-adrenergic signal transduction pathway. *Circulation* 100, 958–966. doi: 10.1161/01.cir.100.9.958

Lu, L., Wei, P., Cao, Y., Zhang, Q., Liu, M., Liu, X. D., et al. (2016). Effect of total peony glucoside pretreatment on NF-kappaB and ICAM-1 expression in myocardial tissue of rat with myocardial ischemia-reperfusion injury. *Genet. Mol. Res.* 15:gmr15048978.

Ma, X. L., Lefer, D. J., Lefer, A. M., and Rothlein, R. (1992). Coronary endothelial and cardiac protective effects of a monoclonal antibody to intercellular adhesion molecule-1 in myocardial ischemia and reperfusion. *Circulation* 86, 937–946. doi: 10.1161/01.cir.86.3.937

Malaer, J. D., and Mathew, P. A. (2017). CS1 (SLAMF7, CD319) is an effective immunotherapeutic target for multiple myeloma. *Am. J. Cancer Res.* 7, 1637–1641.

McDonough, J. L., and Van Eyk, J. E. (2004). Developing the next generation of cardiac markers: disease-induced modifications of troponin I. *Prog. Cardiovasc. Dis.* 47, 207–216. doi: 10.1016/j.pcad.2004.07.001

Metzger, J. M., and Westfall, M. V. (2004). Covalent and noncovalent modification of thin filament action: the essential role of troponin in cardiac muscle regulation. *Circ. Res.* 94, 146–158. doi: 10.1161/01.res.0000110083.17024.60

Mohamed, A. S., Dignam, J. D., and Schlender, K. K. (1998). Cardiac myosin-binding protein C (MyBP-C): identification of protein kinase A and protein kinase C phosphorylation sites. *Arch. Biochem. Biophys.* 358, 313–319. doi: 10.1006/abbi.1998.0857

Montgomery, D. E., Wolska, B. M., Pyle, W. G., Roman, B. B., Dowell, J. C., Buttrick, P. M., et al. (2002). alpha-Adrenergic response and myofilament activity in mouse hearts lacking PKC phosphorylation sites on cardiac TnI. *Am. J. Physiol. Heart Circ. Physiol.* 282, H2397–H2405.

Moreira, R. S., Irigoyen, M., Sanches, T. R., Volpini, R. A., Camara, N. O., Malheiros, D. M., et al. (2014). Apolipoprotein A-I mimetic peptide 4F attenuates kidney injury, heart injury, and endothelial dysfunction in sepsis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 307, R514–R524. doi: 10.1152/ajpregu.00445.2013

Murphy, E., and Steenbergen, C. (2008). Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol. Rev.* 88, 581–609. doi: 10.1152/physrev.00024.2007

Murriel, C. L., Churchill, E., Inagaki, K., Szweda, L. I., and Mochly-Rosen, D. (2004). Protein kinase Cdelta activation induces apoptosis in response to cardiac ischemia and reperfusion damage: a mechanism involving BAD and the mitochondria. *J. Biol. Chem.* 279, 47985–47991. doi: 10.1074/jbc.m405071200

Nguyen Ba-Charvet, K. T., Brose, K., Ma, L., Wang, K. H., Marillat, V., Sotelo, C., et al. (2001). Diversity and specificity of actions of Slit2 proteolytic fragments in axon guidance. *J. Neurosci.* 21, 4281–4289. doi: 10.1523/jneurosci.21-12-04281.2001

Nicolaou, P., Hajjar, R. J., and Kranias, E. G. (2009). Role of protein phosphatase-1 inhibitor-1 in cardiac physiology and pathophysiology. *J. Mol. Cell Cardiol.* 47, 365–371. doi: 10.1016/j.yjmcc.2009.05.010

Niwa, H., Yamamura, K., and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108, 193–199. doi: 10.1016/0378-1119(91)90434-d

Noland, T. A. Jr., Guo, X., Raynor, R. L., Jideama, N. M., Averyhart-Fullard, V., Solaro, R. J., et al. (1995). Cardiac troponin I mutants. Phosphorylation by protein kinases C and A and regulation of Ca(2+)-stimulated MgATPase of reconstituted actomyosin S-1. *J. Biol. Chem.* 270, 25445–25454. doi: 10.1074/jbc.270.43.25445

Park, J. H., Pak, H. J., Riew, T. R., Shin, Y. J., and Lee, M. Y. (2016). Increased expression of Slit2 and its receptors Robo1 and Robo4 in reactive astrocytes of the rat hippocampus after transient forebrain ischemia. *Brain Res.* 1634, 45–56. doi: 10.1016/j.brainres.2015.12.056

Patton, C., Thompson, S., and Epel, D. (2004). Some precautions in using chelators to buffer metals in biological solutions. *Cell Calcium* 35, 427–431. doi: 10.1016/j.ceca.2003.10.006

Pilling, D., Zheng, Z., Vakil, V., and Gomer, R. H. (2014). Fibroblasts secrete Slit2 to inhibit fibrocyte differentiation and fibrosis. *Proc. Natl. Acad. Sci. U.S.A.* 111, 18291–18296. doi: 10.1073/pnas.1417426112

Prasad, A., Stone, G. W., Holmes, D. R., and Gersh, B. (2009). Reperfusion injury, microvascular dysfunction, and cardioprotection: the "dark side" of reperfusion. *Circulation* 120, 2105–2112. doi: 10.1161/circulationaha.108.814640

Roman, B. B., Goldspink, P. H., Spaite, E., Urboniene, D., Mckinney, R., Geenen, D. L., et al. (2004). Inhibition of PKC phosphorylation of cTnI improves cardiac performance *in vivo*. *Am. J. Physiol. Heart Circ. Physiol.* 286, H2089–H2095.

Sadayappan, S., Osinska, H., Klevitsky, R., Lorenz, J. N., Sargent, M., Molkentin, J. D., et al. (2006). Cardiac myosin binding protein C phosphorylation is cardioprotective. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16918–16923.

Samelson, B. K., Gore, B. B., Whiting, J. L., Nygren, P. J., Purkey, A. M., Colledge, M., et al. (2015). A-kinase anchoring protein 79/150 recruits protein kinase C to phosphorylate roundabout receptors. *J. Biol. Chem.* 290, 14107–14119. doi: 10.1074/jbc.M115.637470

Shen, Y., Mani, S., Donovan, S. L., Schwob, J. E., and Meiri, K. F. (2002). Growth-associated protein-43 is required for commissural axon guidance in the developing vertebrate nervous system. *J. Neurosci.* 22, 239–247. doi: 10.1523/jneurosci.22-01-00239.2002

Shukla, S. K., Sikder, K., Sarkar, A., Addya, S., and Rafiq, K. (2018). Molecular network, pathway, and functional analysis of time-dependent gene changes related to cathepsin G exposure in neonatal rat cardiomyocytes. *Gene* 671, 58–66. doi: 10.1016/j.gene.2018.05.110

Singh, R. M., Cummings, E., Pantos, C., and Singh, J. (2017). Protein kinase C and cardiac dysfunction: a review. *Heart Fail. Rev.* 22, 843–859. doi: 10.1007/s10741-017-9634-3

Solaro, R. J. (2008). Multiplex kinase signaling modifies cardiac function at the level of sarcomeric proteins. *J. Biol. Chem.* 283, 26829–26833. doi: 10.1074/jbc.r800037200

Steinberg, S. F. (2012). Cardiac actions of protein kinase C isoforms. *Physiology* 27, 130–139. doi: 10.1152/physiol.00009.2012

Streng, A. S., De Boer, D., Van Der Velden, J., Van Dieijen-Visser, M. P., and Wodzig, W. K. (2013). Posttranslational modifications of cardiac troponin T: an overview. *J. Mol. Cell Cardiol.* 63, 47–56. doi: 10.1016/j.yjmcc.2013.07.004

Takimoto, E., Soergel, D. G., Janssen, P. M., Stull, L. B., Kass, D. A., and Murphy, A. M. (2004). Frequency- and afterload-dependent cardiac modulation in vivo by troponin I with constitutively active protein kinase A phosphorylation sites. *Circ. Res.* 94, 496–504. doi: 10.1161/01.res.0000117307.57798.f5

Tassi, I., and Colonna, M. (2005). The cytotoxicity receptor CRACC (CS-1) recruits EAT-2 and activates the PI3K and phospholipase Cgamma signaling pathways in human NK cells. *J. Immunol.* 175, 7996–8002. doi: 10.4049/jimmunol.175.12.7996

van der Velden, J., and Stienen, G. J. M. (2019). Cardiac disorders and pathophysiology of sarcomeric proteins. *Physiol. Rev.* 99, 381–426. doi: 10.1152/physrev.00040.2017

Verduyn, S. C., Zaremba, R., Van Der Velden, J., and Stienen, G. J. (2007). Effects of contractile protein phosphorylation on force development in permeabilized rat cardiac myocytes. *Basic Res. Cardiol.* 102, 476–487. doi: 10.1007/s00395-007-0663-2

Wong, K., Park, H. T., Wu, J. Y., and Rao, Y. (2002). Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr. Opin. Genet. Dev.* 12, 583–591. doi: 10.1016/s0959-437x(02)00343-x

Yang, F., Aiello, D. L., and Pyle, W. G. (2008). Cardiac myofilament regulation by protein phosphatase type 1alpha and CapZ. *Biochem. Cell Biol.* 86, 70–78. doi: 10.1139/o07-150

Yang, F. H., and Pyle, W. G. (2011). Cardiac actin capping protein reduction and protein kinase C inhibition maintain myofilament function during cardioplegic arrest. *Cell Physiol. Biochem.* 27, 263–272. doi: 10.1159/000327952

Yang, F. H., and Pyle, W. G. (2012). Reduced cardiac CapZ protein protects hearts against acute ischemia-reperfusion injury and enhances preconditioning. *J. Mol. Cell Cardiol.* 52, 761–772. doi: 10.1016/j.yjmcc.2011.11.013

Yang, X. M., Han, H. X., Sui, F., Dai, Y. M., Chen, M., and Geng, J. G. (2010). Slit-Robo signaling mediates lymphangiogenesis and promotes tumor lymphatic metastasis. *Biochem. Biophys. Res. Commun.* 396, 571–577. doi: 10.1016/j.bbrc.2010.04.152

Ye, B. Q., Geng, Z. H., Ma, L., and Geng, J. G. (2010). Slit2 regulates attractive eosinophil and repulsive neutrophil chemotaxis through differential srGAP1 expression during lung inflammation. *J. Immunol.* 185, 6294–6305. doi: 10.4049/jimmunol.1001648

Yeh, C. H., Chen, T. P., Wu, Y. C., Lin, Y. M., and Jing Lin, P. (2005). Inhibition of NFkappaB activation with curcumin attenuates plasma inflammatory cytokines surge and cardiomyocytic apoptosis following cardiac ischemia/reperfusion. *J. Surg. Res.* 125, 109–116. doi: 10.1016/j.jss.2004.11.009

Zhang, X., Li, J., Liu, J., Luo, H., Gou, K., and Cui, S. (2013). Prostaglandin F2alpha upregulates Slit/Robo expression in mouse corpus luteum during luteolysis. *J. Endocrinol.* 218, 299–310. doi: 10.1530/JOE-13-0088

Zhao, H., Anand, A. R., and Ganju, R. K. (2014). Slit2-Robo4 pathway modulates lipopolysaccharide-induced endothelial inflammation and its expression is dysregulated during endotoxemia. *J. Immunol.* 192, 385–393. doi: 10.4049/jimmunol.1302021

Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A. H., Tanaseichuk, O., et al. (2019). Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat. Commun.* 10:1523. doi: 10.1038/s41467-019-09234-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 08 April 2020
doi: 10.3389/fphys.2020.00267

ATF6 as a Nodal Regulator of Proteostasis in the Heart

Christopher C. Glembotski, Adrian Arrieta, Erik A. Blackwood and Winston T. Stauffer*

Department of Biology, College of Sciences, San Diego State University Heart Institute, San Diego State University, San Diego, CA, United States

Proteostasis encompasses a homeostatic cellular network in all cells that maintains the integrity of the proteome, which is critical for optimal cellular function. The components of the proteostasis network include protein synthesis, folding, trafficking, and degradation. Cardiac myocytes have a specialized endoplasmic reticulum (ER) called the sarcoplasmic reticulum that is well known for its role in contractile calcium handling. However, less studied is the proteostasis network associated with the ER, which is of particular importance in cardiac myocytes because it ensures the integrity of proteins that are critical for cardiac contraction, e.g., ion channels, as well as proteins necessary for maintaining myocyte viability and interaction with other cell types, e.g., secreted hormones and growth factors. A major aspect of the ER proteostasis network is the ER unfolded protein response (UPR), which is initiated when misfolded proteins in the ER activate a group of three ER transmembrane proteins, one of which is the transcription factor, ATF6. Prior to studies in the heart, ATF6 had been shown in model cell lines to be primarily adaptive, exerting protective effects by inducing genes that encode ER proteins that fortify protein-folding in this organelle, thus establishing the canonical role for ATF6. Subsequent studies in isolated cardiac myocytes and in the myocardium, *in vivo*, have expanded roles for ATF6 beyond the canonical functions to include the induction of genes that encode proteins outside of the ER that do not have known functions that are obviously related to ER protein-folding. The identification of such non-canonical roles for ATF6, as well as findings that the gene programs induced by ATF6 differ depending on the stimulus, have piqued interest in further research on ATF6 as an adaptive effector in cardiac myocytes, underscoring the therapeutic potential of activating ATF6 in the heart. Moreover, discoveries of small molecule activators of ATF6 that adaptively affect the heart, as well as other organs, *in vivo*, have expanded the potential for development of ATF6-based therapeutics. This review focuses on the ATF6 arm of the ER UPR and its effects on the proteostasis network in the myocardium.

Keywords: ATF6, cardiac myocyte, proteostasis, ER stress, unfolded protein response

OPEN ACCESS

Edited by:
Huabo Su,
Augusta University Medical College of Georgia, United States

Reviewed by:
Margaret Westfall,
University of Michigan, United States
Rajasekaran Namakkal-Soorappan,
The University of Alabama at Birmingham, United States

****Correspondence:***
Christopher C. Glembotski
cglembotski@sdsu.edu

Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *15 January 2020*
Accepted: *09 March 2020*
Published: *08 April 2020*

Citation:
Glembotski CC, Arrieta A, Blackwood EA and Stauffer WT (2020) ATF6 as a Nodal Regulator of Proteostasis in the Heart. Front. Physiol. 11:267. doi: 10.3389/fphys.2020.00267

PROTEOSTASIS AND PROTEOTOXICITY

The integrity of the proteome in cardiac myocytes is critical for normal heart function. Proteome integrity in all eukaryotic cells is maintained by proteostasis, which encompasses the cellular networks that contribute to protein synthesis, folding, trafficking, and degradation (Balch et al., 2008; Sala et al., 2017; **Figure 1**). An imbalance amongst the components of these networks can lead

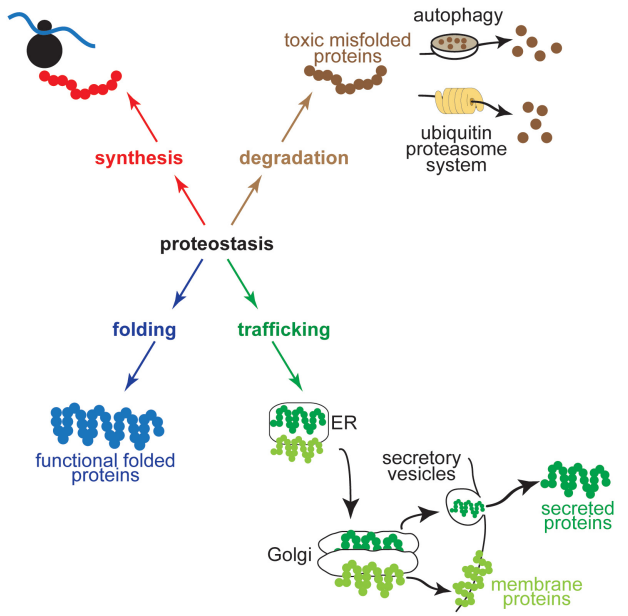


FIGURE 1 | Proteostasis encompasses the cellular networks that contribute to protein synthesis, folding, trafficking, and degradation. Shown for protein synthesis **(red)** is a ribosome in the process of translation, for protein folding **(blue)** is a protein in its folded, functional conformation, for trafficking **(green)** are a secreted protein and a transmembrane protein moving through the ER secretory pathway to the Golgi before being routed to their final destinations in secretory vesicles or embedded in the plasma membrane, and for degradation **(brown)** is a toxic misfolded protein being degraded by an autophagosome by autophagy **(upper)** or by a proteasome via the ubiquitin proteasome system **(lower)**.

to the accumulation of misfolded proteins and proteotoxicity or proteinopathy (Hightower, 1991; Douglas and Cyr, 2010), which in cardiac myocytes is associated with ischemic heart disease, as well as hypertrophic and dilated cardiomyopathies (McLendon and Robbins, 2015; Arrieta et al., 2018). At the least, the misfolding of proteins can impair their functions, but of potentially greater impact is that misfolded proteins can form toxic polypeptides, aggregates, and pre-amyloid oligomers inside and outside of cells that broadly affect cardiac myocyte function and viability, leading to heart failure (Wang and Robbins, 2006; McLendon and Robbins, 2011; Parry et al., 2015). In addition to heart disease, impaired proteostasis has been linked to numerous other pathologies including atherosclerosis, diabetes, fatty liver disease, and neurodegenerative diseases (Wang and Kaufman, 2012, 2014, 2016; Rivas et al., 2015; Hetz and Saxena, 2017; Valenzuela et al., 2018); moreover, impaired proteostasis can occur as a function of the aging process (Kikis et al., 2010; Lakatta, 2015; Hipp et al., 2019).

Since disease- and age-related protein misfolding and proteotoxicity has been found in many organs, numerous studies have focused on discovering components of the proteostasis network in hopes of identifying potential targets for therapies to minimize the untoward effects of proteotoxicity on organ function. These studies have revealed that mechanisms responsible for the surveillance of the structural integrity of nascent and mature proteins, as well as the processes that determine the fate of terminally misfolded proteins, reside in many cellular locations (Labbadia and Morimoto, 2015). One fate of terminally misfolded proteins is degradation by the ubiquitin proteasome system (UPS), the components of which are localized to specific regions of cells (Pohl and Dikic, 2019). For example, proteasomes have been found on cardiac myocyte contractile elements, primarily at the Z-line of sarcomeres and in the cytosol and nucleus, as well as decorating the surface of many organelles, such as the ER, mitochondria, and lysosomes (Brooks et al., 2000; Wojcik and DeMartino, 2003; Glembotski, 2012a; Bard et al., 2018). However, toxic proteins must still be degraded, even if they are generated in cellular regions that do not have proteasomes. For example, terminally misfolded proteins can be degraded in a proteasome-independent manner by autophagy (Chen et al., 2019). Organelle- and subcellular-specific forms of autophagy, such as mitophagy (Gustafsson and Dorn, 2019) and ER-autophagy, or reticulophagy (Wilkinson, 2019), also degrade terminally misfolded proteins and in so doing, they contribute to maintaining proteome integrity.

Most proteins fold co-translationally and in some cases, folding into the final active configuration is a molecular trial-and-error process (Choi et al., 2013). In fact, it is thought that protein-folding process results in as much as 30% of proteins never reaching their active folded configurations; such proteins are degraded either during or soon after translation (Schubert et al., 2000). This suggests that the elements of the proteostasis network that maintain proteome integrity must be physically located near nodal points of protein synthesis. Since secreted and membrane proteins, which account for as much as 40% of proteins made in eukaryotic cells (Fregno and Molinari, 2019), are synthesized at the endoplasmic reticulum (ER), the ER is a natural node for the cellular proteostasis network. In cardiac myocytes, the ER includes an extensive sarcoplasmic reticulum involved in contractile calcium handling (Bers, 2002a,b). However, while it has been less studied than calcium handling, the ER in cardiac myocytes is also important for the synthesis of many membrane and secreted proteins that are important for viability and contractile function, including hormones, growth factors and stem cell homing factors, as well as ion channels and many other proteins that are critical for excitation-contraction coupling (Glembotski, 2012b).

ER STRESS AND THE UNFOLDED PROTEIN RESPONSE

Soluble ER proteins, which include secreted and ER-resident proteins, and membrane proteins are made on ER-bound ribosomes, where they are co-translationally translocated through an ER membrane channel, the translocon, across the ER membrane into the lumen of the ER, or they are embedded in the ER membrane (Lingappa and Blobel, 1980; Kelly, 1985; Nicchitta, 2002; Egea et al., 2005; Viotti, 2016; Glembotski, 2017). This is followed by continued folding of the nascent proteins, as well as post-translational modifications, such as glycosylation,

phosphorylation, disulfide bond formation, and proteolytic processing, most of which also affect the folding process and take place *en route* to their final destinations (Braakman and Bulleid, 2011; Steiner, 2011). Conditions that alter the environment in the ER in ways that impair any of these processes can cause ER stress, which can lead to the accumulation of potentially proteotoxic misfolded proteins in the ER lumen or membrane (Paschen and Doutheil, 1999; Welihinda et al., 1999; Hampton, 2000; Urano et al., 2000; Berridge, 2002; Rutkowski and Kaufman, 2004). Conditions that place higher demands on the ER protein-synthesis, -trafficking and -routing machinery, such as high levels of protein synthesis at the ER, can also lead to ER stress (Oakes, 2017; Su and Dai, 2017). For example, β-cells of the pancreas make so much insulin, which is synthesized and trafficking by the ER/Golgi secretory pathway that they are continually under ER stress (Iwawaki et al., 2004; Eizirik and Cnop, 2010; Hodish et al., 2011).

When the ER environment is altered in ways that cause ER stress, ER protein misfolding activates the ER unfolded protein response (UPR) (**Figure 2A**). There are three main branches of the UPR that are regulated by ER transmembrane protein sensors of ER stress; ATF6α (activation of transcription factor 6, called ATF6 from here on), PERK [protein kinase R (PKR)-like kinase, and IRE1 (inositol requiring enzyme 1)] (Glembotski, 2007; Hetz and Glimcher, 2011; Walter and Ron, 2011). ATF6 is a transcription factor (Wang et al., 2000; Glembotski, 2014). IRE1 is a nuclease that splices the XBP1 mRNA so it encodes an active transcription factor called XBP1 spliced (XBP1s) (Urano et al., 2000). PERK is a kinase that phosphorylates the translation initiation factor, eIF2α on Ser-51, which causes global translational arrest, but allows for the continued translation of a select subset of mRNAs that encode proteins that are necessary for the adaptive UPR (Schroder and Kaufman, 2006). Many of the genes induced by ATF6 and XBP1s, as well as other events that lie down stream of PERK, are initially oriented toward restoring ER protein folding. Such genes that are upregulated during the initial phases of UPR activation encode ER chaperones, protein disulfide isomerases (PDIs), and proteins involved in ER associated protein degradation (ERAD). ERAD is a specialized form of protein degradation that involves the retrotranslocation of misfolded proteins out of the ER lumen or membrane, followed by their ubiquitylation by ER-bound E3-ubiquitin ligases on the cytosolic face of the ER, which marks them for degradation by proteasomes, also located on the cytosolic face of the ER (Hampton, 2002; McCracken and Brodsky, 2003; Ahner and Brodsky, 2004; Meusser et al., 2005; Kuhnle et al., 2019).

Many early studies of the UPR used chemical inducers of ER stress, such as thapsigargin and tunicamycin (D'Amico et al., 1992; Wong et al., 1993), which are more robust activators of the UPR than physiological or pathological conditions that activate the UPR. These early studies showed that many of the effects of the different branches of the UPR overlap, i.e., induction of ER chaperones and ERAD components by ATF6 and IRE1 branches, while others did not, e.g., induction of ATF4 and CHOP by PERK activation (**Figure 2A**). However, as the field matured it was found that the effects of each branch of the UPR can be quite different when observed under more subtle

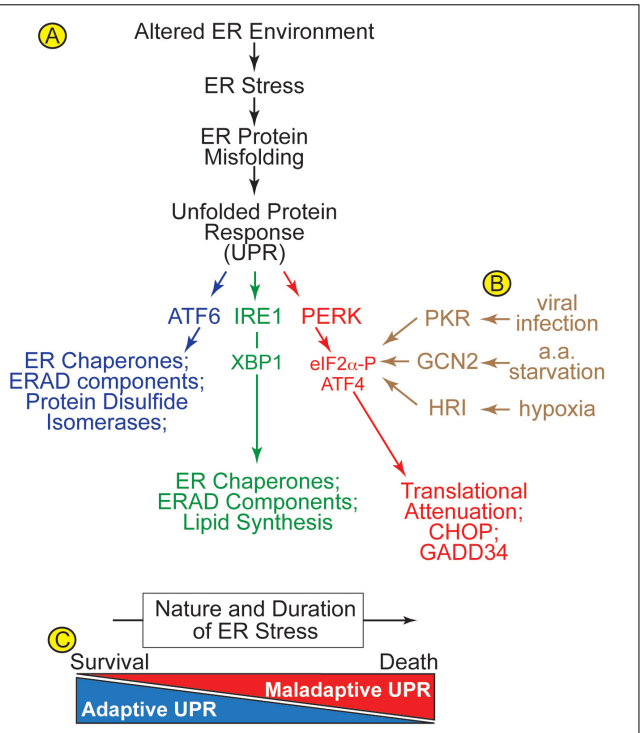


FIGURE 2 | (A) Alterations in the ER environment cause ER stress and ER protein misfolding, which activates three arms of the unfolded protein response (UPR) via three ER transmembrane proteins, ATF6, IRE1, and PERK. **(B)** The integrated stress response includes ER stress, viral infection, amino acid starvation, and hypoxia, which activate four different kinases, PKR-like ER kinase (PERK), protein kinase double stranded RNA-dependent (PKR), general control non-derepressible-2 (GCN2), and heme-regulated inhibitor (HRI), respectively, all of which converge on the phosphorylation of eIF2α on Ser-51. **(C)** Acute ER stress activates survival-oriented adaptive aspects of the UPR, while chronic ER stress leads to death-oriented maladaptive aspects of the UPR. The balance between adaptive and maladaptive UPR pathways is determined by the nature and duration of the ER stress.

stress conditions, including those that occur during pathology. For example, amongst the three branches of the UPR, the IRE1/XBP1 arm is a specific inducer of genes involved in protein O-GlcNAcylation in the ischemic heart (Wang et al., 2014), while ATF6 is an inducer of certain antioxidant genes during myocardial ischemia/reperfusion (I/R) (Jin et al., 2017). Studies such as these provide evidence that the downstream effects of the three branches of the UPR probably overlap less than originally observed when thapsigargin and tunicamycin were used to elicit ER stress. Contributing further to this complexity is the finding that ATF6 can induce XBP1 (Lee et al., 2002), and ATF6 can induce itself (Misra et al., 2013), underscoring the not-well-understood autoregulation that exists amongst the UPR branches (Brewer, 2014). Further complicating matters is the fact that, in addition to PERK, there are at least three other kinases known to phosphorylate eIF2α as part of the integrated stress response, one component of which is ER stress (**Figure 2B**; Donnelly et al., 2013). Moreover, the temporal dynamics with which the three branches of the UPR are activated differs, depending on the cell type, as well as the nature, strength, and duration of

the stress (Marton et al., 2017; Sharma et al., 2019). These temporal dynamics appear to dictate whether the UPR is adaptive or maladaptive (**Figure 2C**). The adaptive UPR is the first to be activated, and its major role is to restore ER proteostasis, improve protein folding and avoid proteotoxicity. However, if the adaptive UPR does not sufficiently fortify the ER protein-folding machinery, then continued ER stress is associated with a conversion of UPR signaling from adaptive to maladaptive, as the downstream events that are regulated by the UPR shift from being survival oriented to cell death oriented (Sano and Reed, 2013).

ATF6 IS AN ADAPTIVE RESPONDER TO ER STRESS

Mechanism of ATF6 Activation During ER Stress

The focus of this review is the ATF6 arm of the UPR, which has major adaptive effects in numerous cell types (Wu et al., 2007; Yamamoto et al., 2007; Wang et al., 2018; Glembotski et al., 2019). The activation of ATF6 as a transcription factor takes place very soon after the onset of ER stress. At this time ATF6 regulates a gene program that fosters adaptive UPR responses, in part because so many ER-resident protein-folding proteins, such as chaperones, are induced at this early time by ATF6 (Mao et al., 2006; Martindale et al., 2006). Moreover, several studies have examined the effects of ATF6 in the heart in mice, *in vivo*, and have revealed the adaptive effects of ATF6 during physiological and pathological conditions (Martindale et al., 2006; Jin et al., 2017; Yu et al., 2017; Blackwood et al., 2019a,b).

A number of studies have elucidated the mechanism of ATF6 activation. In the absence of ER stress, inactive ATF6 is a 90 kD ER transmembrane protein (Haze et al., 1999) (**Figure 3A**). Upon ER stress, ATF6 senses the accumulation of misfolded proteins in the ER. In part, this sensing mechanism involves the ER chaperone, GRP78. In the absence of ER stress, GRP78 binds to the ER luminal domain of ATF6 and, by virtue of the ER-retention sequence at the C-terminus of GRP78 it anchors ATF6 in the ER, as ATF6 has no known ER-retention sequence (Chen et al., 2002; Shen et al., 2005). Upon ER stress GRP78 dissociates from, and thus, releases ATF6, allowing it to relocate to the next destination in the secretory pathway, the Golgi (**Figure 3B**; Shen et al., 2002). In the Golgi, ATF6 is cleaved by S1P and S2P proteases by regulated intramembrane proteolysis (Ye et al., 2000). This cleavage liberates the N-terminal 50 kD cytosolic portion of ATF6, which has a nuclear localization sequence, facilitating its movement to the nucleus, where it acts as a transcription factor to induce genes that encode proteins that fortify the ER protein-folding environment (**Figure 3C**; Haze et al., 1999). In the heart, ATF6 has been shown to induce many genes that are known to serve roles in the adaptive UPR (Belmont et al., 2008; Blackwood et al., 2019b). These canonical ATF6-inducible genes encode proteins, most of which are ER-resident chaperones (e.g., GRP78), PDIs, and ERAD components (**Figure 3D**) that localize to the ER where they contribute to restoring ER protein folding (**Figure 3E**). However, it was found in the heart that ATF6 also induces non-canonical adaptive genes that were not previously known to be ER stress-response genes, many of which encode proteins that do not even reside in the ER (**Figure 3F**; Jin et al., 2017).

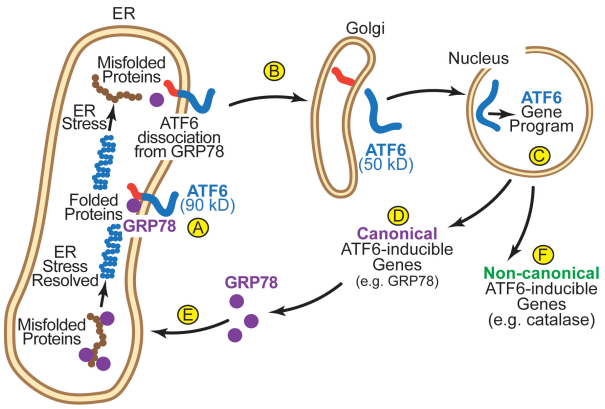


FIGURE 3 | (A) In its inactivated state, ATF6 is a 90 kD ER transmembrane protein that is anchored in the membrane by GRP78. **(B)** Upon ER stress, GRP78 dissociates from the ER luminal domain of ATF6, which allows the 90 kD form of ATF6 to translocate to the Golgi, where is it cleaved by S1P and S2P to liberate the N-terminal approximately 400 amino acids (50 kD) of ATF6 from the ER membrane. **(C)** The clipped form of ATF6 has a nuclear localization sequence, which facilitates its movement to the nucleus where it binds to specific regulatory elements in ATF6-responsive genes, such as ER stress response elements (ERSEs), and induces the ATF6 gene program. **(D)** The canonical ATF6 gene program comprises genes that encode proteins that localize to the ER, such as the chaperone, GRP78 **(E)**, where they fortify ER protein folding. **(F)** The non-canonical ATF6 gene program comprises genes that encode proteins not typically categorized as ER stress-response proteins, such as catalase, which localize to regions of the cell outside the ER.

ATF6 Is Rapidly Degraded When Activated

One of the most fascinating findings regarding ATF6 was the discovery in model cell lines that the active form of ATF6 is rapidly degraded (Thuerauf et al., 2002). In fact, the degradation is so rapid that it is difficult to find the cleaved form of ATF6 by immunoblotting unless proteasome inhibitors are used. The rapid degradation of ATF6 suggests that its activity is designed to be transient; although the reasons for this are not yet known, it is apparent that this transient activation leads to transient induction of ATF6 responsive genes, and that this temporal sequence must be an important determinant of the effects of these genes. In addition to ATF6, there are a number of other transcription factors that are rapidly degraded, once they are activated (Geng et al., 2012). Thus, while ATF6 is not unique in this regard, functional mapping of the domains of ATF6 have led to novel findings regarding the mechanism by which ATF6 transcriptional activity and degradation are regulated. These mapping studies, done in cell lines, demonstrated that within the N-terminal transcriptional activation domain of ATF6 is a stretch of 8 amino acids with a sequence that is very similar to a sequence of amino acids found in the rapidly degraded viral transcription factor, VP16 (Thuerauf et al., 2002). In VP16, this eight amino acid region is responsible for its

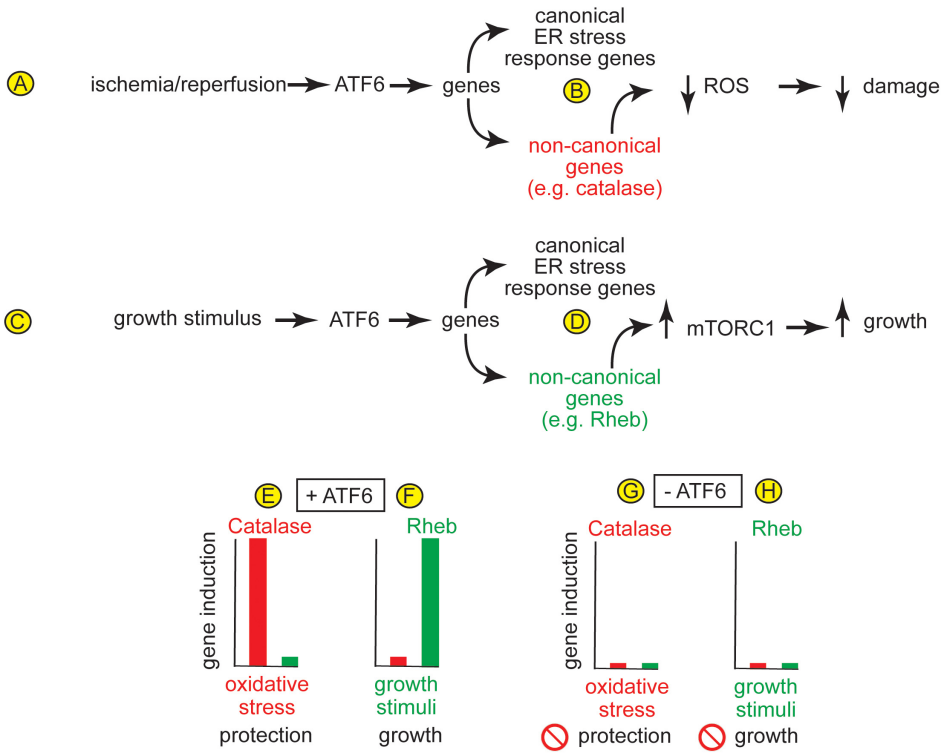


FIGURE 4 | (A) In the heart, ischemia/reperfusion activates ATF6, which **(B)** induces canonical ER stress-response genes, as well as non-canonical genes, such as the peroxisomal antioxidant, catalase, which resides in peroxisomes and protects from the ROS that are generated during I/R. **(C)** In the heart, growth stimuli activate ATF6, which **(D)** induces canonical ER stress-response genes, as well as non-canonical genes, such as the small GTP-binding protein, Rheb, which is an activator of the growth-promoting kinase complex, mTORC1. **(E,F)** In the heart, different stimuli induce different ATF6-dependent genes. For example, **(E)** oxidative stress induces catalase but not rheb, and catalase protects the heart from damage, while **(F)** growth stimuli induce rheb but not catalase, which is required for physiological and pathological hypertrophic growth of the heart. **(G,H)** The absence of induction of any of these genes when ATF6 is deleted from cardiac myocytes supports the view that gene induction by both oxidative stress and growth stimuli is dependent upon ATF6.

potent transcriptional activity, as well as its rapid degradation (Tanaka, 1996). Mutation analyses showed that as in VP16, this region of ATF6 is responsible for its transcriptional activity and its rapid degradation (Thuerauf et al., 2002). In fact, detailed functional mapping of ATF6 showed that the transcriptional activation domain also serves as the signal for rapid degradation. Additional studies demonstrated that ATF6 was rapidly degraded only when it was actively engaged in transcriptional activation, and that any mutations introduced into ATF6 that decreased it transcriptional activity coordinately increased its half-life (Thuerauf et al., 2007). Finally, a different form of ATF6, called ATF6β, which is also activated during ER stress, does not have the VN8 region but is similar to ATF6 in many other regions. It was further shown that it was because ATF6β does not have the VN8, it has very low transcription factor activity and it is slowly degraded, thus it has molecular characteristics that oppose those of ATF6a. In fact, since ATF6 functions as a dimer, and since it can dimerize with ATF6β, the relative amounts of these two forms of ATF6 can dictate the composition of ATF6 dimers in ways that determine the strength with which the ATF6 gene program is induced (Thuerauf et al., 2007). For example, in that study it was shown that the transcription factor activity and the stability of dimers decreased coordinately in the following order: ATF6-ATF6 > ATF6-ATF6β > ATF6β-ATF6β. Thus, it is possible that if ATF6β is activated by ER stress at a slightly later time than ATF6 it may contribute to decreasing the transcriptional induction effects of ATF6, thus ensuring the transient and, thus, adaptive nature of ATF6-mediated gene induction.

Transgenic Mice Reveal Adaptive Roles for ATF6 *in vivo*

While Mice Reveal Adaptive Roles for ATF6 *in vivo* the precise reasons for the transient nature of ATF6 activation are yet to be determined, and while the importance of the functional and physical interactions between ATF6 and ATF6β are not completely understood, it is apparent that the relatively brief time of ATF6 activation after ER stress must be important for its adaptive properties. Based on this premise, the ability to carefully regulate the extent of ATF6 activation was a consideration when ATF6 transgenic mice were prepared (Martindale et al., 2006). Accordingly, in those studies the transgenic mice were designed so they express the active form of ATF6 fused to the mutant mouse estrogen receptor (MER), which can bind tamoxifen (Zhang et al., 1996). Based on other studies with MER fusion protein expression in mice, it was reasoned that in the absence of

tamoxifen, ATF6-MER would assume a conformation that blocks its transcriptional activity. However, when tamoxifen binds to ATF6-MER, the conformation would change to an active form of ATF6. Accordingly, to study the function of activated ATF6 in cardiac myocytes, *in vivo*, the construct used to generate the mice was prepared so that ATF6-MER expression would be under the control of the αMHC gene, which specifies cardiac myocyte-specific expression (Martindale et al., 2006). Indeed, tamoxifen administration for short periods of time led to the transcriptional induction of known ATF6 gene targets through regulatory regions in the genes called ER stress response elements, or ERSEs. Interestingly, ATF6-MER was not only a robust transcription factor, but it was rapidly degraded; however, both of these properties were dependent upon tamoxifen administration. This demonstrated that, in addition to cultured model cell lines, the degraded-when-active property of ATF6 could also be observed in cardiac myocytes, *in vivo*. Accordingly, this ATF6-transgenic (TG) mouse model allowed the precise temporal regulation of ATF6 activation in the heart in the absence of ER stress, so the function of only the ATF6 arm of the UPR in cardiac myocytes could be determined. Moreover, this mouse model facilitated the identification of genes that are regulated by ATF6 in the mouse heart, *in vivo*.

ROLES FOR ATF6 IN THE HEART

ATF6 Is Activated by Ischemia/Reperfusion and Is Protective

Soon after the ATF6-MER transgenic mice were developed they were used to determine the role for activated ATF6 in cardiac myocytes, *in vivo*. It was found that activated ATF6 induced canonical ATF6-dependent ER stress response genes, such as ER chaperones, and conferred protection from I/R damage in *ex vivo* isolated perfused heart preparations and maintained contractile function (Martindale et al., 2006). This was the first report that activated ATF6 could be protective in any tissue, *in vivo*. These findings were coupled with other studies showing that in wild type mice, I/R could activate ATF6 (Blackwood et al., 2019a), leading to the hypothesis that when I/R activates ATF6, the genes induced by ATF6 contribute to protection against I/R damage and, thus play a role in maintaining cardiac function (**Figure 4A**). A subsequent study that also indicated that ATF6 reduces damage in the heart, examined the effects of ATF6 inhibition using either a chemical inhibitor of ATF6 or transgenic overexpression of dominant negative ATF6 in a mouse model of myocardial infarction (Toko et al., 2010). That study showed that inhibiting ATF6 increased the damage after MI. Another study examined roles for ATF6 outside the heart, where ATF6-MER was expressed specifically in mouse forebrain neurons *in vivo*. In that study, activated ATF6 protected neurons from damage a mouse model of ischemic stroke (Yu et al., 2017). These findings stimulated the search for the mechanism of protection from I/R damage. Since I/R damage is caused mainly by the generation of damaging reactive oxygen species (ROS) by mitochondria during reperfusion, it was not obvious how the canonical roles for ATF6, such as the induction of ER chaperones, like GRP78, could contribute to protection. Accordingly, transcriptomics approaches were undertaken in hopes of finding genes that might contribute to the adaptive effects of ATF6, *in vivo*.

MECHANISMS OF THE ADAPTIVE EFFECTS OF ATF6 IN THE HEART

Discovery of Non-canonical Roles for ATF6 in the Heart

Initial microarray studies to identify the genes induced by ATF6 in the hearts of ATF6-MER mice provided a wealth of information, the most provocative being that there were numerous genes induced by ATF6 that encoded proteins that were not previously known to be ATF6- or ER stress-inducible, and did not code for proteins that reside in the ER. These non-canonical ATF6-inducible genes provided the first clue that in the mouse myocardium, the function of ATF6 was much broader than the canonical ER stress response (Belmont et al., 2008). For example, in that study it was shown that ATF6 might affect myocardial growth by inducing the NFAT regulator, RCAN. Another study used a micro-RNA array analysis to define the microRNAs regulated by ATF6, and in the process demonstrated that one ATF6-downregulated microRNA was specific for the ER luminal calcium-binding protein, calreticulin, which implicated ATF6 as a regulator of the expression of calcium-handling proteins in the heart (Belmont et al., 2012).

ATF6 Induces Antioxidant Genes in the Heart

As a complement to the ATF6 gain-of-function approach afforded by the ATF6-MER mice, ATF6 gene deletion was used to determine roles for endogenous ATF6 in mouse hearts under physiological and pathological conditions. It was found that ATF6 knockout mice (ATF6 KO) exhibited greater damage in mouse models of *ex vivo* and *in vivo* myocardial I/R, which was also consistent with the hypothesis that in the heart, I/R-mediated ATF6 activation led to the induction of genes that could protect against I/R damage (Jin et al., 2017). In that same study, transcriptome analyses provided a surprising result, that in cardiac myocytes, in addition to canonical ATF6-inducible genes, like GRP78, ATF6 induced numerous antioxidant genes, one of which encodes the potent antioxidant enzyme, catalase (**Figure 4B**). This study went on to show that catalase is a previously unidentified ER stress response gene, and that it is induced in the heart in an ATF6-dependent manner during I/R. Supporting this assertion was the finding that the addition of exogenous catalase restores protection against I/R damage to the hearts of mice in which ATF6 and been deleted, demonstrating that it is a least partly by virtue of inducing catalase induction that ATF6 mitigates the generation of ROS and reduces I/R damage in the mouse heart (Jin et al., 2017). It is interesting to note that catalase is not an ER-resident protein, but is instead normally expressed in peroxisomes (Poole et al., 1969). Accordingly, the catalase in peroxisomes serves to neutralize some of the ROS generated in mitochondria during I/R in the heart.

ATF6 Is Required for Hypertrophic Growth of the Heart

Taking the ATF6 gene deletion studies a step further were studies in which ATF6 was conditionally deleted specifically from cardiac myocytes (ATF6 cKO) (Blackwood et al., 2019b). Compared to wild type mice, myocardial damage was exacerbated in ATF6 cKO mice subjected to I/R *in vivo* (Blackwood et al., 2019b). These findings corroborated the studies with ATF6 KO mice and further supported that ATF6-induced genes that protected from I/R damage (Jin et al., 2017). However, another surprise finding was that cardiac specific deletion of ATF6 decreased hypertrophic growth of the heart *in vivo* upon short times of pressure overload-induced pathological hypertrophy and decreased cardiac function (Blackwood et al., 2019b). In fact, physiological cardiac hypertrophy in mice subjected to free-wheel exercise was lower in the hearts of ATF6 cKO mice compared to control mice, supporting the hypothesis that growth stimuli activate ATF6-dependent genes that are required for cardiac myocyte growth (**Figure 4C**). In that study, transcript profiling revealed that, in addition to canonical ATF6-inducible genes, like GRP78, some of the non-canonical genes induced by ATF6 in mouse hearts were growth regulators, one of which was the small GTP-binding protein, Rheb (**Figure 4D**). Rheb had previously been shown to be a required activator of the growth-promoting kinase, mTORC1 in neurons (Yamagata et al., 1994). In the heart, it was shown that ATF6 was required to induce Rheb and, thus, mTORC1-dependent growth in acute models of pressure-overload induced pathological hypertrophy and in longer-term freewheel exercise-induced physiological hypertrophy (Blackwood et al., 2019b). In that study, further mechanistic examination showed that it is the increase in protein synthesis during hypertrophic growth of the heart that increases the protein-folding demand in cardiac myocytes, leading to activation of ATF6 and the induction of Rheb and activation of mTORC1. Similar results were found in a more recent publication, where it was shown that deletion of ATF6α or ATF6β resulted in a reduction of pathological cardiac hypertrophy at early times after pressure overload (Correll et al., 2019). In that report it was concluded that deletion of either ATF6α or ATF6β had a similar impact on hypertrophy, suggesting some redundancy in their functions.

DIFFERENT ATF6 ACTIVATORS INDUCE DIFFERENT ATF6-DEPENDENT GENE PROGRAMS

While activation of ATF6 in the ATF6-MER TG mice was a useful model system for identifying numerous genes that could be induced specifically by the ATF6 arm of the UPR in the heart, it was of interest to determine what ATF6-dependent genes were induced under physiological and pathophysiological conditions that activate endogenous ATF6. Such studies led to one of the most remarkable discoveries, that different activators of ATF6 could induce different ATF6-dependent gene programs. For example, it was found that in mice treatments that cause oxidative stress, such as I/R, activate ATF6, which induces the antioxidant, catalase, but not the growth-promoter, Rheb (**Figure 4E**). In contrast, treatments that stimulate growth, such as pressure overload or exercise, which also activate ATF6, induce Rheb but not catalase (**Figure 4F**). Importantly, ATF6 gene deletion showed that each of these stimulus-specific gene programs is ATF6-dependent (**Figures 4G,H**; Blackwood et al., 2019b). Further promoter analysis of these genes demonstrated that ATF6 bound specifically to ERSEs in the catalase and rheb gene promoters, but this binding occurred only when cells were subjected to oxidative stress or growth stimuli, respectively. These studies suggest that the ATF6 gene program is tailored to suit the needs of cells, which differ, depending on the form of stress. Further support of this is a study in yeast and mammalian cell lines showing that ATF6 can be activated by specific sphingolipids, such as dihydrosphingosine (DHS) and dihydroceramide (DHC), and this occurs in the absence of ER protein misfolding (Tam et al., 2018). It seems possible that different stress conditions that all activate ATF6 may differentially activate other, as yet unidentified components of ER stress-inducible transcriptional programs, the latter of which may be responsible for imparting stress-specific gene regulation to ATF6.

ATF6-BASED SMALL MOLECULE THERAPIES FOR HEART DISEASE

In more recent studies a chemical biology approach has been taken in an effort to capitalize on the beneficial effects of ATF6 activation for the development of potential therapeutics for heart disease. These studies began with the screening of a small molecule library of over 600,000 different compounds for those that would activate ATF6 in 293 cells (Plate et al., 2016). This study used a highly rigorous multiplex screening approach in hopes of finding compounds that would activate only the ATF6 arm of the UPR, without activating other signaling pathways and without cause cell death. While several compounds were initially identified, one of them, compound 147, was the most selective for ATF6 activation in 293 cells, as well as isolated cardiac myocytes. In subsequent studies it was shown that compound 147 could be administered to mice without any untoward effects in the absence of pathology; but in mice subjected to *in vivo* myocardial I/R, 147 administration decreased cardiac damage and improved contractile function (Blackwood et al., 2019a). In that study, it was shown that compound 147 also decreased damage and improved motor function in a mouse model of cerebral I/R and decreased kidney damage in a mouse model of renal I/R while improving glomerular filtration. A subsequent study of the mechanism of action of compound 147 showed that this compound itself is not the active species, but it is converted to the active compound by a cytochrome P450 enzyme that is specifically expressed in the ER (Paxman et al., 2018), which probably accounts for the high selectivity of compound 147 as an activator of ATF6. The results obtained so far with compound 147 suggest that it may be a good candidate for consideration as a treatment for ischemic disease and other pathologies involving protein misfolding, including cardiomyopathy and heart failure.

Accordingly, next steps in moving compound 147 toward clinical application would involve animal studies of the pharmacological properties of compound 147, including pharmacokinetics and toxicology, as well as examining the effects of 147 in large animal models of disease, such as pig models.

CONCLUSION AND FUTURE DIRECTIONS

Like many signaling pathways, the UPR exhibits both adaptive and maladaptive activities depending upon the nature and duration of the conditions that activate the UPR. While it was important to discover the conditions leading to either the adaptive and maladaptive UPR, this dual nature of the UPR makes it difficult to design UPR-based therapeutics. The ATF6 arm of the UPR provides fertile ground for developing adaptive therapeutics because it is mostly known for its adaptive effects. However, even ATF6 probably has both adaptive and maladaptive properties, which may be why it exhibits a "degraded-when-active" property. Thus, it is reasonable to posit that a useful ATF6-based therapy should exhibit a rapid onset, as well as a relatively transient action, thus mimicking, to some extent, the way the adaptive effects of ATF6 are activated during ER stress. The development of gene therapy approaches to increase activated ATF6, while useful in experimental animal systems (Jin et al., 2017), may not represent the best therapeutic approach because of the relatively long-term effects of such therapies. However, the ability of a small molecule, like compound 147, to activate endogenous ATF6 in a relatively transient manner mimics the adaptive effects of ATF6 *in vivo* and as such, may represent a worthwhile direction for future therapeutic development (Blackwood et al., 2019a). Indeed, the lack of untoward effects of compound 147, coupled with its relatively brief action *in vivo* set the stage for a bright future for such an approach. Moreover, the search for better ATF6 activators, such as those that might capitalize on the stimulus-specific activation properties of ATF6 and induce the ATF6 gene program only during I/R, or pressure overload might be sought. Also, selective activators of the other arms of the UPR will likely result in the discovery of lead compounds, like 147, that can be used in therapeutic approaches aimed at capitalizing on the adaptive effects of the UPR in all tissue and cell types.

AUTHOR CONTRIBUTIONS

All of the authors participated in all aspects of preparing this manuscript.

FUNDING

This work was supported by NIH grants 1HL135893, 1HL141463, and 1HL149931 to CG and NIH grant 1F31HL140850 and the Inamori Foundation Graduate Fellowship to EB, and the ARCS Foundation, Inc., San Diego Chapter to EB and WS. AA, EB, and WS are Rees-Stealy Research Foundation Phillips Gausewitz, M.D., Scholars of the SDSU Heart Institute.

REFERENCES

Ahner, A., and Brodsky, J. L. (2004). Checkpoints in ER-associated degradation: excuse me, which way to the proteasome? *Trends Cell Biol.* 14, 474–478. doi: 10.1016/j.tcb.2004.07.013

Arrieta, A., Blackwood, E. A., and Glembotski, C. C. (2018). ER protein quality control and the unfolded protein response in the heart. *Curr. Top. Microbiol. Immunol.* 414, 193–213. doi: 10.1007/82_2017_54

Balch, W. E., Morimoto, R. I., Dillin, A., and Kelly, J. W. (2008). Adapting proteostasis for disease intervention. *Science* 319, 916–919. doi: 10.1126/science.1141448

Bard, J. A. M., Goodall, E. A., Greene, E. R., Jonsson, E., Dong, K. C., and Martin, A. (2018). Structure and function of the 26S proteasome. *Annu. Rev. Biochem.* 87, 697–724.

Belmont, P. J., Chen, W. J., Thuerauf, D. J., and Glembotski, C. C. (2012). Regulation of microRNA expression in the heart by the ATF6 branch of the ER stress response. *J. Mol. Cell. Cardiol.* 52, 1176–1182. doi: 10.1016/j.yjmcc.2012.01.017

Belmont, P. J., Tadimalla, A., Chen, W. J., Martindale, J. J., Thuerauf, D. J., Marcinko, M., et al. (2008). Coordination of growth and endoplasmic reticulum stress signaling by regulator of calcineurin 1 (RCAN1), a novel ATF6-inducible gene. *J. Biol. Chem.* 283, 14012–14021. doi: 10.1074/jbc.M709776200

Berridge, M. J. (2002). The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium* 32, 235–249. doi: 10.1016/s0143416002001823

Bers, D. M. (2002a). Cardiac excitation-contraction coupling. *Nature* 415, 198–205.

Bers, D. M. (2002b). Sarcoplasmic reticulum Ca release in intact ventricular myocytes. *Front. Biosci.* 7, d1697–d1711.

Blackwood, E. A., Azizi, K., Thuerauf, D. J., Paxman, R. J., Plate, L., Kelly, J. W., et al. (2019a). Pharmacologic ATF6 activation confers global protection in widespread disease models by reprograming cellular proteostasis. *Nat. Commun.* 10:187. doi: 10.1038/s41467-018-08129-2

Blackwood, E. A., Hofmann, C., Santo Domingo, M., Bilal, A. S., Sarakki, A., Stauffer, W., et al. (2019b). ATF6 regulates cardiac hypertrophy by transcriptional induction of the mTORC1 activator. *Rheb. Circ. Res.* 124, 79–93. doi: 10.1161/CIRCRESAHA.118.313854

Braakman, I., and Bulleid, N. J. (2011). Protein folding and modification in the mammalian endoplasmic reticulum. *Annu. Rev. Biochem.* 80, 71–99. doi: 10.1146/annurev-biochem-062209-093836

Brewer, J. W. (2014). Regulatory crosstalk within the mammalian unfolded protein response. *Cell. Mol. Life Sci.* 71, 1067–1079. doi: 10.1007/s00018-013-1490-2

Brooks, P., Fuertes, G., Murray, R. Z., Bose, S., Knecht, E., Rechsteiner, M. C., et al. (2000). Subcellular localization of proteasomes and their regulatory complexes in mammalian cells. *Biochem. J.* 346(Pt 1), 155–161. doi: 10.1042/bj3460155

Chen, R. H., Chen, Y. H., and Huang, T. Y. (2019). Ubiquitin-mediated regulation of autophagy. *J. Biomed. Sci.* 26:80. doi: 10.1186/s12929-019-0569-y

Chen, X., Shen, J., and Prywes, R. (2002). The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the golgi. *J. Biol. Chem.* 277, 13045–13052. doi: 10.1074/jbc.m110636200

Choi, S. I., Kwon, S., Son, A., Jeong, H., Kim, K. H., and Seong, B. L. (2013). Protein folding in vivo revisited. *Curr. Protein Pept. Sci.* 14, 721–733. doi: 10.2174/13892037140813122717054 4

Correll, R. N., Grimes, K. M., Prasad, V., Lynch, J. M., Khalil, H., and Molkentin, J. D. (2019). Overlapping and differential functions of ATF6alpha versus ATF6beta in the mouse heart. *Sci. Rep.* 9:2059. doi: 10.1038/s41598-019-39515-5

D'Amico, L., Valsasina, B., Daminati, M. G., Fabbrini, M. S., Nitti, G., Bollini, R., et al. (1992). Bean homologs of the mammalian glucose-regulated proteins: induction by tunicamycin and interaction with newly synthesized seed storage

proteins in the endoplasmic reticulum. *Plant J.* 2, 443–455. doi: 10.1046/j.1365-313x.1992.t01-19-00999.x

Donnelly, N., Gorman, A. M., Gupta, S., and Samali, A. (2013). The eIF2alpha kinases: their structures and functions. *Cell. Mol. Life Sci.* 70, 3493–3511. doi: 10.1007/s00018-012-1252-6

Douglas, P. M., and Cyr, D. M. (2010). Interplay between protein homeostasis networks in protein aggregation and proteotoxicity. *Biopolymers* 93, 229–236. doi: 10.1002/bip.21304

Egea, P. F., Stroud, R. M., and Walter, P. (2005). Targeting proteins to membranes: structure of the signal recognition particle. *Curr. Opin. Struct. Biol.* 15, 213–220. doi: 10.1016/j.sbi.2005.03.007

Eizirik, D. L., and Cnop, M. (2010). ER stress in pancreatic beta cells: the thin red line between adaptation and failure. *Sci. Signal.* 3:e7. doi: 10.1126/scisignal.3110pe7

Fregno, I., and Molinari, M. (2019). Proteasomal and lysosomal clearance of faulty secretory proteins: ER-associated degradation (ERAD) and ER-to-lysosome-associated degradation (ERLAD) pathways. *Crit. Rev. Biochem. Mol. Biol.* 54, 153–163. doi: 10.1080/10409238.2019.1610351

Geng, F., Wenzel, S., and Tansey, W. P. (2012). Ubiquitin and proteasomes in transcription. *Annu. Rev. Biochem.* 81, 177–201. doi: 10.1146/annurev-biochem-052110-120012

Glembotski, C. C. (2007). Endoplasmic reticulum stress in the heart. *Circ. Res.* 101, 975–984.

Glembotski, C. C. (2012a). Clarifying the cardiac proteasome paradox: protein quality control. *Circ. Res.* 111, 509–512. doi: 10.1161/circresaha.112.275917

Glembotski, C. C. (2012b). Roles for the sarco-/endoplasmic reticulum in cardiac myocyte contraction, protein synthesis, and protein quality control. *Physiology* 27, 343–350. doi: 10.1152/physiol.00034.2012

Glembotski, C. C. (2014). Roles for ATF6 and the sarco/endoplasmic reticulum protein quality control system in the heart. *J. Mol. Cell. Cardiol.* 71, 11–15. doi: 10.1016/j.yjmcc.2013.09.018

Glembotski, C. C. (2017). Expanding the paracrine hypothesis of stem cell-mediated repair in the heart: when the unconventional becomes conventional. *Circ. Res.* 120, 772–774. doi: 10.1161/circresaha.116.310298

Glembotski, C. C., Rosarda, J. D., and Wiseman, R. L. (2019). Proteostasis and beyond: ATF6 in ischemic disease. *Trends Mol. Med.* 25, 538–550. doi: 10.1016/j.molmed.2019.03.005

Gustafsson, A. B., and Dorn, G. W. II (2019). Evolving and expanding the roles of mitophagy as a homeostatic and pathogenic process. *Physiol. Rev.* 99, 853–892. doi: 10.1152/physrev.00005.2018

Hampton, R. Y. (2000). ER stress response: getting the UPR hand on misfolded proteins. *Curr. Biol.* 10, R518–R521.

Hampton, R. Y. (2002). ER-associated degradation in protein quality control and cellular regulation. *Curr. Opin. Cell Biol.* 14, 476–482. doi: 10.1016/s0955-0674(02)00358-7

Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol. Biol. Cell* 10, 3787–3799. doi: 10.1091/mbc.10.11.3787

Hetz, C., and Glimcher, L. H. (2011). Protein homeostasis networks in physiology and disease. *Curr. Opin. Cell Biol.* 23, 123–125. doi: 10.1016/j.ceb.2011.01.004

Hetz, C., and Saxena, S. (2017). ER stress and the unfolded protein response in neurodegeneration. *Nat. Rev. Neurol.* 13, 477–491. doi: 10.1038/nrneurol.2017.99

Hightower, L. E. (1991). Heat shock, stress proteins, chaperones, and proteotoxicity. *Cell* 66, 191–197. doi: 10.1016/0092-8674(91)90611-2

Hipp, M. S., Kasturi, P., and Hartl, F. U. (2019). The proteostasis network and its decline in ageing. *Nat. Rev. Mol. Cell Biol.* 20, 421–435. doi: 10.1038/s41580-019-0101-y

Hodish, I., Absood, A., Liu, L., Liu, M., Haataja, L., Larkin, D., et al. (2011). In vivo misfolding of proinsulin below the threshold of frank diabetes. *Diabetes* 60, 2092–2101. doi: 10.2337/db10-1671

Iwawaki, T., Akai, R., Kohno, K., and Miura, M. (2004). A transgenic mouse model for monitoring endoplasmic reticulum stress. *Nat. Med.* 10, 98–102. doi: 10.1038/nm970

Jin, J. K., Blackwood, E. A., Azizi, K., Thuerauf, D. J., Fahem, A. G., Hofmann, C., et al. (2017). ATF6 decreases myocardial ischemia/reperfusion damage and links ER stress and oxidative stress signaling pathways in the heart. *Circ. Res.* 120, 862–875. doi: 10.1161/CIRCRESAHA.116.310266

Kelly, R. B. (1985). Pathways of protein secretion in eukaryotes. *Science* 230, 25–32. doi: 10.1126/science.2994224

Kikis, E. A., Gidalevitz, T., and Morimoto, R. I. (2010). Protein homeostasis in models of aging and age-related conformational disease. *Adv. Exp. Med. Biol.* 694, 138–159. doi: 10.1007/978-1-4419-7002-2_11

Kuhnle, N., Dederer, V., and Lemberg, M. K. (2019). Intramembrane proteolysis at a glance: from signalling to protein degradation. *J. Cell Sci.* 132:jcs217745. doi: 10.1242/jcs.217745

Labbadia, J., and Morimoto, R. I. (2015). The biology of proteostasis in aging and disease. *Annu. Rev. Biochem.* 84, 435–464. doi: 10.1146/annurev-biochem-060614-033955

Lakatta, E. G. (2015). So! What's aging? Is cardiovascular aging a disease? *J. Mol. Cell Cardiol.* 83, 1–13. doi: 10.1016/j.yjmcc.2015.04.005

Lee, K., Tirasophon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., et al. (2002). IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev.* 16, 452–466. doi: 10.1101/gad.964702

Lingappa, V. R., and Blobel, G. (1980). Early events in the biosynthesis of secretory and membrane proteins: the signal hypothesis. *Recent Prog. Horm. Res.* 36, 451–475. doi: 10.1016/b978-0-12-571136-4.50018-8

Mao, C., Tai, W. C., Bai, Y., Poizat, C., and Lee, A. S. (2006). In vivo regulation of Grp78/BiP transcription in the embryonic heart: role of the endoplasmic reticulum stress response element and GATA. *J. Biol. Chem.* 281, 8877–8887. doi: 10.1074/jbc.m505784200

Martindale, J. J., Fernandez, R., Thuerauf, D., Whittaker, R., Gude, N., Sussman, M. A., et al. (2006). Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF. *Circ. Res.* 98, 1186–1193. doi: 10.1161/01.res.0000220643.65941.8d

Marton, M., Kurucz, A., Lizak, B., Margittai, E., Banhegyi, G., and Kapuy, O. (2017). A Systems biological view of life-and-death decision with respect to endoplasmic reticulum stress-the role of PERK pathway. *Int. J. Mol. Sci.* 18:58. doi: 10.3390/ijms18010058

McCracken, A. A., and Brodsky, J. L. (2003). Evolving questions and paradigm shifts in endoplasmic-reticulum-associated degradation (ERAD). *Bioessays* 25, 868–877. doi: 10.1002/bies.10320

McLendon, P. M., and Robbins, J. (2011). Desmin-related cardiomyopathy: an unfolding story. *Am. J. Physiol. Heart Circ. Physiol.* 301, H1220–H1228. doi: 10.1152/ajpheart.00601.2011

McLendon, P. M., and Robbins, J. (2015). Proteotoxicity and cardiac dysfunction. *Circ. Res.* 116, 1863–1882. doi: 10.1161/CIRCRESAHA.116.305372

Meusser, B., Hirsch, C., Jarosch, E., and Sommer, T. (2005). ERAD: the long road to destruction. *Nat. Cell Biol.* 7, 766–772. doi: 10.1038/ncb0805-766

Misra, J., Kim, D. K., Choi, W., Koo, S. H., Lee, C. H., Back, S. H., et al. (2013). Transcriptional cross talk between orphan nuclear receptor ERRgamma and transmembrane transcription factor ATF6alpha coordinates endoplasmic reticulum stress response. *Nucleic Acids Res.* 41, 6960–6974. doi: 10.1093/nar/gkt429

Nicchitta, C. V. (2002). A platform for compartmentalized protein synthesis: protein translation and translocation in the ER. *Curr. Opin. Cell Biol.* 14, 412–416. doi: 10.1016/s0955-0674(02)00353-8

Oakes, S. A. (2017). Endoplasmic reticulum proteostasis: a key checkpoint in cancer. *Am. J. Physiol. Cell Physiol.* 312, C93–C102. doi: 10.1152/ajpcell.00266.2016

Parry, T. L., Melehani, J. H., Ranek, M. J., and Willis, M. S. (2015). Functional amyloid signaling via the inflammasome, necrosome, and signalosome: new therapeutic targets in heart failure. *Front. Cardiovasc. Med.* 2:25. doi: 10.3389/fcvm.2015.00025

Paschen, W., and Doutheil, J. (1999). Disturbances of the functioning of endoplasmic reticulum: a key mechanism underlying neuronal cell injury? *J. Cereb. Blood Flow Metab.* 19, 1–18. doi: 10.1097/00004647-199901000-00001

Paxman, R., Plate, L., Blackwood, E. A., Glembotski, C., Powers, E. T., Wiseman, R. L., et al. (2018). Pharmacologic ATF6 activating compounds are metabolically activated to selectively modify endoplasmic reticulum proteins. *eLife* 7:e37168. doi: 10.7554/eLife.37168

Plate, L., Cooley, C. B., Chen, J. J., Paxman, R. J., Gallagher, C. M., Madoux, F., et al. (2016). Small molecule proteostasis regulators that reprogram the ER to reduce extracellular protein aggregation. *eLife* 5:e15550.

Pohl, C., and Dikic, I. (2019). Cellular quality control by the ubiquitin-proteasome system and autophagy. *Science* 366, 818–822. doi: 10.1126/science.aax3769

Poole, B., Leighton, F., and De Duve, C. (1969). The synthesis and turnover of rat liver peroxisomes. II. Turnover of peroxisome proteins. *J. Cell Biol.* 41, 536–546. doi: 10.1083/jcb.41.2.536

Rivas, A., Vidal, R. L., and Hetz, C. (2015). Targeting the unfolded protein response for disease intervention. *Expert Opin. Ther. Targets* 19, 1203–1218. doi: 10.1517/14728222.2015.1053869

Rutkowski, D. T., and Kaufman, R. J. (2004). A trip to the ER: coping with stress. *Trends Cell Biol.* 14, 20–28. doi: 10.1016/j.tcb.2003.11.001

Sala, A. J., Bott, L. C., and Morimoto, R. I. (2017). Shaping proteostasis at the cellular, tissue, and organismal level. *J. Cell Biol.* 216, 1231–1241. doi: 10.1083/jcb.201612111

Sano, R., and Reed, J. C. (2013). ER stress-induced cell death mechanisms. *Biochim. Biophys. Acta* 1833, 3460–3470. doi: 10.1016/j.bbamcr.2013.06.028

Schroder, M., and Kaufman, R. J. (2006). Divergent roles of IRE1alpha and PERK in the unfolded protein response. *Curr. Mol. Med.* 6, 5–36. doi: 10.2174/156652406775574569

Schubert, U., Anton, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W., and Bennink, J. R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404, 770–774. doi: 10.1038/35008096

Sharma, R. B., Snyder, J. T., and Alonso, L. C. (2019). Atf6alpha impacts cell number by influencing survival, death and proliferation. *Mol. Metab.* 27S, S69–S80. doi: 10.1016/j.molmet.2019.06.005

Shen, J., Chen, X., Hendershot, L., and Prywes, R. (2002). ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev. Cell* 3, 99–111. doi: 10.1016/s1534-5807(02)00203-4

Shen, J., Snapp, E. L., Lippincott-Schwartz, J., and Prywes, R. (2005). Stable binding of ATF6 to BiP in the endoplasmic reticulum stress response. *Mol. Cell Biol.* 25, 921–932. doi: 10.1128/mcb.25.3.921-932.2005

Steiner, D. F. (2011). On the discovery of precursor processing. *Methods Mol. Biol.* 768, 3–11. doi: 10.1007/978-1-61779-204-5_1

Su, K. H., and Dai, C. (2017). mTORC1 senses stresses: coupling stress to proteostasis. *Bioessays* 39:201600268.

Tam, A. B., Roberts, L. S., Chandra, V., Rivera, I. G., Nomura, D. K., Forbes, D. J., et al. (2018). The UPR activator ATF6 responds to proteotoxic and lipotoxic stress by distinct mechanisms. *Dev. Cell* 46, 327.e7–343.e7.

Tanaka, M. (1996). Modulation of promoter occupancy by cooperative DNA binding and activation-domain function is a major determinant of transcriptional regulation by activators in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 93, 4311–4315. doi: 10.1073/pnas.93.9.4311

Thuerauf, D. J., Marcinko, M., Belmont, P. J., and Glembotski, C. C. (2007). Effects of the isoform-specific characteristics of ATF6 alpha and ATF6 beta on endoplasmic reticulum stress response gene expression and cell viability. *J. Biol. Chem.* 282, 22865–22878. doi: 10.1074/jbc.m701213200

Thuerauf, D. J., Morrison, L. E., Hoover, H., and Glembotski, C. C. (2002). Coordination of ATF6-mediated transcription and ATF6 degradation by a domain that is shared with the viral transcription factor. *J. Biol. Chem.* 277, 20734–20739. doi: 10.1074/jbc.m201749200

Toko, H., Takahashi, H., Kayama, Y., Okada, S., Minamino, T., Terasaki, F., et al. (2010). ATF6 is important under both pathological and physiological states in the heart. *J. Mol. Cell Cardiol.* 49, 113–120. doi: 10.1016/j.yjmcc.2010.03.020

Urano, F., Bertolotti, A., and Ron, D. (2000). IRE1 and efferent signaling from the endoplasmic reticulum. *J. Cell Sci.* 113(Pt 21), 3697–3702.

Valenzuela, V., Jackson, K. L., Sardi, S. P., and Hetz, C. (2018). Gene therapy strategies to restore ER proteostasis in disease. *Mol. Ther.* 26, 1404–1413. doi: 10.1016/j.ymthe.2018.04.004

Viotti, C. (2016). ER to Golgi-dependent protein secretion: the conventional pathway. *Methods Mol. Biol.* 1459, 3–29. doi: 10.1007/978-1-4939-3804-9_1

Walter, P., and Ron, D. (2011). The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 334, 1081–1086. doi: 10.1126/science.1209038

Wang, M., and Kaufman, R. J. (2016). Protein misfolding in the endoplasmic reticulum as a conduit to human disease. *Nature* 529, 326–335. doi: 10.1038/nature17041

Wang, S., and Kaufman, R. J. (2012). The impact of the unfolded protein response on human disease. *J. Cell Biol.* 197, 857–867. doi: 10.1083/jcb.201110131

Wang, S., and Kaufman, R. J. (2014). How does protein misfolding in the endoplasmic reticulum affect lipid metabolism in the liver? *Curr. Opin. Lipidol.* 25, 125–132. doi: 10.1097/mol.0000000000000056

Wang, X., and Robbins, J. (2006). Heart failure and protein quality control. *Circ. Res.* 99, 1315–1328. doi: 10.1161/01.res.0000252342.61447.a2

Wang, X., Xu, L., Gillette, T. G., Jiang, X., and Wang, Z. V. (2018). The unfolded protein response in ischemic heart disease. *J. Mol. Cell Cardiol.* 117, 19–25. doi: 10.1016/j.yjmcc.2018.02.013

Wang, Y., Shen, J., Arenzana, N., Tirasophon, W., Kaufman, R. J., and Prywes, R. (2000). Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. *J. Biol. Chem.* 275, 27013–27020.

Wang, Z. V., Deng, Y., Gao, N., Pedrozo, Z., Li, D. L., Morales, C. R., et al. (2014). Spliced X-box binding protein 1 couples the unfolded protein response to hexosamine biosynthetic pathway. *Cell* 156, 1179–1192. doi: 10.1016/j.cell.2014.01.014

Welihinda, A. A., Tirasophon, W., and Kaufman, R. J. (1999). The cellular response to protein misfolding in the endoplasmic reticulum. *Gene Expr.* 7, 293–300.

Wilkinson, S. (2019). ER-phagy: shaping up and destressing the endoplasmic reticulum. *FEBS J.* 286, 2645–2663. doi: 10.1111/febs.14932

Wojcik, C., and DeMartino, G. N. (2003). Intracellular localization of proteasomes. *Int. J. Biochem. Cell Biol.* 35, 579–589. doi: 10.1016/s1357-2725(02)00380-1

Wong, W. L., Brostrom, M. A., Kuznetsov, G., Gmitter-Yellen, D., and Brostrom, C. O. (1993). Inhibition of protein synthesis and early protein processing by thapsigargin in cultured cells. *Biochem. J.* 289(Pt 1), 71–79. doi: 10.1042/bj2890071

Wu, J., Rutkowski, D. T., Dubois, M., Swathirajan, J., Saunders, T., Wang, J., et al. (2007). ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress. *Dev. Cell* 13, 351–364. doi: 10.1016/j.devcel.2007.07.005

Yamagata, K., Sanders, L. K., Kaufmann, W. E., Yee, W., Barnes, C. A., Nathans, D., et al. (1994). rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. *J. Biol. Chem.* 269, 16333–16339.

Yamamoto, K., Sato, T., Matsui, T., Sato, M., Okada, T., Yoshida, H., et al. (2007). Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP. *Dev. Cell* 13, 365–376. doi: 10.1016/j.devcel.2007.07.018

Ye, J., Rawson, R. B., Komuro, R., Chen, X., Dave, U. P., Prywes, R., et al. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol. Cell* 6, 1355–1364. doi: 10.1016/s1097-2765(00)00133-7

Yu, Z., Sheng, H., Liu, S., Zhao, S., Glembotski, C. C., Warner, D. S., et al. (2017). Activation of the ATF6 branch of the unfolded protein response in neurons improves stroke outcome. *J. Cereb. Blood Flow Metab.* 37, 1069–1079. doi: 10.1177/0271678X16650218

Zhang, Y., Riesterer, C., Ayrall, A. M., Sablitzky, F., Littlewood, T. D., and Reth, M. (1996). Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic Acids Res.* 24, 543–548. doi: 10.1093/nar/24.4.543

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 23 April 2020
doi: 10.3389/fphys.2020.00389

Molecular Mechanism of Hippo–YAP1/TAZ Pathway in Heart Development, Disease, and Regeneration

Xiaoqing Chen[1,2], *Yilang Li*[1,2], *Jiandong Luo*[1,2]* *and Ning Hou*[1,2]*

[1] *Key Laboratory of Molecular Target & Clinical Pharmacology, School of Pharmaceutical Sciences and the Fifth Affiliated Hospital, Guangzhou Medical University, Guangzhou, China,* [2] *Guangzhou Institute of Cardiovascular Disease, Guangzhou Key Laboratory of Cardiovascular Disease, and The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, China*

OPEN ACCESS

Edited by:
Mark J. Ranek, Johns Hopkins University, United States

Reviewed by:
Hyun Woo Park, Yonsei University, South Korea
Dominic Del Re, Rutgers, The State University of New Jersey, United States

***Correspondence:**
Ning Hou
houning@gzhmu.edu.cn
Jiandong Luo
jiandongluo@hotmail.com

Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *15 November 2019*
Accepted: *01 April 2020*
Published: *23 April 2020*

Citation:
Chen X, Li Y, Luo J and Hou N (2020) Molecular Mechanism of Hippo–YAP1/TAZ Pathway in Heart Development, Disease, and Regeneration. Front. Physiol. 11:389. doi: 10.3389/fphys.2020.00389

The Hippo–YAP1/TAZ pathway is a highly conserved central mechanism that controls organ size through the regulation of cell proliferation and other physical attributes of cells. The transcriptional factors Yes-associated protein 1 (YAP1) and PDZ-binding motif (TAZ) act as downstream effectors of the Hippo pathway, and their subcellular location and transcriptional activities are affected by multiple post-translational modifications (PTMs). Studies have conclusively demonstrated a pivotal role of the Hippo–YAP1/TAZ pathway in cardiac development, disease, and regeneration. Targeted therapeutics for the YAP1/TAZ could be an effective treatment option for cardiac regeneration and disease. This review article provides an overview of the Hippo–YAP1/TAZ pathway and the increasing impact of PTMs in fine-tuning YAP1/TAZ activation; in addition, we discuss the potential contributions of the Hippo–YAP1/TAZ pathway in cardiac development, disease, and regeneration.

Keywords: YAP1/TAZ, Hippo pathway, post-translational modification, heart development, cardiac disease, regeneration

INTRODUCTION

The Hippo pathway, originally identified in the *Drosophila* genus, is a highly conserved kinase cascade that regulates organ size (Harvey et al., 2003; Pan, 2007; Hayashi et al., 2015). The transcriptional coactivator Yes-associated protein 1 (YAP1; homolog of Yorkie) was first identified as a binding partner of the SH3 domain of c-yes; the YAP1 and its paralog PDZ-binding motif (TAZ; also known as WW–domain-containing transcription regulator 1 [WWTR1]), are both downstream effectors of Hippo signaling (Sudol, 1994). This pathway has been implicated in diverse biological functions, both in *Drosophila* and in mammals; these include cell proliferation, apoptosis, organ-size control, and cancer progression (Mo, 2017). Recent reports have revealed the critical role of YAP1/TAZ in cardiac development, regeneration, and stress response; however, there are some inconsistent and even contradictory results that warrant further investigation (Zhou et al., 2015). Interestingly, recent studies have identified a variety of post-translational modifications (PTMs)

to YAP1/TAZ, such as phosphorylation, O-GlcNacylation, methylation, and ubiquitination, which offers an opportunity to control the Hippo–YAP1/TAZ pathway. In this review, we summarize the overall picture of the Hippo–YAP1/TAZ pathway; in particular, we highlight the novel discoveries with regard to PTM-related regulation and the function of the YAP1/TAZ pathway in cardiac development, disease and regeneration.

OVERVIEW OF THE HIPPO–YAP1/TAZ PATHWAY

The Canonical Hippo–YAP1/TAZ Pathway in Mammals

The components of the Hippo pathway in mammals are highly consistent with those in *Drosophila*, including sterile 20-like protein kinases (MST1/2; homologs of D. Hpo), salvador family protein 1 (SAV1; which contains a WW domain), large tumor suppressors (LATS1/2; homologs of Wts), Mps one binder kinase activator-like 1A/1B (MOB1; orthologs of Mats), YAP1 /TAZ, and other peripheral proteins (**Figure 1**).

The upstream signals and stressors of the Hippo pathway include cell polarity, energy stresses, G–protein-coupled receptors (GPCRs), and stiffness of the extracellular matrix (ECM) (Meng et al., 2016). On stimulation, MST1/2 and its adaptor protein SAV1 are phosphorylated leading to their activation; subsequently, these stimulate the phosphorylation, and activation of the LATS1/2–MOB1 complex. Through an interaction between the PPxY (PY) motifs of LATS1/2 and the WW domains of YAP1/TAZ, the activated LATS1/2 can induce phosphorylation of YAP1 (mouse YAP1 at Ser112, human YAP1 at Ser127) and TAZ (Ser89) (Zhao et al., 2007). After phosphorylation, YAP1/TAZ binds to the 14-3-3 protein, which induces cytoplasmic retention of YAP1/TAZ. Moreover, phosphorylated YAP1/TAZ is likely to undergo ubiquitination degradation that is dependent on the β-transducin repeat-containing E3 ubiquitin protein ligase complex ($SCF^{\beta-TRCP}$) (Dong et al., 2007; Zhao et al., 2007).

When Hippo signaling is inactive, the YAP1/TAZ are activated and mainly localized in the nucleus, where they produce downstream biological effects. The YAP1/TAZ do not harbor DNA-binding domains; instead, these interact with transcriptional partner proteins to initiate or impede transcription. The transcriptional partners of YAP1/TAZ that have been identified thus far include transcriptional enhanced associate domain proteins (TEADs), members of the Smad family (Anorga et al., 2018; Ben Mimoun and Mauviel, 2018; Miskolczi et al., 2018; Qin et al., 2018), and p63/p73 (Levy et al., 2008; Kim et al., 2018). The onus of the YAP1/TAZ biological output rests with the transcriptional partners and the target genes that they modulate.

Downstream Output of YAP1/TAZ

As orthologs of *Drosophila* Scalloped, the TEAD family of proteins in mammals comprise YAP1/TAZ- and DNA-binding domains, which facilitates the construction of a YAP1/TAZ–TEADs co-transcription complex (Li et al., 2010). Recent studies investigating YAP1/TAZ mechanisms have identified some target genes of the YAP1/TAZ–TEADs complex. The *glucose transporter 1* (*Glut1*) promotor contains TEAD-binding motifs that allow *Glut1* to be regulated by the YAP1–TEAD1 transcriptional complex, which enhances cell glycolysis in breast cancer cells (Valis et al., 2016; Lin and Xu, 2017). Moreover, GLUT1, a transmembranous protein, participates in cell glucose uptake (Wang et al., 2017). Left ventricular hypertrophy is characterized by enhanced GLUT1 expression and basal glucose uptake in heart tissues. Cardiac-specific overexpression of GLUT1 can retard the progression of heart failure and reduce mortality associated with pressure overload (PO) (Liao et al., 2002). Thus, YAP1/TAZ may have a cardioprotective effect by enhancing GLUT1 expression. In breast cancer cells, the *G2 and S phase-expressed 1* (*GTSE1*) acts as a novel target gene of the YAP1–TEAD4 complex, which forms cell protrusions and boosts cell migration (Stelitano et al., 2017). The GTSE1 is a microtubule-associated protein that is involved in cell proliferation, invasion, and migration (Liu et al., 2019). Therefore, through regulation of GTSE1, YAP1 facilitates epithelial-to-mesenchymal transition (EMT) in cancer cells.

Furthermore, the YAP1/TAZ–TEAD complex can recruit and bind to transcription enhancers and/or mediators to potentiate and blunt transcription. Prox1, a major regulator of lymphangiogenesis, is regulated by the YAP1/TAZ–TEAD complex (Harvey et al., 2005; Cho et al., 2019). The YAP1/TAZ–TEAD transcriptional complex recruits the nucleosome remodeling and histone deacetylase (NuRD) complex to negatively modulate *Prox1*; this was shown to reduce pathological lymphangiogenesis and maintain the lymphatic valve postnatally (Cho et al., 2019). In addition, the YAP1/TAZ, together with histone deacetylase 7 (HDAC7) and TEAD, binds to the *COX-2* promoter and attenuates *COX-2* transcription in order to inhibit IL-1β-induced cell migration and invasion (Zhang et al., 2018a). Based on its tyrosine phosphorylation status, parafibromin (a nuclear scaffold protein) selectively interacts with YAP1 or TAZ. Phosphorylated parafibromin binds to the YAP1-TEAD complex, whereas dephosphorylated parafibromin combines with the TAZ-TEAD transcriptional activator (Tang et al., 2018). YAP1 and TAZ have largely overlapping transcriptional functions, such as cell proliferation and cell migration; however, both have unique biological functions (Lai et al., 2018; Liu et al., 2018; Negron-Perez and Hansen, 2018). For example, YAP1 knockout (KO), rather than TAZ KO, in mice leads to embryonic death (Hossain et al., 2007; Makita et al., 2008). Conversely, parafibromin regulates the activity of YAP1 and TAZ in different status, which may explain the unique functions of YAP1 and TAZ.

Newly discovered co-transcription proteins reportedly contribute to YAP1/TAZ functions. Pyruvate kinase M2 (PKM2) is a relevant glycolytic protein; the interaction of YAP1 with hypoxia-inducible factor 1α (HIF-1α) promotes tumor cell glycolysis by triggering the transcription of the *Pkm2* gene (Zhang et al., 2018b). YAP1-mediated PKM2 expression enhances cell glycolysis and adapts tumor cells for

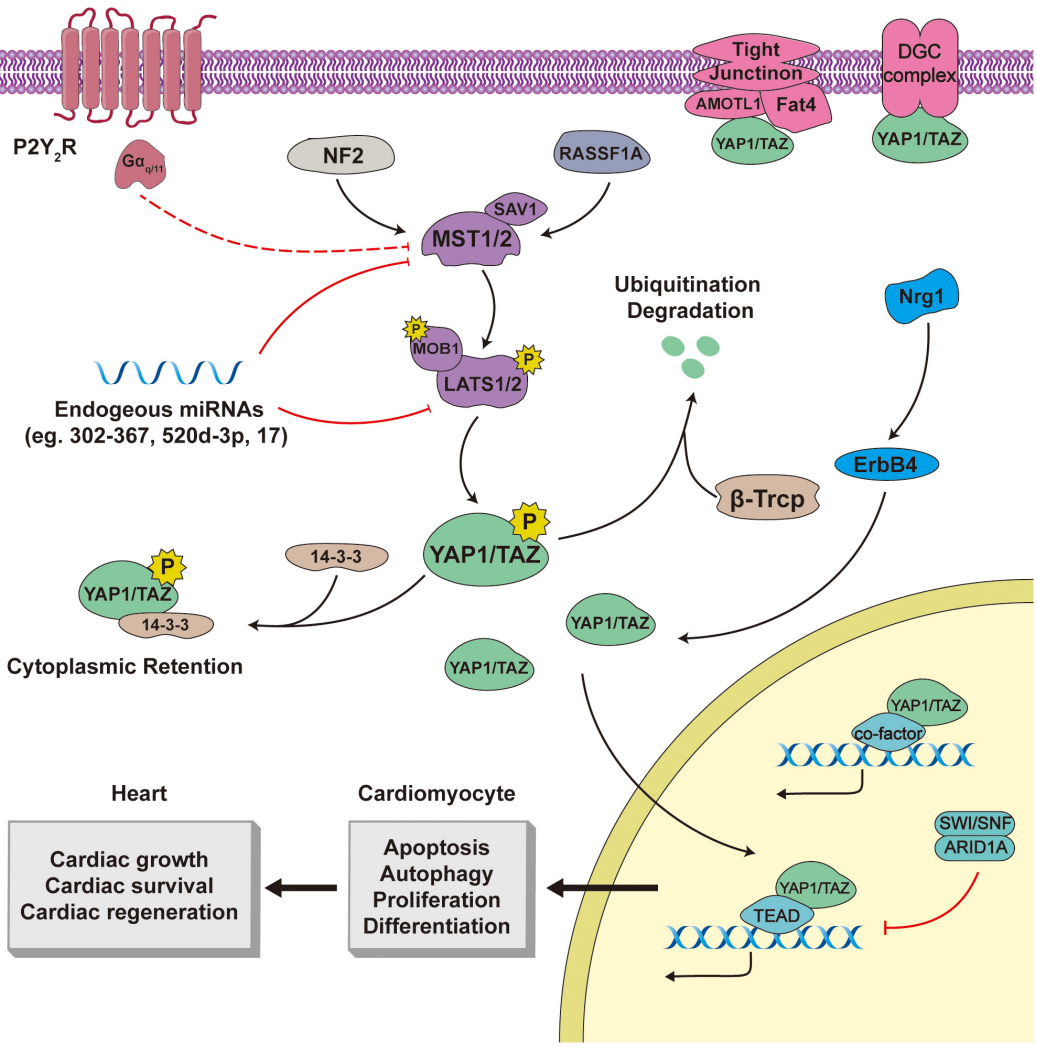


FIGURE 1 | The Hippo–YAP1/TAZ pathway in cardiac biology. Both YAP1 and TAZ are phosphorylated by core components, including Mst1/2, SAV1, Lats1/2, and MOB1, of the canonical Hippo pathway, and are subsequently degraded or stranded in the cytoplasm. Active YAP1/TAZ translocate to the nucleus, bind to transcriptional partners, and modulate downstream output in cardiac tissue. In addition, the Hippo-YAP1/TAZ pathway is regulated by several upstream pathways and cell-cell junctions. Line arrows indicate activation, whereas connector lines imply inhibition.

abnormal growth. The SWI/SNF (Brg/Brahma-associated factors [BAF]) and ARID1A (BAF250A) proteins are inhibitors of the YAP1/TAZ-TEADs complex; in addition, these are capable of occupying the TEAD-binding site of YAP1/TAZ (Chang et al., 2018). Although the ARID1A–SWI/SNF complex is inactive during tumor growth, this negative association highlights the role of the ARID1A–SWI/SNF complex in cancer development through the suppression of oncogenic YAP1/TAZ activity (Kadoch and Crabtree, 2015).

Collectively, novel transcription mechanisms have been reported for explaining partly YAP1/TAZ biological output; these findings bode well for future YAP1/TAZ studies. However, the diversity of transcription partners and the target genes of YAP1/TAZ make it difficult to clarify the biological effects of YAP1/TAZ; therefore, future research should consider the entire molecular network of the YAP1/TAZ pathway.

REGULATION OF THE YAP1/TAZ BY POST-TRANSLATIONAL MODIFICATIONS

Regulation of the YAP1/TAZ by Phosphorylation

Yes-associated protein 1 and TAZ, two salient downstream effectors of Hippo signaling, are found both in the cytoplasm and nucleus. Since these two effectors trigger target gene transcription in the nucleus, nuclear accumulation of YAP1/TAZ is a key step in their mechanism of action. This nuclear accumulation is mediated by several events: (1) post-translational modifications (PTMs) including phosphorylation, methylation, and ubiquitination; (2) other related protein interactions with YAP1/TAZ; and (3) translational interference

(Piccolo et al., 2014; Mo, 2017). In most scenarios, YAP1/TAZ are mediated by PTMs that determine their subcellular localization and content (**Table 1**).

In the canonical Hippo pathway, phosphorylation plays a pivotal role in the regulation of YAP1/TAZ; they can be phosphorylated at multiple sites, such as Ser61, Ser109, Ser127 (Ser89 in TAZ), Ser164, and Ser381 (Ser311 in TAZ) (Zhao et al., 2010). Among these, Ser127 and Ser381 are the main phosphorylation sites by LATS1/2. Following phosphorylation at Ser381, YAP1 is prone to further phosphorylation by the casein kinase I isoform δ/ε (CKI δ/ε), which eventually promotes $SCF^{\beta-TRCP}$-induced ubiquitination degradation of YAP1 (Zhao et al., 2010). YAP1 binds to 14-3-3 protein through the Ser127 phosphorylation-dependent way and becomes sequestered in the cytoplasm (Zhao et al., 2007). Notably, the combination of YAP1 and 14-3-3 can be blocked by phosphorylation at the YAP1 Ser128 site by Nemo-like kinase (NLK); this phenomenon can also be seen in *Drosophila* Nemo (Moon et al., 2017). This indicates that NLK/Nemo are highly conserved in Hippo-YAP1/TAZ signaling to mediate the YAP1/Yki subcellular compartmentation via phosphorylation.

Phosphorylation-dependent cytoplasmic retention of YAP1/TAZ may hamper their transcriptional activity. In the transgenic heart, MST1 overexpression was shown to enhance the phosphorylation of YAP1 Ser127 and induce inactivation of YAP1; this induced cardiomyocyte (CM) apoptosis and myocardial fibrosis (Yamamoto et al., 2003; Zhao et al., 2019). In LATS1/2 cardiac knock out (CKO) of the embryonic epicardium, LAST1/2 deficiency was shown to inhibit the phosphorylation of YAP1 Ser127 and increase the YAP1 transcriptional activity; eventually, this resulted in the failure of subepicardial cells to differentiate into cardiac fibroblasts and induced coronary vessel defects (Xiao et al., 2018). Thus, the canonical Hippo pathway inhibits the YAP1/TAZ activity in cardiac tissues through phosphorylation. Interestingly, YAP1/TAZ was shown to increase the concentration and activity of endogenous LATS2 by directly inducing TEAD-dependent *LATS2* transcription and/or indirectly activating neurofibromin 2 (NF2)-mediated LATS2 activity, which subsequently enhances the phosphorylation of YAP1/TAZ (Moroishi et al., 2015; Dai et al., 2017). This physiological negative feedback mechanism is expected to maintain the dynamic homeostasis of the Hippo pathway, and may help elucidate the complex mechanism of cardiac development and disease.

Notably, YAP1/TAZ can be dephosphorylated by a catalytic subunit of protein phosphatase-1 (PP1A) at Ser127 (Ser89 and Ser311 in TAZ), and is then translocated into the nucleus, thereby causing leak activation of the target gene (Liu et al., 2011; Wang et al., 2011). This abnormal activation can be potentially prevented by some mechanisms. The pre-mRNA splicing factor 4 kinase (PRP4K) promotes phosphorylation of Yki/YAP1/TAZ in the nucleus as well as its subsequent nuclear export; this acts as a safety threshold for the rescue of aberrant Yki/YAP1/TAZ activities and cell growth (Cho et al., 2018).

Besides serine phosphorylation, the subcellular distribution and activities of YAP1/TAZ are affected by the phosphorylation of tyrosine residues. Several members of the SRC family of kinases (SFK), such as Yes, Src, and c-Abl, participate in

TABLE 1 | Post-translational modifications that modulate YAP1/TAZ.

Modification types	Regulative sites	Enzymes	Effects
Phosphorylation	Ser109	LATS1/2	YAP1/TAZ cytoplasm retention (Zhao et al., 2010)
	Ser127 (Ser89 in TAZ)	LATS1/2	Binding YAP1 to 14-3-3 and inducing cytoplasm retention (Zhao et al., 2007)
	Ser128	NLK	Blocking YAP1 binding to 14-3-3 (Moon et al., 2017)
	Ser381 (Ser311 in TAZ)	LATS1/2	β–Trcp-dependent ubiquitination degradation (Zhao et al., 2010)
	NS	PRP4K	YAP1/TAZ nuclear export (Cho et al., 2018)
	Tyr357	c-Abl	Stabilizing YAP1 stable and increasing YAP1–p73 interaction inducing cell apoptosis (Levy et al., 2008)
	Tyr357	Src	Enhancing YAP1 nuclear retention and target gene *CTGF* expression (Taniguchi et al., 2015)
	one or more tyrosine residues	Yes	Promoting YAP1 translocation and *Oct-3/4* transcription (Tamm et al., 2011)
Dephosphorylation	Ser127 (Ser89 and Ser311 in TAZ)	PP1A	YAP1/TAZ nuclear translocation (Liu et al., 2011; Wang et al., 2011).
O-GlcNacylation	Ser109	OGT	Impeding LATS1/2-induced YAP1 Ser109 phosphorylation and potentiating YAP1 activity (Peng et al., 2017)
	Thr241	OGT	Attenuating LATS1/2-dependent YAP1 Ser127 phosphorylation and enhancing YAP1 activity (Zhang et al., 2017)
Methylation	Lys342	SET1A	Dampening YAP1–CRM1 fusion and enhancing YAP1 nuclear retention (Fang et al., 2018)
	Lys494	SET7	Enhancing YAP1 translocation to cytosol and cytoplasmic membrane Oudhoff et al., 2013)
Ubiquitination	NS	$SCF^{\beta-TRCP}$	YAP1/TAZ protein degradation (Dong et al., 2007; Zhao et al., 2007)
	NS	Fbxw7	YAP1 protein degradation (Tu et al., 2014; Zhang et al., 2016)
	Lys321, Lys497	SKP2	Accumulating YAP1 nuclear localization and association between YAP1 and TEAD (Yao et al., 2018)
De-ubiquitination	NS	USP9X	Stabilizing YAP1 and enhancing its activity (Li et al., 2018a).
	NS	OTUD1	Reducing YAP1 stability and attenuating the cell proliferation activity of YAP1 (Yao et al., 2018).

NS, not specified, where the specific regulator sites have not been validated.

tyrosine phosphorylation of YAP1. In response to DNA damage, YAP1 Tyr357 phosphorylation by c-Abl stabilizes YAP1 and increases the nuclear interaction of YAP1-p73 to induce apoptosis (Levy et al., 2008). This phosphorylation may inhibit the cell proliferation and oncogenic ability of YAP1 that are necessary to sustain its normal biological functions. The activated Yes phosphorylates YAP1 on one or more tyrosine residues; the phosphorylated YAP1 is subsequently translocated to the nucleus to trigger the transcription of the pluripotency factor *Oct-3/4*, which is critical for maintaining the self-renewal ability of the mouse and human embryonic stem cells (hESCs) (Tamm et al., 2011). Furthermore, Src phosphorylated by the IL6–GP130 complex phosphorylates YAP1 at Tyr357, which increases YAP1 nuclear retention and expression of its target gene *connective tissue growth factor* (*CTGF*); this Src-induced YAP phosphorylation facilitates the regeneration of the injured intestinal mucosa (Taniguchi et al., 2015). The above-mentioned research shows that tyrosine phosphorylation participates in the regulation of YAP1/TAZ in specific cell and tissue regeneration. This suggests that the tyrosine phosphorylation of YAP1/TAZ may be conductive to embryonic cardiac development and post-injury cardiac regeneration; these findings provide new insights into cardiac development and regeneration.

Regulation of the YAP1/TAZ by O-GlcNAcylation

Besides phosphorylation, several PTMs, such as O-GlcNAcylation, are involved in the regulation of YAP1/TAZ. O-GlcNAc transferase (OGT) directly induces O-GlcNAcylation at YAP1 Ser109 and Thr241, and potentiates the pro-proliferation activity of YAP1 in pancreatic cancer and liver cancer cells (Peng et al., 2017; Zhang et al., 2017). Mechanistically, YAP1 O-GlcNAcylation can affect YAP1 phosphorylation. As mentioned in Section "Regulation of the YAP1/TAZ by Phosphorylation," LATS1/2 can phosphorylate YAP1 Ser109 (Zhao et al., 2010). Therefore, O-GlcNAcylation and phosphorylation may act competitively at Ser109. The stoichiometry of Ser109 phosphorylation is much lower than that of O-GlcNAcylation, which suggests that O-GlcNAcylation plays a key role in PTM at this site rather than phosphorylation (Peng et al., 2017). O-GlcNAcylation at YAP1 Thr241 can attenuate the phosphorylation of YAP1 at Ser127 via interrupting the LATS1/2 interaction (Zhang et al., 2017). In addition, these studies reveal that YAP1 not only acts as a substrate of OGT but also triggers *OGT* transcription to regulate its expression, thereby forming a positive feedback loop (Peng et al., 2017; Zhang et al., 2017). This positive feedback loop further regulates YAP1 activity and output.

Therefore, YAP1 may participate in cancer glycosylation metabolism through O-GlcNAcylation and OGT expression in order to maintain the abnormal proliferation and survival of cancer cells. *In vitro*, OGT KO inhibits O-GlcNAcylation at the YAP1 Ser109, thereby inhibiting cell-colony formation in pancreatic cancer; *in vivo*, OGT knockdown downregulates tumor growth (Peng et al., 2017). High glucose stimulation improves the level of O-GlcNAcylation at YAP1 Thr241; in addition, O-GlcNAcylation enhances the stability and activity of YAP1, which subsequently induces cell proliferation and transformative phenotypes in hepatocellular carcinoma cells (HCC, THLE-3, and HL-7702 cell lines) (Zhang et al., 2017). In addition to liver cancer, high glucose level affects diabetic cardiomyopathy. Elevated cardiac concentrations of active YAP1 have been demonstrated in mice fed with high-fat diet and in patients with diabetic cardiomyopathy (Ikeda et al., 2019b). However, the relationship between YAP1 and diabetic cardiomyopathy is not completely clear. Thus, YAP1 O-GlcNAcylation might be a promising target for the study of diabetic cardiomyopathy.

Regulation of the YAP1/TAZ by Ubiquitination

The stability and content of YAP1/TAZ are partly regulated by ubiquitination-induced degradation; therefore, ubiquitination cannot be neglected in research into YAP1/TAZ. $SCF^{\beta-TRCP}$-dependent ubiquitination is a well-known YAP1/TAZ degradation mechanism in the canonical Hippo pathway that is dependent on YAP1/TAZ phosphorylation (Dong et al., 2007; Zhao et al., 2007). Similarly, Fbxw7, another subunit of E3 ligase, participates in the regulation of YAP1 protein level and activity via ubiquitination and proteasomal degradation (Tu et al., 2014; Zhang et al., 2016). Fbxw7 expression exhibits a negative correlation with YAP1 content in human HCC and pancreatic ductal adenocarcinoma tissues; however, the regulatory mechanisms are yet to be elucidated. This proteolytic ubiquitination can be reversed by deubiquitinases; the deubiquitinase USP9X was shown to directly deubiquitinate and stabilize YAP1, leading to enhanced YAP1 activity and promotion of tumor growth (Li et al., 2018a). LATS1/2, activated by mono-ubiquitinated angiomotin-like 2 (AMOTL2), triggers YAP1 phosphorylation and suppresses its activity (Kim et al., 2016). USP9X deubiquitinates AMOTL2 to inactivate LATS1/2, which indirectly activates YAP1 (Kim et al., 2016). Therefore, ubiquitination appears to be a negative regulatory factor for YAP1/TAZ in the canonical Hippo pathway.

Intriguingly, recent studies have provided new insights into ubiquitination independent of the Hippo pathway. The SCF^{SKP2} E3 ligase complex (SKP2) facilitates K63-linkage-specific ubiquitination of YAP1 at Lys321 and Lys497, which does not reduce YAP1 abundance (Yao et al., 2018). Moreover, these ubiquitination promotes nuclear translocation of YAP1, and cements the association between YAP1 and TEAD to enhance the cell proliferation activity of YAP1 (Yao et al., 2018). This non-proteolytic ubiquitination can be reversed by the binding and interaction between deubiquitinase OTUD1 and YAP1; OTUD1 decreases YAP1 stability, and attenuates the cell proliferation and tumor-growth activity of YAP1 (Yao et al., 2018). Obviously, this non-proteolytic ubiquitination is beneficial for YAP1 activation and output. The above-mentioned studies delineate the controversial role of ubiquitination in YAP1/TAZ in the canonical Hippo–YAP1/TAZ pathway as well as in the Hippo-independent pathway. YAP1/TAZ is degraded by

ubiquitinases that exert proteolytic effects, whereas it is stabilized by ubiquitinases with non-proteolytic activities; these events can be reversed by the related deubiquitinases. Undoubtedly, ubiquitination plays an essential role in mediating YAP1/TAZ content and stability that should be further studied. The biological effects of ubiquitinated/deubiquitinated YAP1/TAZ have been investigated mainly in tumorigenesis, and less so in cardiogenesis and cardiac disease.

Regulation of the YAP1/TAZ by Methylation

Chromosomal maintenance 1 (CRM1), a nuclear export protein, triggers the nuclear export of YAP1. The methyltransferase SET1A targets YAP1 at Lys342 through mono-methylation, which inhibits CRM1-induced nuclear export of YAP1 with resultant enhancement of YAP1 nuclear retention and transcriptional activity (Fang et al., 2018). Mono-methylation at Lys342 in YAP1 contributes to *in vivo* cell proliferation and tumorigenesis (Fang et al., 2018). However, this result was discordant with that from a previous study on mono-methylated YAP1. The SET7 is a methyltransferase that connects the cell membrane and the cytoskeleton (Garbino et al., 2009). Mono-methylation of YAP1 Lys494 by SET7 is necessary for the retention of YAP1 in the cytosol and at the cytoplasmic membrane, which may impede interactions between YAP1 and PDZ-dependent-binding partners (Oudhoff et al., 2013). Thus, methylation at YAP1 Lys494 represses YAP1 activity to downregulate *CTGF* transcription. Evidently, methylation can exert certain effects on YAP1/TAZ, but there is a paucity of studies on YAP1/TAZ methylation and their biological output. In addition, the mechanisms by which methylated YAP1/TAZ affect cardiac biology remain unknown.

Several types of PTMs alter the subcellular translocation of YAP1/TAZ to regulate their activities and output, regardless of how YAP1/TAZ are modified by PTMs. Therefore, it is important to study the nuclear–cytoplasmic trafficking of YAP1/TAZ. An increasing body of evidence supports the mechanisms of YAP1/TAZ nucleocytoplasmic shuttling. In a stiff cultured environment, the nucleus encounters mechanical forces and becomes flattened; in addition, the nuclear pores are stretched, subsequently creating conditions for nuclear import of YAP1 (Elosegui-Artola et al., 2017). A nuclear localization sequence (NLS) and nuclear export sequence (NES) were identified within TAZ, which contributes to the nucleocytoplasmic distribution of TAZ in different ways: TEAD binding interaction partly conceals the NES; RhoA enhances NLS-induced nuclear import; and TEAD and 14-3-3 competitively bind with TAZ (Kofler et al., 2018).

Collectively, PTMs can alter YAP1/TAZ stability and/or YAP1/TAZ conformation to change its affinity for other proteins; these eventually mediate YAP1/TAZ subcellular location and content. PTMs mediate YAP1/TAZ through interdependent, synergistic, or competitive mechanisms. A growing body of evidence has revealed the underlying mechanisms of PTMs and YAP1/TAZ activity; however, several important nuances are yet to be elucidated.

HIPPO–YAP1/TAZ SIGNALING IN CARDIAC DEVELOPMENT

Cardiogenesis involves events that alter the spatiotemporal and morphological mechanisms. In the gastrulation period, the mesoderm, which emanates from the anterior primitive streak (APS), produces cardiac progenitor cells (Tam et al., 1997). Subsequently, cardiac progenitor cells form two different heart fields: the primary heart field gives rise to the left ventricle and left/right atria, while the secondary heart field generates the right ventricle, left/right atria, and the outflow tract (Xin et al., 2013b). Postnatally, there is a decrease in CM proliferation alongside an increase in hypertrophy which helps attain the physiologic cardiac size. At approximately postnatal Day 4 (P4), myocardial cell numbers peak, and then stop increasing (Li et al., 1996). Myocardial proliferation can be blunted as early as E11.5 with the help of cardiac gene reprogram; this results in abnormal cardiac growth and perinatal lethality (Wang et al., 2014).

Role of Hippo–YAP1/TAZ Signaling and Its Proliferation Output in Heart Development

The Hippo pathway is a highly conserved mechanism for the regulation of heart size and maturation (Harvey et al., 2003; Pan, 2007; Hayashi et al., 2015). Its downstream transcriptional factors, YAP1/TAZ, are key regulators of embryonic and neonatal cardiac development, and this has been validated by multiple lines of evidence (**Tables 2**, **3**). Mouse embryonic heart with SAV1 CKO (*Nkx2.5*^{cre}: *SAV*$^{f/f}$) shows ventricular septal defect and abnormal heart growth, including thickening of ventricular walls, expansion of trabecular and ventricular myocardial layers, and enlargement of ventricular chambers (Heallen et al., 2011). Similarly, LATS2 and MST1/2 CKO hearts show the same abnormal heart phenotypes (Matsui et al., 2008; Heallen et al., 2011). The SAV CKO heart with reduced phosphorylation of YAP1 exhibit enhanced proliferation of CM and unchanged cell size (Heallen et al., 2011). A WWTR1 (or TAZ) deficiency in zebrafish heart results in arrested CM proliferation, reduced cardiac trabeculation, and immature trabecular bridges (Lai et al., 2018). YAP1 knockdown using Tnnt2-Cre in mouse fetal heart causes cardiac hypoplasia with reduced ventricular chamber size, ventricular septal defects, peripheral edema, and pericardial effusion; these embryos cannot survive beyond E16.5 (von Gise et al., 2012). YAP1 inactivation represses fetal CM proliferation; however, it does not affect the cell size. Consistent with this, YAP1 deletion in the postnatal heart using Tnnt2-Cre does not significantly affect physical cardiac hypertrophy (von Gise et al., 2012). Thus, the Hippo pathway regulates the normal cardiac structure and size by mediating the cell proliferation activities of YAP1/TAZ in the fetal and postnatal phase.

Yes-associated protein 1/TAZ, as vital effectors of the Hippo pathway, mainly affect target genes transcription and downstream signaling to exhibit their pro-proliferation activities in heart development (**Table 3**). Neuregulin 1 (Nrg1), secreted by endocardial cells, is a crucial factor in myocardial growth; it induces CM proliferation and re-entry of differentiated CMs

TABLE 2 | Cardiac output of the Hippo–YAP1/TAZ pathway in cell and animal models.

Gene	Model		Promoter	Output
YAP1	Mouse	CKO	Tnnt2-Cre	Cardiac hypoplasia (reduced ventricular chamber size, ventricular septal defects, peripheral edema, and pericardial effusion) (von Gise et al., 2012)
	Mouse	CKO	Nfatc1$^{IRES-Cre/+}$	Less CM proliferation, impaired compact myocardium, and early postnatal lethality (Artap et al., 2018)
	Mouse	CKO	α-MHC-Cre	Blunted cardiac hypertrophy and amplified CM apoptosis and fibrosis; cardiac dilatation and dysfunction after TAC (Byun et al., 2019)
	Mouse	CKO	Nkx2.5-cre	Lack of healthy myocardial tissue in the left ventricle wall and an enhancing fibrotic infarct zone following MI (Xin et al., 2013a)
	Mouse	CKO	α-MHC-Cre	Thinned septal and posterior wall, and chamber dilation (Del Re et al., 2013).
	Mouse	CKO	α-MHC-Cre	Increasing CM apoptosis, fibrosis, enlarging infarct size, and impairing cardiac function (Del Re et al., 2013)
	Mouse	Overexpression	Adeno-associated virus subtype 9: human YAP1	Alleviating MI injury and ameliorating cardiac function (Lin et al., 2014)
	Mouse	YAP5SA (active YAP1) overexpression	αMyHC-Cre-ERT2	Re-entering the cell cycle and reprogramming into more primitive and fetal cell states; thickened ventricular walls and smaller chambers (Monroe et al., 2019)
	Mouse	YAPS112A (active YAP1) overexpression	α-MHC	Increased myocardial tissue and reduced LV fibrosis in neonatal heart (Xin et al., 2013a)
	AC16 human CMs	Overexpression	Lentiviral vectors	Reducing CM apoptosis, cell hypertrophy, and ROS generation after IR (Khan et al., 2019)
WWTR1 (TAZ)	Zebrafish	CKO	CRISPR/CAS9	CM proliferation arrest, reduced cardiac trabeculation, and immature trabecular bridges (Lai et al., 2018)
WW45	Mouse	CKO	Myh6-Cre	Sustained YAP1 activation in CMs with cell-cycle re-entry, increased de-differentiation, and decreased apoptosis; cardiac dysfunction, severe heart failure, and enhanced mortality in response to TAC (Ikeda et al., 2019a,b)
SAV1	Mouse	CKO	Nkx2.5-cre	Ventricular septal defect and abnormal heart growth (thickening of ventricular walls, expansion of trabecular and ventricular myocardial layers, and enlargement of ventricular chambers) (Heallen et al., 2011)
	Mouse	CKO	Myh6^{CreERT2} αMHC-mcm	Presenting renewal capacity: increase in cell number and myocardial regeneration following cardiac apex resection in postnatal hearts; increased LV CMs, less fibrosis, and improved cardiac function after MI in adult heart (Heallen et al., 2013; Leach et al., 2017)
RASSF1A	Mouse	cardiomyocyte-specific KO	α-MHC-Cre	Basal nondistinctive cardiac phenotype or functional abnormality; reduced apoptosis, fibrosis, and hypertrophy after TAC (Del Re et al., 2010)
	Mouse	KO	NS	Increased hypertrophic response, reduced cardiomyocytes apoptosis, and increased fibrosis after TAC (Del Re et al., 2010)
MST1/2	Mouse	CKO	Nkx2.5-cre	Ventricular septal defect and abnormal heart growth (Heallen et al., 2011)
MST1	Mouse	DN-MST1 overexpression	CMV-Cre	Reducing the size of MI in the area at risk, and decreasing CM apoptosis (Nakamura et al., 2016)
	Mouse	DN-MST1 overexpression	α-MHC	Reduced left ventricular remodeling, improved left ventricular function, and enhanced survival rate after MI (Maejima et al., 2013)
LATS2	Mouse	LATS2 overexpression	α-MHC	Reduced left ventricular systolic and diastolic dysfunction, and smaller left/right ventricle (Matsui et al., 2008)
	Mouse	DN-LATS2 overexpression	α-MHC	Reduced CM apoptosis and enhanced biventricular hypertrophy following TAC (Matsui et al., 2008)
	Mouse	CKO	Nkx2.5-cre	Ventricular septal defect and abnormal heart growth (Heallen et al., 2011)
LATS1/2	Mouse	LATS1/2 CKO; YAP1/TAZ CKO	Wt^{CreERT2} allele	Successful survival past E15.5 without defects in coronary vasculature (Xiao et al., 2018)
	Mouse	CKO	Wt^{CreERT2} allele	Failing to survive past E15.5; smaller hearts with less compacted myocardium (Xiao et al., 2018)

(Continued)

TABLE 2 | Continued

Gene	Model		Promoter	Output
	Mouse	CKO	$Myh6^{CreERT2}$	Presenting renewal capacity: increasing cell number and regenerating the myocardium following cardiac apex resection in postnatal hearts; increased LV CMs, less fibrosis, and improved cardiac function after MI in adult heart (Heallen et al., 2013)
	Zebrafish	DKO	Myh6; Myl7	Enhanced Hand2 expression and CM differentiation (Schindler et al., 2014; Fukui et al., 2018)

NS, not specified, where the specific promotors have not been validated; YAP1, Yes-associated protein 1; TAZ, PDZ-binding motif; CKO, cardiac knockout; KO, knockout; DN, dominant negative, MI, myocardial infarction; CM, cardiomyocytes; TAC, transverse aortic constriction; IR, ischemia–reperfusion; ROS, reactive oxygen species.

in the cell-cycle via Nrg1/ErbB2 signaling (Bersell et al., 2009; Gemberling et al., 2015). Targeting at *Nrg1*, YAP1/TAZ activates Nrg1/ErbB2 signaling in the endocardium; in addition, loss of YAP1 in the endocardium contributes to less CM proliferation, impaired development of compact myocardium, and early postnatal mortality (Artap et al., 2018). Moreover, Nrg1/ErbB4 signaling can reduce the phosphorylation of YAP1 Ser127, and promote YAP1-dependent *CTGF* transcription through the production of the ErbB4–YAP1–TEAD complex in breast cancer cells (Haskins et al., 2014). Thus, it is hypothesized that YAP1 proliferatively increases output not only by directly mediating *CTGF* transcription, but also indirectly by activating Nrg1/ErbB4 signaling. However, it is unclear whether Nrg1/ErbB4 signaling-dependent YAP1 activation exists in the endocardium.

TABLE 3 | Target genes and cardiac output of the Hippo–YAP1/TAZ pathway.

Target genes	Type of Regulation	Outcome
Bmp2b	Promotion	Activating the BMP pathway to enhance the number of CPCs in the secondary heart field (Fukui et al., 2018)
Cyr61 and *CTGF*	Promotion	Promoting CM proliferation (Badouel et al., 2015; Ragni et al., 2017; Zou et al., 2018)
Dpp4 and *Dhrs3*	Promotion	Inhibiting subepicardial cells from differentiating into cardiac fibroblasts, thus inducing coronary vessel defects (Xiao et al., 2018)
hand2	Promotion	Regulating differentiation of LPM cells that develop into the atrium of the heart (Fukui et al., 2018)
miR-152	Promotion	Promoting CM proliferation (Wang et al., 2018)
miR-206	Promotion	Promoting cell hypertrophy and cell survival by downregulating FoxP1 after TAC (Yang et al., 2015)
Nrg1	Promotion	Activating Nrg1/ErbB2 signaling and reducing CM proliferation, to impair compact myocardium development (Artap et al., 2018).
Oct-3/4	Promotion	Maintaining mouse and human embryonic stem cell self-renewal (Tamm et al., 2011)
OSM	Promotion	Regulating CM de-differentiation (Ikeda et al., 2019a)
Park2	Promotion	Promoting the clearance of impaired mitochondria through autophagy, subsequently enhancing CM resistance to stress (Kubli et al., 2013; Leach et al., 2017).
Pik3cb	Promotion	Activating PI3K-AKT signaling to trigger CM proliferation and survival (Xin et al., 2011; Lin et al., 2015)
Sox17	Inhibition	Disrupting CM differentiation (Hsu et al., 2018)

CM, cardiomyocytes; PI3K, phosphoinositol 3-kinase; TAC, transverse aortic constriction; LPM, lateral-plate mesoderm, CPC, cardiac precursor cell.

Through differential gene expression analysis, *Pik3cb* [which encodes a catalytic subunit of phosphoinositol-3-kinase (PI3K)] has been identified as the target gene of YAP1. YAP1 promotes *Pik3cb* transcription to activate PI3K-AKT signaling and trigger CM proliferation and survival (Xin et al., 2011; Lin et al., 2015). In cases with Pik3cb deficiency, the mouse embryo cannot survive beyond E10.5 (Bi et al., 2002). Moreover, PI3K-AKT signaling inhibits glycogen synthase kinase 3β (GSK3β)-dependent phosphorylation of β-catenin; the consequent activation of β-catenin promotes CM proliferation (Xin et al., 2011). The interaction between the Hippo pathway, Wnt/β-catenin signaling, and PI3K-AKT signaling facilitates the proliferation of CMs. Besides the aforementioned signaling, YAP1/TAZ can also affect bone morphogenetic protein (BMP) pathway to regulate CM proliferation. By upregulating *Bmp2b* transcription, YAP1/TAZ activate the BMP pathway to enhance the number of cardiac precursor cells (CPCs) in the secondary cardiac field (Fukui et al., 2018). More importantly, BMP2 induces EMT in cardiac cushion, which facilitates the development of valves and septa (Gomez-Puerto et al., 2019). These mechanisms delineate the crosstalk between YAP1/TAZ and BMP pathway in cardiac development. Collectively, the above-mentioned findings suggest that the pro-proliferation activities of YAP1/TAZ participate in cardiac development from the fetal to postnatal periods, including the trabeculation and formation of the ventricles and endocardium. Thus, the functions of the Hippo pathway and YAP1/TAZ are shown to be highly conserved, as is the crosstalk between the Hippo pathway and other signaling pathways in cardiac development.

Role of Hippo–YAP1/TAZ Signaling and Its Differentiation Output in Heart Development

Besides cell proliferation, cardiogenesis involves cell differentiation; in addition, different cell types are involved in salient events during cardiac development. YAP1/TAZ are the key inhibitors of hESC differentiation into cardiac mesoderm. The CKO of LATS1/2 expression in the epicardium by using

$Wt^{CreERT2}$ prevents embryos from surviving past E15.5 (Xiao et al., 2018). Due to the deletion of LATS1/2, activated YAP1 inhibits the differentiation of subepicardial cells into cardiac fibroblasts which induces coronary vascular defects; however, the LATS1/2 CKO and YAP1/TAZ CKO embryos do not exhibit defects of the coronary vasculature. This indicates that LATS1/2 plays an important role in restricting YAP1/TAZ activities and that YAP1/TAZ are essential for normal heart growth (Xiao et al., 2018). In LATS1/2 CKO heart, downstream genes *Dpp4* and *Dhrs3* transcription is upregulated through the YAP1-TEAD complex, which regulates the differentiation events of subepicardial cells and the coronary vessel remodeling, respectively (Xiao et al., 2018). Dehydrogenase/reductase superfamily 3 (Dhrs3), a negative modulator of retinoic acid generation, inhibits retinoic acid signaling, which inhibits the differentiation of cardiac fibroblasts (Billings et al., 2013; Xiao et al., 2018). In response to LATS1/2 deficiency, Dipeptidyl peptidase-4 (Dpp4; a serine protease) alters the composition of the ECM and is involved in abnormal vessel development, such as increasing blood islands and defects of mean lacunarity (Ghersi et al., 2006; Xiao et al., 2018).

Furthermore, YAP1/TAZ regulate the transcription of other target genes to affect the progression of cell differentiation in cardiogenesis, such as *Sox17* and *hand2*. *Sox17*, a member of *SOX* genes, plays a critical role in cardiac development. YAP1 selectively represses the expression of APS cell genes *Sox17* to disrupt the differentiation of CMs (Hsu et al., 2018). Inhibition of YAP1 by dasatinib induces the differentiation of hESCs into APS-derived endoderm and cardiac mesoderm. In addition to cardiac mesoderm, Sox17 is also required for the development of endocardium. Sox17 deficiency leads to abnormal endocardium with impaired ventricular trabeculation and thickened myocardium (Lange et al., 2014). YAP1/TAZ, through facilitation of *hand2* transcription, can regulate the differentiation of the lateral-plate mesoderm (LPM) cells, which finally develop into the atrium of the heart (Fukui et al., 2018). Hand2 can facilitate CM differentiation, and the expression of hand2 can be potentiated by LATS1/2 double KO (DKO) (Schindler et al., 2014; Fukui et al., 2018). Therefore, YAP1/TAZ can facilitate de-differentiation and differentiation activity in different cell types during various phases of cardiac development.

Regulation of YAP1/TAZ by Diverse Mechanisms in Heart Development

During cardiac development, the content and activity of YAP1/TAZ are not always in a stable level. At approximately P4, myocardial cell numbers peak and this increase is reconciled with the percentage of nuclear YAP1 (Li et al., 1996). During the late maturation of trabeculae, CMs need to withdraw from the cell cycle and differentiate; if not, non-compaction cardiomyopathy occurs. YAP1 may modulate these events (Hertig et al., 2018). These studies indicate that during the early stage of development, there are high levels of YAP1/TAZ activity and abundance, contributing to cell proliferation and de-differentiation; however, in the late stage, the proliferation effects of YAP1/TAZ are blunted for normal cardiac development, which underscores the importance of the spatiotemporal modulation of YAP1/TAZ. Thus, YAP1/TAZ activities need to be fine-tuned spatiotemporally depending upon the pace of cardiogenesis.

A diverse range of mechanisms are involved in the precise modulation of YAP1/TAZ activities in cardiac homeostasis and appropriate cardiac size. As noted previously, it is imperative to focus on the PTMs of YAP1/TAZ, as these affect the subcellular localization and activity of YAP1/TAZ. Previous studies have mainly shown that the phosphorylation of YAP1/TAZ is dependent on the canonical Hippo pathway. In the SAV1 CKO heart, YAP1 that is phosphorylated at Ser127 is reduced, thereby causing CM proliferation (Heallen et al., 2011). Moreover, Nrg1/ErbB4 signaling can inhibit LATS1-dependent phosphorylation at YAP1 Ser127 and promote YAP1-dependent *CTGF* transcription (Haskins et al., 2014). Cullin-RING ligases (CRLs), also known as the ubiquitin ligases, consist of Cullins, substrate-recognition, and RING proteins that mediate proteolysis of cellular proteins (Petroski and Deshaies, 2005). CRL-dependent ubiquitination appears to play an important role in temporal regulation of the Hippo–YAP1/TAZ signaling during cardiac development. In the developing heart, CRLs trigger the ubiquitination-induced degradation of MST1; this, in turn, induces dephosphorylation and nuclear translocation of YAP1, thereby ensuring CM proliferation (Zou et al., 2018). Nonetheless, the ubiquitination effect of CRLs depends on the neddylation of a ubiquitin-like protein, NEDD8. Consequently, cardiac-specific inhibition of NEDD8 attenuates the activity of CRLs and thereafter blunts the cell-proliferation activity of YAP1; this causes ventricular hypoplasia and non-compaction and, eventually, heart failure and neonatal death in mice (Zou et al., 2018).

In the developing heart, endogenous microRNAs (miRNAs) regulate the Hippo pathway and YAP1/TAZ to maintain heart homeostasis; miRNAs, small non-coding RNA molecules, silence mRNAs and suppress mRNA translation to control cardiac development (Ambros, 2004; Bartel, 2004). miR302-367 restrains MST1, LATS2, and MOB1b, which reduces the phosphorylation of YAP1 Ser127 and enhances the nuclear translocation of YAP1 (Tian et al., 2015). miR302-367, through activation of YAP1, promotes cell proliferation in embryonic and postnatal CMs (Tian et al., 2015). Using functional screen and computational approaches, some miRNAs were found to promote YAP1-dependent CM proliferation by targeting and inhibiting the members of Hippo signaling (Tian et al., 2015; Diez-Cunado et al., 2018). For example, the predicted targets of miR-520d-3p are TAOK1/2 and LATS2; in addition, members of the miR-17 family repress TAOK1/2/3, MST2, SAV1, LATS2, MOBKL1A, and others (Diez-Cunado et al., 2018). Subsequently, miR-520d-3p, miR-17 family and other detected miRNAs enhance the nuclear localization of YAP1 and induce pro-proliferation activity. However, inactivation of a single miRNA (such as miR-520d-3p or miR-17 family) does not have any obvious effect on YAP1 activity; this suggests that none of these miRNAs can individually maintain YAP1 nuclear location and cell proliferation. This indicates that multiple endogenous miRNAs synergistically, but not individually, regulate Hippo signaling-induced cell proliferation during cardiac development.

In the early postnatal period, cell–cell junctions in CMs progressively mature and modulate nuclear-cytoplasmic localization of YAP1/TAZ. The dystrophin-glycoprotein complex (DGC), a kind of transmembrane complex, serves as a link between the cellular cytoskeletal system and the ECM (Srivastava and Yu, 2006). DGC directly binds to YAP1 at the cell membrane, suppressing YAP1 activity. This interaction is synergistically augmented by the activation of the Hippo pathway; this suggests that Hippo signaling and DGC work in concert to inhibit the proliferation of murine CM (Morikawa et al., 2017). A cell junction protein Fat4 binds angiomotin-like 1 (AMOTL1, a isoform of angiomotin [AMOT] family) with YAP1 at the cell junction, leading to impaired cell proliferation in postnatal mouse heart; this is independent of the canonical Hippo pathway (Ragni et al., 2017). In the absence of Fat4, YAP1 translocates to the nucleus, promoting YAP1-dependent *CTGF* transcription; this leads to thicker ventricular myocardium and interventricular septum in the mouse heart with increased cell proliferation (Badouel et al., 2015; Ragni et al., 2017). Similar to this, AMOTL1 can bind to LATS2 at tight junction, and activate LATS2 to enhance YAP1 phosphorylation and repress YAP1 activity (Paramasivam et al., 2011). Therefore, cell junction related proteins may induce cytoplasmic sequestration of YAP1/TAZ; the consequent inhibition of YAP1 activity impairs heart growth after birth. Interestingly, AMOTL1 exhibits a dichotomous effect on the regulation of YAP1 activity. YAP1, forming a complex with AMOTL1, co-translocates to the nucleus and triggers proliferation activity as a result of Fat4 deficiency (Ragni et al., 2017); in addition, AMOTL1 inhibits YAP1 with the help of other junction proteins or the Hippo pathway (Paramasivam et al., 2011).

The above-mentioned studies reveal that the protein level and stability of YAP1/TAZ play a pivotal role in cardiac function and development. YAP1/YAZ imbalance leads to abnormal cardiac development, dysfunction, and even mortality. And YAP1/TAZ are mediated by several complex mechanisms such as PTMs, endogenous miRNAs, and cell–cell junctions, which makes it difficult to understand the complete molecular network and remains to be studied.

ROLE OF HIPPO–YAP1/TAZ SIGNALING IN CARDIAC DISEASE

The heart can be damaged by several stimuli such as inadequate nutrition, oxygen deficiency, and hemodynamic stress. Among these, ischemic heart disease plays a major role in cardiac disease. The regulation of the Hippo–YAP1/TAZ pathway appears to be cardiac protective, with relevant effects on cell proliferation, apoptosis, and cardiac remodeling (**Tables 2**, **3**).

Role of Hippo–YAP1/TAZ Signaling in Cardiac Ischemia-Reperfusion and Myocardial Infarction

An increasing body of evidence has implicated YAP1/TAZ in the causation of cardiac ischemia-reperfusion (IR) damage. IR induces activation of MST1 in mouse heart; however, after overexpressing dominant-negative MST1 (DN-MST1), the inactive MST1 induces reduction in myocardial infarction (MI) size in areas at risk of mouse heart (Nakamura et al., 2016). Endogenous MST1 potentiates IR-induced myocardial injury mainly by increasing apoptosis and partly by inhibiting autophagy, which is independent of the canonical Hippo pathway (Yamamoto et al., 2003; Nakamura et al., 2016). Mechanistically, the Ras-association domain family 1 isoform A (RASSF1A) enhances MST1 activity and translocation to the mitochondria; thereafter, activated MTS1 augments Bax- or Bcl-xL-induced apoptosis and suppresses Beclin1-mediated autophagy (Yamamoto et al., 2003; Nakamura et al., 2016). In this setting, CM autophagy, as a protective mechanism, degrades and recycles harmful cytoplasmic components, which contributes to repair of IR damage and promotes cell survival; in contrast, apoptosis was shown to perform an anti-survival role in IR damage (Nishida et al., 2008). Similar results have been observed in models of MI. After MI, endogenous MST1 potentiates left ventricular (LV) enlargement, impairs LV ejection fraction, and reduces the survival rate by suppressing autophagy (Odashima et al., 2007; Maejima et al., 2013). MI-mediated MST1 activation stabilizes the Beclin1 homodimer, and suppresses the downstream output of Beclin1 to inhibit autophagy; this impairs the recycling of harmful components and protein quality control (Maejima et al., 2013). Besides the MST1–Bax–Bcl-xL axis, MST1 mediates the canonical Hippo pathway to exert pro-apoptotic activity. In cultured CMs, LATS2 was shown to increase cell apoptosis in a dose-dependent manner; in contrast, dominant-negative LATS2 was found to suppress MST1-induced apoptosis (Matsui et al., 2008). IR induces activation of NF2 and potentiation of MST1 activity, which increases CM apoptosis; hearts lacking NF2 by $Cre^{\alpha MHC}$ exhibit diminished phosphorylation of MST1 and activation of YAP1 (Matsuda et al., 2016). In the NF2 CKO heart, enhanced YAP1 activity protects against IR damage. Collectively, these findings suggest that MST1 participates in IR and MI injury by enhancing apoptosis and reducing autophagy; moreover, MST1 works as a negative regulator in cardiac protection through Hippo-dependent or -independent mechanisms. Based on the available evidence, it can be indirectly inferred that the Hippo pathway may be an anti-survival mediator in cardiac IR and MI damage.

As mentioned previously, IR and MI stress induce activation of the Hippo pathway and inactivation of YAP1/TAZ. Activated YAP1 has a protective effect on IR-damaged heart. Lentivirus-generated YAP1 overexpression in AC16 human CMs decreases CM apoptosis, cell hypertrophy, and generation of reactive oxygen species (ROS), which protects CMs from IR injury (Khan et al., 2019). Interestingly, downregulation of the hypertrophy effect of YAP1 is not consistent with the results of previous studies that showed that YAP1 promotes CM hypertrophy after IR stress (Yang et al., 2015; Zhou et al., 2015). This downregulation of the hypertrophy effect may be secondary to decreased apoptosis and ROS generation instead of direct regulation by YAP1; therefore, there exists a difference. Several mechanisms explain the cardioprotective function of YAP1. YAP1 inhibits the ataxia–telangiectasia

mutated (ATM)/ATM-and Rad3-related (ATR) DNA-damage cascade to decrease apoptosis (Khan et al., 2019). ATM and ATR are sensors of DNA lesions that recognize failed DNA repairs and excessive DNA lesions and consequently trigger apoptosis (Roos and Kaina, 2013). In addition, YAP1 mediates mitochondrial homeostasis to resist ischemic stress. YAP1 can activate optic atrophy 1 (OPA-1)-mediated mitochondrial fusion, which ensures mitochondrial homeostasis (Ma and Dong, 2019). Mitochondrial fusion can reduce IR-induced mitochondrial fragmentation and mitochondrial oxidative stress, and suppress mitochondrial apoptosis; this promotes cell survival and alleviates cardiac IR injury (Anzell et al., 2018; Ma and Dong, 2019). *Park2* (a YAP1 target gene) encodes Parkin, which is an important E3 ubiquitin ligase in the outer mitochondrial membrane. Parkin promotes the clearance of impaired mitochondria through mitochondrial autophagy, which enhances stress resistance of CMs (Kubli et al., 2013; Leach et al., 2017). Moreover, YAP1 binding to Forkhead box protein O1 (FoxO1) was shown to facilitate antioxidant catalase and manganese superoxide dismutase transcription; the consequent decrease in ROS generation was shown to protect CMs against IR-induced oxidative stress (Shao et al., 2014). In the setting of MI, heterozygous inactivation of YAP1 in heart enhances CM apoptosis, fibrosis, enlarges infarct size, and impairs cardiac function; YAP1 activation using adeno-associated virus subtype 9: human YAP1 (AAV9: hYAP1) can alleviate MI injury and ameliorate cardiac function (Lin et al., 2014). In contrast to IR, YAP1 exerts anti-apoptosis activity via activation of PI3K-AKT signaling and increases CM proliferation against MI damage. Therefore, YAP1 mainly suppresses cell apoptosis through several downstream pathways and outputs to protect CMs and the heart from IR and MI damage.

Role of Hippo–YAP1/TAZ Signaling in Cardiac Pressure Overload

Similar to IR damage, endogenous RASSF1A phosphorylates and activates MST1 to promote CM apoptosis and impair cardiac function following transverse aortic constriction (TAC) - a form of PO (Del Re et al., 2010). This RASSF1A-induced apoptosis can be inhibited by MST1 deficiency. In the heart with CM-specific KO of RASSF1A, endogenous MST1 activation is abolished, and there is reduced apoptosis, fibrosis, and hypertrophy after TAC (Del Re et al., 2010). Thus, the RASSF1A-MST1 pathway is implicated in causing heart damage in response to PO stress. Another component of the Hippo pathway, LATS2, is elevated and activated after TAC. Cardiac overexpression of LATS2 was shown to be associated with diminished left ventricular systolic and diastolic dysfunction and smaller left and right ventricles (Matsui et al., 2008). Overexpression of dominant negative LATS2 (DN-LATS2) in the murine heart was found to repress CM apoptosis and promote hypertrophy following TAC (Matsui et al., 2008). In addition, a recent study showed activation of endogenous YAP1 (the terminal effector of the Hippo pathway) in the compensated phase in response to TAC (Byun et al., 2019). Heterozygous cardiac-specific YAP1 KO mice, when subjected to TAC, present blunted cardiac hypertrophy and amplified CM apoptosis and fibrosis, with resultant cardiac dilatation and dysfunction (Byun et al., 2019). The Hippo pathway, as a deleterious regulator, has been shown to exacerbate PO-induced cardiac damage; therefore, inhibition of the Hippo pathway may have a cardioprotective effect against PO injury. Mechanistically, YAP1 directly triggers the transcription of target genes to mediate downstream output and to protect the heart against PO damage. *miR-206* is identified as the novel target gene of YAP1; thus, YAP1-induced miR-206 expression promotes cell hypertrophy and cell survival by downregulating the expression of the Forkhead box protein P1 (FoxP1) (Yang et al., 2015). YAP1, through its regulation of miR-206, promotes cell hypertrophy and exerts a cardioprotective effect in response to TAC. Moreover, YAP1 promotes *Pik3cb* transcription to activate PI3K-AKT signaling and trigger CM proliferation and survival (Xin et al., 2011; Lin et al., 2015). Collectively, these studies demonstrate the cardioprotective role of YAP1/TAZ against several stressors. Thus, YAP1/TAZ may serve as a therapeutic target in many cardiac diseases.

Pernicious Role of YAP1/TAZ in Cardiac Disease

Although YAP/TAZ undertake the cardioprotective role against cardiac damage, high levels of YAP1/TAZ activity are not always beneficial. The effects of long-term suppression of Hippo signaling or activation of YAP1/TAZ in cardiac disease are opposite to those of short-term suppression. Clinical ischemic heart disease and idiopathic dilated cardiomyopathy (DCM) is characterized by enhanced YAP1/TAZ protein levels and increased transcriptional activity with the resultant upregulation of target genes, such as *ankyrin repeat domain 1*, *CTGF*, and *CYR61*; moreover, this phenomenon was observed in the murine heart with desmin-related cardiomyopathy (Hou et al., 2017). The WW45 CKO by Myh6-Cre mice show sustained YAP1 activation in CMs, which induces cardiac dysfunction, severe heart failure, and enhanced mortality in response to TAC (Ikeda et al., 2019a,b). CMs are subject to cell-cycle re-entry, increased de-differentiation, and decreased apoptosis; however, heart with active YAP1 cannot be rescued from PO damage (Ikeda et al., 2019a,b). This may be explained by the dual role of oncostatin M (OSM), which is identified as a novel target of the YAP1-TEAD1 complex. OSM promotes CM de-differentiation and induces the expression of stem cell markers to improve CM resistance to damage; however, excessive OSM stimulation inhibits CM contractility and induces cardiac remodeling (Kubin et al., 2011). In addition, YAP1-dependent OSM expression further activates YAP1 by inhibiting LATS2-induced phosphorylation, thereby forming a positive feedback loop (Ikeda et al., 2019a). This positive feedback mechanism results in sustained YAP1 activation and redundant OSM, which exacerbates cardiac injury and heart failure upon PO. Furthermore, the YAP1/TEAD1-OSM feedback cycle develops in the heart of high-fat-diet (HFD)-fed mice following PO, and contributes to the progression of diabetic cardiomyopathy (Ikeda et al., 2019b).

These observations suggest a pleiotropic role of YAP1 (Ikeda et al., 2019b). YAP1 activation during the acute phase of CM injury can induce compensatory cell proliferation and de-differentiation, which temporarily improves the tolerance of CM to damage. In later periods, persistent activation of YAP1 produces cardiac dysfunction and heart failure, which may be partly caused by the YAP1/TEAD1–OSM positive feedback loop and cardiac remodeling. However, these events do not completely explain the cardiac pathology, and there is a need to further explore the underlying mechanisms. The available evidence suggests the significance of sustaining the appropriate YAP1/TAZ protein level in therapy of cardiac disease.

The involvement of the Hippo pathway in cardiac fibrosis and remodeling explains the pernicious role of YAP1/TAZ in cardiac disease. In a murine DCM model, endogenous Angiotensin II (Ang II) was shown to activate YAP1 and promote the proliferation of cardiac fibroblasts and their transdifferentiation to myofibroblasts; subsequently, these changes were found to induce cardiac remodeling and impaired cardiac contractility (Jin et al., 2019). The Hippo–YAP1/TAZ pathway, as a mechano-sensor, is affected by mechanical signaling through cell geometry and alterations in cytoskeletal tension; this may help elucidate the pathological mechanism of chronic heart failure (Dupont et al., 2011). The subcellular localization and activity of YAP1/TAZ varies in cells with different matrix stiffness. In a stiff matrix, there is nuclear translocation and higher transcriptional activity of YAP1/TAZ; in contrast, a soft matrix is characterized by cytoplasmic sequestration and reduced activity of YAP1/TAZ (Nasrollahi and Pathak, 2017). The above studies potentially support the hypothesis that, in the context of cardiac ECM remodeling and/or fibrosis, the ECM stiffness and/or cytoskeletal tension may change and affect YAP1 activity; furthermore, activated YAP1 modulates the progression of ECM remodeling and fibrosis, which further exacerbates cardiac injury and even contributes to the development of chronic heart failure. However, the relationship between mechanical stimuli, remodeling/fibrosis, and YAP1/TAZ is not well characterized; therefore, studies to identify YAP1/TAZ activity and effects in cardiac disease are urgently needed.

ROLE OF HIPPO–YAP1/TAZ SIGNALING IN CARDIAC REGENERATION

Several endogenous and exogenous factors may cause cardiac damage and induce cardiac dysfunction. The heart is a highly differentiated organ with limited regenerative activity; this makes it difficult to restore normal function after any injury (Moya and Halder, 2018). Moreover, the regenerative capability of the adult heart is less than that of the neonatal heart. Consequently, cardiac regeneration can be activated through re-entry of terminally differentiated cells into cell cycle, reactivation of cell proliferation, and/or differentiation of stem cells into CMs, which contributes to post-damage recovery (**Tables 2**, **3**; Ponnusamy et al., 2017; Moya and Halder, 2018).

As a pathway that controls organ size, Hippo–YAP1/TAZ signaling is essential for cardiac regeneration and could be applied for cardiac regeneration and targeted therapeutics in cardiac disease. The downstream effectors of YAP1/TAZ have clear biological effects in cardiac regeneration and CM protection. Studies have shown that adult and postnatal hearts with the deletion of SAV or LATS1/2 exhibit renewal capacity, which contributes to recovery after MI and cardiac apex resection (Heallen et al., 2013; Leach et al., 2017). Postnatal hearts lacking Hippo exhibit increase in cell number and myocardial regeneration following cardiac apex resection; post-MI, SAV CKO hearts show increased LV CMs, less fibrosis, and improved cardiac function (Heallen et al., 2013; Leach et al., 2017). Mechanically, SAV deletion was found to upregulate cell cycle genes and heart growth-related genes, which increases DNA synthesis and induces cell-cycle re-entry (Leach et al., 2017). Among these upregulated genes, *Park2* (encoding Parkin) was found to promote cell regeneration following stress. Parkin participates in the clearance of damaged mitochondria through mitochondria autophagy and apoptosis, and decreases the CM sensitivity to ischemic stress (Kubli et al., 2013; Leach et al., 2017). Additionally, studies have demonstrated enhanced expression of Paired-like homeodomain transcription factor 2 (Pitx2) in the Hippo-deficient heart (Tao et al., 2016). Pitx2, as a transcriptional factor, has the capacity to regulate several target genes encoding mitochondrial, oxidation-reduction, and respiratory chain proteins via binding to YAP1 or other undefined co-factors. Therefore, Pitx2 and YAP1 may synergistically trigger anti-oxidative effect and proliferation activity to improve the stress resistance and regeneration of adult and neonatal heart (Shao et al., 2014; Tao et al., 2016). These findings suggest that the Hippo pathway plays the role of a pernicious mediator in the context of cardiac regeneration.

The YAP1 CKO neonatal heart lacks healthy myocardial tissue in the LV wall and shows enhanced fibrotic infarct zone following an MI; neonatal heart expressing active form of YAP1 exhibits increased myocardial tissue and reduced LV fibrosis (Xin et al., 2013a). Thus, the proliferative activity of YAP1 is essential for cardiac regeneration, and its regenerative activity is involved in proliferative gene programs. Mechanically, glycolytic YAP1 activation upregulates miR-152 expression to increase CM proliferation in neonatal mice (Wang et al., 2018). Furthermore, miR-152 suppresses the expression of cell-cycle inhibitory proteins, such as p27kip and DNA methyltransferase1 (DNMT1), leading to re-entry of neonatal CMs in the cell cycle. YAP1 activation promotes cell proliferation not only directly through targeting of the *CTGF* but also indirectly through inhibition of cell-cycle-inhibitory proteins; this contributes to neonatal cardiac regeneration and repair after MI injury (Haskins et al., 2014; Wang et al., 2018). As mentioned in Section "Role of Hippo–YAP1/TAZ Signaling and Its Differentiation Output in Heart Development," YAP1/TAZ have the capacity to induce proliferation and de-differentiation through targeting of the related genes (*Pik3cb*, *Dpp4*, *Dhrs3*, *SOX17*, *Bmp2b*, and others), which may induce CMs to re-enter the cell cycle and regeneration (Billings et al., 2013; Khurana et al., 2013; Lin et al., 2015; Estaras et al., 2017; Fukui et al., 2018; Hsu et al., 2018; Xiao et al., 2018).

In addition to their action on highly differentiated CMs, YAP1/TAZ regulate stem cells to undergo differentiation and proliferation to achieve cardiac regeneration. $P2Y_2$ receptor ($P2Y_2R$) is a pro-regenerative GPCR that regulates cell regeneration after damage (Vassort, 2001). Activated $P2Y_2R$ promotes the proliferation and migration of human c-Kit^+ cardiac progenitor cells (hCPCs) via activation of YAP1 (Khalafalla et al., 2017). Moreover, hCPCs improves cardiac function in MI animal models, which supports the use of hCPCs for stem cell therapy in cardiac failure (Ellison et al., 2013). YAP5SA, an active version of YAP1, is overexpressed in the mouse heart. YAP5SA overexpression causes CMs to partially re-enter the cell cycle; in addition, CMs can be reprogrammed into a more primitive and fetal cell state, whereby the heart exhibits thickened ventricular walls and smaller chambers (Monroe et al., 2019). Active Yes phosphorylates YAP1 on one or more tyrosine residues; subsequently, phosphorylated YAP1 translocates into the nucleus and induces the transcription of the key pluripotency factor *Oct-3/4* (Tamm et al., 2011). Oct-3/4 is critical for maintaining the self-renewal capacity of mouse and human ESCs. It promotes the cell cycle re-entry of ESCs via suppressing the transcription of cyclin-dependent kinase inhibitors (Lee et al., 2010). These findings indicate that YAP1 plays a critical role in maintaining the proliferative and de-differentiation capacity of stem cells and promoting adult CM renewal following injury.

Yes-associated protein 1/TAZ have the capacity to reactivate CM proliferation and may play a role in cardiac regeneration and therapy for cardiac disease. Nonetheless, the complete mechanism of action and the effects of YAP1/TAZ in cardiac development and disease remain an enormous puzzle. Therefore, further research is required to unravel the contributory mechanisms in order to clearly understand the therapeutic potential of targeted activation of the YAP1/TAZ signaling pathway in myocardial disease and cardiac regeneration.

COMPOUNDS THAT REGULATE HIPPO–YAP1/TAZ SIGNALING

Several reports have focused on the role of Hippo–YAP1/TAZ signaling in cardiac disease to explore the efficacy of leading therapeutic compounds and agents. The YAP1/TAZ–TEAD complex is considered a novel drug target to affect its biological output. Verteporfin, an inhibitor of YAP1, has been widely used to study the Hippo–YAP1/TAZ pathway (Khalafalla et al., 2017; Ikeda et al., 2019b). Verteporfin combined with YAP1 prevents the interaction of the YAP1-TEADs complex; verteporfin alters the conformation of YAP1 to enhance its binding with trypsin as well as tryptic cleavage (Liu-Chittenden et al., 2012; Tang et al., 2019). As mentioned in Section "Role of Hippo–YAP1/TAZ Signaling and Its Differentiation Output in Heart Development," YAP1/TEAD1-OSM feedback cycle exacerbates heart failure and the progression of diabetic cardiomyopathy in HFD-fed mice following TAC (Ikeda et al., 2019b). Verteporfin treatment via suppressing YAP1/TEAD1 complex alleviates cardiac dysfunction and improves the survival rate of HFD-fed mice by increasing the expression of CM dedifferentiation protein and by attenuating myocardial infiltration. $P2Y_2R$-induced YAP1 activation promotes the proliferation and migration of hCPCs, which can be repressed by verteporfin (Khalafalla et al., 2017). Therefore, verteporfin may abolish the capacity of hCPCs in repairing MI injury (Ellison et al., 2013). YAP1/TAZ luciferase reporter assays have identified apigenin as a potential YAP1 inhibitor; it was found to disrupt the interaction between YAP1 and TEAD (Li et al., 2018b). Apigenin, a kind of flavonoid, was found to exhibit a protective effect against cardiovascular diseases. *In vivo* and *vitro*, apigenin suppresses ROS production, loss of mitochondrial membrane potential (MMP), and apoptosis via PI3K/AKT signaling as well as mitochondrial Notch1/Hes1 signaling; this was found to protect H9C2 cells and rat hearts against IR injury (Hu et al., 2015; Zhou et al., 2018). However, there is no evidence to suggest that apigenin can mediate the Hippo–YAP1/TAZ pathway to defend heart against stress.

Yes-associated protein 1/TAZ are affected by other pathways; in addition, certain drugs have been shown to target the upstream regulation of YAP1. Dasatinib, which targets the mitogen-activated protein kinase (MAPK) pathway (a pro-proliferative pathway, in contrast to Hippo signaling), may affect YAP1 phosphorylation and inhibit its activity (Rosenbluh et al., 2012; He et al., 2016). Inhibition of YAP1 by dasatinib was found to induce differentiation of hESCs into APS-derived endoderm and cardiac mesoderm, consequently affecting the progression of cell differentiation in cardiogenesis (Hsu et al., 2018). GPCRs are targeted by extracellular ligands and regulate YAP1 activity. As mentioned in Section "Role of Hippo–YAP1/TAZ Signaling and Its Differentiation Output in Heart Development," Ang II and melatonin activate YAP1 through GPCRs to induce corresponding changes in cardiac function (Jin et al., 2019; Ma and Dong, 2019). YAP1 is activated by endogenous Ang II to promote the proliferation of cardiac fibroblasts and their transdifferentiation to myofibroblasts, inducing cardiac remodeling and DCM (Jin et al., 2019). In addition, melatonin facilitates OPA-1-mediated mitochondrial fusion via activating YAP1, which attenuates IR-induced mitochondrial apoptosis and alleviates cardiac IR damage (Ma and Dong, 2019). Dobutamine enhances YAP1 Ser127 phosphorylation and cytoplasmic sequestration through β-adrenergic receptor (a class of GPCRs) rather than the Hippo pathway (Bao et al., 2011). A35 (an antitumor compound) induces mutation of YAP1 Ser127, which induces the recovery of proliferative inhibition and apoptosis compared to wild-type YAP1 (Zhao et al., 2018). Although Dobutamine and A35 can mediate YAP activity in cancer cells, their effects on YAP1 in CMs and heart tissue are yet to be elucidated.

In conclusion, drugs and compounds that control kinases upstream and/or downstream of YAP1/TAZ can regulate YAP1/TAZ activity and subsequently influence cardiac injury. Undoubtedly, YAP1/TAZ is a potential therapeutic target for cancer and cardiac disease. However, the latent regulatory network of YAP1/TAZ has not been completely elucidated in previous research, especially in the context of cardiac disease.

AUTHOR CONTRIBUTIONS

NH, XC, and YL participated in research design. NH and JL supervised in research design. XC and YL performed the data analysis. NH, JL, XC, and YL wrote or contributed to the writing of the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (grant Nos. 81773720, 81402928, and 81573433) and Guangzhou Municipal Science and Technology Project (grant No. 201804010490).

REFERENCES

Ambros, V. (2004). The functions of animal microRNAs. *Nature* 431, 350–355. doi: 10.1038/nature02871

Anorga, S., Overstreet, J. M., Falke, L. L., Tang, J., Goldschmeding, R. G., Higgins, P. J., et al. (2018). Deregulation of Hippo-TAZ pathway during renal injury confers a fibrotic maladaptive phenotype. *FASEB J.* 32, 2644–2657. doi: 10.1096/fj.201700722R

Anzell, A. R., Maizy, R., Przyklenk, K., and Sanderson, T. H. (2018). Mitochondrial quality control and disease: insights into ischemia-reperfusion injury. *Mol. Neurobiol.* 55, 2547–2564. doi: 10.1007/s12035-017-0503-9

Artap, S., Manderfield, L. J., Smith, C. L., Poleshko, A., Aghajanian, H., See, K., et al. (2018). Endocardial Hippo signaling regulates myocardial growth and cardiogenesis. *Dev. Biol.* 440, 22–30. doi: 10.1016/j.ydbio.2018.04.026

Badouel, C., Zander, M. A., Liscio, N., Bagherie-Lachidan, M., Sopko, R., Coyaud, E., et al. (2015). Fat1 interacts with Fat4 to regulate neural tube closure, neural progenitor proliferation and apical constriction during mouse brain development. *Development* 142, 2781–2791. doi: 10.1242/dev.123539

Bao, Y., Nakagawa, K., Yang, Z., Ikeda, M., Withanage, K., Ishigami-Yuasa, M., et al. (2011). A cell-based assay to screen stimulators of the Hippo pathway reveals the inhibitory effect of dobutamine on the YAP-dependent gene transcription. *J. Biochem.* 150, 199–208. doi: 10.1093/jb/mvr063

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297.

Ben Mimoun, S., and Mauviel, A. (2018). Molecular mechanisms underlying TGF-beta/Hippo signaling crosstalks – role of baso-apical epithelial cell polarity. *Int. J. Biochem. Cell Biol.* 98, 75–81. doi: 10.1016/j.biocel.2018.03.006

Bersell, K., Arab, S., Haring, B., and Kuhn, B. (2009). Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* 138, 257–270. doi: 10.1016/j.cell.2009.04.060

Bi, L., Okabe, I., Bernard, D. J., and Nussbaum, R. L. (2002). Early embryonic lethality in mice deficient in the p110beta catalytic subunit of PI 3-kinase. *Mamm. Genome* 13, 169–172. doi: 10.1007/bf02684023

Billings, S. E., Pierzchalski, K., Tjaden, N. E. B., Pang, X. Y., Trainor, P. A., Kane, M. A., et al. (2013). The retinaldehyde reductase DHRS3 is essential for preventing the formation of excess retinoic acid during embryonic development. *Faseb. J.* 27, 4877–4889. doi: 10.1096/fj.13-227967

Byun, J., Del Re, D. P., Zhai, P., Ikeda, S., Shirakabe, A., Mizushima, W., et al. (2019). Yes-associated protein (YAP) mediates adaptive cardiac hypertrophy in response to pressure overload. *J. Biol. Chem.* 294, 3603–3617. doi: 10.1074/jbc.RA118.006123

Chang, L., Azzolin, L., Di Biagio, D., Zanconato, F., Battilana, G., Xiccato, R. L., et al. (2018). The SWI/SNF complex is a mechanoregulated inhibitor of YAP and TAZ. *Nature* 563, 265–269. doi: 10.1038/s41586-018-0658-1

Cho, H., Kim, J., Ahn, J. H., Hong, Y. K., Makinen, T., Lim, D. S., et al. (2019). YAP and TAZ negatively regulate Prox1 during developmental and pathologic lymphangiogenesis. *Circ. Res.* 124, 225–242. doi: 10.1161/CIRCRESAHA.118.313707

Cho, Y. S., Zhu, J., Li, S., Wang, B., Han, Y., and Jiang, J. (2018). Regulation of Yki/Yap subcellular localization and Hpo signaling by a nuclear kinase PRP4K. *Nat. Commun.* 9:1657. doi: 10.1038/s41467-018-04090-2

Dai, X., Liu, H., Shen, S., Guo, X., Yan, H., Ji, X., et al. (2017). YAP activates the Hippo pathway in a negative feedback loop. *Cell Res.* 27:1073. doi: 10.1038/cr.2017.96

Del Re, D. P., Matsuda, T., Zhai, P., Gao, S., Clark, G. J., Van Der Weyden, L., et al. (2010). Proapoptotic Rassf1A/Mst1 signaling in cardiac fibroblasts is protective against pressure overload in mice. *J. Clin. Invest.* 120, 3555–3567. doi: 10.1172/JCI43569

Del Re, D. P., Yang, Y., Nakano, N., Cho, J., Zhai, P., Yamamoto, T., et al. (2013). Yes-associated protein isoform 1 (Yap1) promotes cardiomyocyte survival and growth to protect against myocardial ischemic injury. *J. Biol. Chem.* 288, 3977–3988. doi: 10.1074/jbc.M112.436311

Diez-Cunado, M., Wei, K., Bushway, P. J., Maurya, M. R., Perera, R., Subramaniam, S., et al. (2018). miRNAs that induce human cardiomyocyte proliferation converge on the hippo pathway. *Cell Rep.* 23, 2168–2174. doi: 10.1016/j.celrep.2018.04.049

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S. A., et al. (2007). Elucidation of a universal size-control mechanism in *Drosophila* and mammals. *Cell* 130, 1120–1133. doi: 10.1016/j.cell.2007.07.019

Dupont, S., Morsut, L., Aragona, M., Enzo, E., Giulitti, S., Cordenonsi, M., et al. (2011). Role of YAP/TAZ in mechanotransduction. *Nature* 474, 179–183. doi: 10.1038/nature10137

Ellison, G. M., Vicinanza, C., Smith, A. J., Aquila, I., Leone, A., Waring, C. D., et al. (2013). Adult c-kit(pos) cardiac stem cells are necessary and sufficient for functional cardiac regeneration and repair. *Cell* 154, 827–842. doi: 10.1016/j.cell.2013.07.039

Elosegui-Artola, A., Andreu, I., Beedle, A. E. M., Lezamiz, A., Uroz, M., Kosmalska, A. J., et al. (2017). Force triggers YAP nuclear entry by regulating transport across nuclear pores. *Cell* 171, 1397–1410.e14. doi: 10.1016/j.cell.2017.10.008

Estaras, C., Hsu, H.-T., Huang, L., and Jones, K. A. (2017). YAP repression of the WNT3 gene controls hESC differentiation along the cardiac mesoderm lineage. *Genes Dev.* 31, 2250–2263. doi: 10.1101/gad.307512.117

Fang, L., Teng, H., Wang, Y., Liao, G., Weng, L., Li, Y., et al. (2018). SET1A-mediated mono-methylation at K342 regulates YAP activation by blocking its nuclear export and promotes tumorigenesis. *Cancer Cell* 34, 103–118.e9. doi: 10.1016/j.ccell.2018.06.002

Fukui, H., Miyazaki, T., Chow, R. W.-Y., Ishikawa, H., Nakajima, H., Vermot, J., et al. (2018). Hippo signaling determines the number of venous pole cells that originate from the anterior lateral plate mesoderm in zebrafish. *eLife* 7:e29106. doi: 10.7554/eLife.29106

Garbino, A., Van Oort, R. J., Dixit, S. S., Landstrom, A. P., Ackerman, M. J., and Wehrens, X. H. (2009). Molecular evolution of the junctophilin gene family. *Physiol. Genomics* 37, 175–186. doi: 10.1152/physiolgenomics.00017.2009

Gemberling, M., Karra, R., Dickson, A. L., and Poss, K. D. (2015). Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish. *eLife* 4:e05871. doi: 10.7554/eLife.05871

Ghersi, G., Zhao, Q., Salamone, M., Yeh, Y., Zucker, S., and Chen, W. T. (2006). The protease complex consisting of dipeptidyl peptidase IV and seprase plays a role in the migration and invasion of human endothelial cells in collagenous matrices. *Cancer Res.* 66, 4652–4661. doi: 10.1158/0008-5472.can-05-1245

Gomez-Puerto, M. C., Iyengar, P. V., Garcia De Vinuesa, A., Ten Dijke, P., and Sanchez-Duffhues, G. (2019). Bone morphogenetic protein receptor signal transduction in human disease. *J. Pathol.* 247, 9–20. doi: 10.1002/path.5170

Harvey, K. F., Pfleger, C. M., and Hariharan, I. K. (2003). The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* 114, 457–467. doi: 10.1016/s0092-8674(03)00557-9

Harvey, N. L., Srinivasan, R. S., Dillard, M. E., Johnson, N. C., Witte, M. H., Boyd, K., et al. (2005). Lymphatic vascular defects promoted by Prox1 haploinsufficiency cause adult-onset obesity. *Nat. Genet.* 37, 1072–1081. doi: 10.1038/ng1642

Haskins, J. W., Nguyen, D. X., and Stern, D. F. (2014). Neuregulin 1-activated ERBB4 interacts with YAP to induce Hippo pathway target genes and promote cell migration. *Sci. Signal.* 7:ra116. doi: 10.1126/scisignal.2005770

Hayashi, S., Yokoyama, H., and Tamura, K. (2015). Roles of Hippo signaling pathway in size control of organ regeneration. *Dev. Growth Differ.* 57, 341–351. doi: 10.1111/dgd.12212

He, M., Zhou, Z., Shah, A. A., Hong, Y., Chen, Q., and Wan, Y. (2016). New insights into posttranslational modifications of Hippo pathway in carcinogenesis and therapeutics. *Cell Div.* 11:4. doi: 10.1186/s13008-016-0013-6

Heallen, T., Morikawa, Y., Leach, J., Tao, G., Willerson, J. T., Johnson, R. L., et al. (2013). Hippo signaling impedes adult heart regeneration. *Development* 140, 4683–4690. doi: 10.1242/dev.102798

Heallen, T., Zhang, M., Wang, J., Bonilla-Claudio, M., Klysik, E., Johnson, R. L., et al. (2011). Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science* 332, 458–461. doi: 10.1126/science.1199010

Hertig, V., Matos-Nieves, A., Garg, V., Villeneuve, L., Mamarbachi, M., Caland, L., et al. (2018). Nestin expression is dynamically regulated in cardiomyocytes during embryogenesis. *J. Cell. Physiol.* 233, 3218–3229. doi: 10.1002/jcp.26165

Hossain, Z., Ali, S. M., Ko, H. L., Xu, J., Ng, C. P., Guo, K., et al. (2007). Glomerulocystic kidney disease in mice with a targeted inactivation of Wwtr1. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1631–1636. doi: 10.1073/pnas.0605266104

Hou, N., Wen, Y., Yuan, X., Xu, H., Wang, X., Li, F., et al. (2017). Activation of Yap1/Taz signaling in ischemic heart disease and dilated cardiomyopathy. *Exp. Mol. Pathol.* 103, 267–275. doi: 10.1016/j.yexmp.2017.11.006

Hsu, H.-T., Estaras, C., Huang, L., and Jones, K. A. (2018). Specifying the anterior primitive streak by modulating YAP1 levels in human pluripotent stem cells. *Stem Cell Rep.* 11, 1357–1364. doi: 10.1016/j.stemcr.2018.10.013

Hu, J., Li, Z., Xu, L. T., Sun, A. J., Fu, X. Y., Zhang, L., et al. (2015). Protective effect of apigenin on ischemia/reperfusion injury of the isolated rat heart. *Cardiovasc. Toxicol.* 15, 241–249. doi: 10.1007/s12010-014-9290-y

Ikeda, S., Mizushima, W., Sciarretta, S., Abdellatif, M., Zhai, P., Mukai, R., et al. (2019a). Hippo deficiency leads to cardiac dysfunction accompanied by cardiomyocyte dedifferentiation during pressure overload. *Circ. Res.* 124, 292–305. doi: 10.1161/CIRCRESAHA.118.314048

Ikeda, S., Mukai, R., Mizushima, W., Zhai, P., Oka, S. I., Nakamura, M., et al. (2019b). Yes-associated protein (YAP) facilitates pressure overload-induced dysfunction in the diabetic heart. *JACC Basic Transl. Sci.* 4, 611–622. doi: 10.1016/j.jacbts.2019.05.006

Jin, B., Zhu, J., Shi, H.-M., Wen, Z.-C., and Wu, B.-W. (2019). YAP activation promotes the transdifferentiation of cardiac fibroblasts to myofibroblasts in matrix remodeling of dilated cardiomyopathy. *Br. J. Med. Biol. Res.* 52:e7914. doi: 10.1590/1414-431X20187914

Kadoch, C., and Crabtree, G. R. (2015). Mammalian SWI/SNF chromatin remodeling complexes and cancer: mechanistic insights gained from human genomics. *Sci. Adv.* 1:e1500447. doi: 10.1126/sciadv.1500447

Khalafalla, F. G., Greene, S., Khan, H., Ilves, K., Monsanto, M. M., Alvarez, R. Jr., et al. (2017). P2Y(2) nucleotide receptor prompts human cardiac progenitor cell activation by modulating hippo signaling. *Circ. Res.* 121, 1224–1236. doi: 10.1161/circresaha.117.310812

Khan, K., Makhoul, G., Yu, B., Schwertani, A., and Cecere, R. (2019). The cytoprotective impact of yes-associated protein 1 after ischemia-reperfusion injury in AC16 human cardiomyocytes. *Exp. Biol. Med. (Maywood)* 244, 802–812. doi: 10.1177/1535370219851243

Khurana, S., Margamuljana, L., Joseph, C., Schouteden, S., Buckley, S. M., and Verfaillie, C. M. (2013). Glypican-3-mediated inhibition of CD26 by TFPI: a novel mechanism in hematopoietic stem cell homing and maintenance. *Blood* 121, 2587–2595. doi: 10.1182/blood-2012-09-456715

Kim, M., Kim, M., Park, S. J., Lee, C., and Lim, D. S. (2016). Role of angiomotin-like 2 mono-ubiquitination on YAP inhibition. *EMBO Rep.* 17, 64–78. doi: 10.15252/embr.201540809

Kim, M. K., Jang, J. W., and Bae, S. C. (2018). DNA binding partners of YAP/TAZ. *BMB Rep.* 51, 126–133. doi: 10.5483/bmbrep.2018.51.3.015

Kofler, M., Speight, P., Little, D., Di Ciano-Oliveira, C., Szaszi, K., and Kapus, A. (2018). Mediated nuclear import and export of TAZ and the underlying molecular requirements. *Nat. Commun.* 9:4966. doi: 10.1038/s41467-018-07450-0

Kubin, T., Poling, J., Kostin, S., Gajawada, P., Hein, S., Rees, W., et al. (2011). Oncostatin M is a major mediator of cardiomyocyte dedifferentiation and remodeling. *Cell Stem Cell* 9, 420–432. doi: 10.1016/j.stem.2011.08.013

Kubli, D. A., Zhang, X., Lee, Y., Hanna, R. A., Quinsay, M. N., Nguyen, C. K., et al. (2013). Parkin protein deficiency exacerbates cardiac injury and reduces survival following myocardial infarction. *J. Biol. Chem.* 288, 915–926. doi: 10.1074/jbc.M112.411363

Lai, J. K. H., Collins, M. M., Uribe, V., Jimenez-Amilburu, V., Guenther, S., Maischein, H.-M., et al. (2018). The Hippo pathway effector Wwtr1 regulates cardiac wall maturation in zebrafish. *Development* 145:dev159210. doi: 10.1242/dev.159210

Lange, A. W., Haitchi, H. M., Lecras, T. D., Sridharan, A., Xu, Y., Wert, S. E., et al. (2014). Sox17 is required for normal pulmonary vascular morphogenesis. *Dev. Biol.* 387, 109–120. doi: 10.1016/j.ydbio.2013.11.018

Leach, J. P., Heallen, T., Zhang, M., Rahmani, M., Morikawa, Y., Hill, M. C., et al. (2017). Hippo pathway deficiency reverses systolic heart failure after infarction. *Nature* 550, 260–264. doi: 10.1038/nature24045

Lee, J., Go, Y., Kang, I., Han, Y. M., and Kim, J. (2010). Oct-4 controls cell-cycle progression of embryonic stem cells. *Biochem. J.* 426, 171–181. doi: 10.1042/BJ20091439

Levy, D., Adamovich, Y., Reuven, N., and Shaul, Y. (2008). Yap1 phosphorylation by c-Abl is a critical step in selective activation of proapoptotic genes in response to DNA damage. *Mol. Cell* 29, 350–361. doi: 10.1016/j.molcel.2007.12.022

Li, F., Wang, X., Capasso, J. M., and Gerdes, A. M. (1996). Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J. Mol. Cell Cardiol.* 28, 1737–1746. doi: 10.1006/jmcc.1996.0163

Li, L., Liu, T., Li, Y., Wu, C., Luo, K., Yin, Y., et al. (2018a). The deubiquitinase USP9X promotes tumor cell survival and confers chemoresistance through YAP1 stabilization. *Oncogene* 37, 2422–2431. doi: 10.1038/s41388-018-0134-2

Li, Y. W., Xu, J., Zhu, G. Y., Huang, Z. J., Lu, Y., Li, X. Q., et al. (2018b). Apigenin suppresses the stem cell-like properties of triple-negative breast cancer cells by inhibiting YAP/TAZ activity. *Cell Death Discov.* 4:105. doi: 10.1038/s41420-018-0124-8

Li, Z., Zhao, B., Wang, P., Chen, F., Dong, Z., Yang, H., et al. (2010). Structural insights into the YAP and TEAD complex. *Genes Dev.* 24, 235–240. doi: 10.1101/gad.1865810

Liao, R., Jain, M., Cui, L., D'agostino, J., Aiello, F., Luptak, I., et al. (2002). Cardiac-specific overexpression of GLUT1 prevents the development of heart failure attributable to pressure overload in mice. *Circulation* 106, 2125–2131. doi: 10.1161/01.cir.0000034049.61181.f3

Lin, C., and Xu, X. (2017). YAP1-TEAD1-Glut1 axis dictates the oncogenic phenotypes of breast cancer cells by modulating glycolysis. *Biomed. Pharmacother.* 95, 789–794. doi: 10.1016/j.biopha.2017.08.091

Lin, Z., Von Gise, A., Zhou, P., Gu, F., Ma, Q., Jiang, J., et al. (2014). Cardiac-specific YAP activation improves cardiac function and survival in an experimental murine MI model. *Circ. Res.* 115, 354–363. doi: 10.1161/CIRCRESAHA.115.303632

Lin, Z., Zhou, P., Von Gise, A., Gu, F., Ma, Q., Chen, J., et al. (2015). Pi3kcb links Hippo-YAP and PI3K-AKT signaling pathways to promote cardiomyocyte proliferation and survival. *Circ. Res.* 116, 35–45. doi: 10.1161/CIRCRESAHA.115.304457

Liu, A., Zeng, S., Lu, X., Xiong, Q., Xue, Y., Tong, L., et al. (2019). Overexpression of G2 and S phase-expressed-1 contributes to cell proliferation, migration, and invasion via regulating p53/FoxM1/CCNB1 pathway and predicts poor prognosis in bladder cancer. *Int. J. Biol. Macromol.* 123, 322–334. doi: 10.1016/j.ijbiomac.2018.11.032

Liu, C. Y., Lv, X., Li, T., Xu, Y., Zhou, X., Zhao, S., et al. (2011). PP1 cooperates with ASPP2 to dephosphorylate and activate TAZ. *J. Biol. Chem.* 286, 5558–5566. doi: 10.1074/jbc.M110.194019

Liu, S., Bou, G., Zhao, J., Guo, S., Guo, J., Weng, X., et al. (2018). Asynchronous CDX2 expression and polarization of porcine trophoblast cells reflects a species-specific trophoderm lineage determination progress model. *Mol. Reprod. Dev.* 85, 590–598. doi: 10.1002/mrd.22994

Liu-Chittenden, Y., Huang, B., Shim, J. S., Chen, Q., Lee, S. J., Anders, R. A., et al. (2012). Genetic and pharmacological disruption of the TEAD-YAP complex suppresses the oncogenic activity of YAP. *Genes Dev.* 26, 1300–1305. doi: 10.1101/gad.192856.112

Ma, S., and Dong, Z. (2019). Melatonin attenuates cardiac reperfusion stress by improving OPA1-related mitochondrial fusion in a Yap-Hippo pathway-dependent manner. *J. Cardiovasc. Pharmacol.* 73, 27–39. doi: 10.1097/FJC.0000000000000626

Maejima, Y., Kyoi, S., Zhai, P., Liu, T., Li, H., Ivessa, A., et al. (2013). Mst1 inhibits autophagy by promoting the interaction between Beclin1 and Bcl-2. *Nat. Med.* 19, 1478–1488. doi: 10.1038/nm.3322

Makita, R., Uchijima, Y., Nishiyama, K., Amano, T., Chen, Q., Takeuchi, T., et al. (2008). Multiple renal cysts, urinary concentration defects, and pulmonary emphysematous changes in mice lacking TAZ. *Am. J. Physiol. Renal. Physiol.* 294, F542–F553. doi: 10.1152/ajprenal.00201.2007

Matsuda, T., Zhai, P., Sciarretta, S., Zhang, Y., Jeong, J. I., Ikeda, S., et al. (2016). NF2 activates Hippo signaling and promotes ischemia/reperfusion injury in the heart. *Circ. Res.* 119, 596–606. doi: 10.1161/CIRCRESAHA.116.308586

Matsui, Y., Nakano, N., Shao, D., Gao, S., Luo, W., Hong, C., et al. (2008). Lats2 is a negative regulator of myocyte size in the heart. *Circ. Res.* 103, 1309–1318. doi: 10.1161/CIRCRESAHA.108.180042

Meng, Z., Moroishi, T., and Guan, K. L. (2016). Mechanisms of Hippo pathway regulation. *Genes Dev.* 30, 1–17. doi: 10.1101/gad.274027.115

Miskolczi, Z., Smith, M. P., Rowling, E. J., Ferguson, J., Barriuso, J., and Wellbrock, C. (2018). Collagen abundance controls melanoma phenotypes through lineage-specific microenvironment sensing. *Oncogene* 37, 3166–3182. doi: 10.1038/s41388-018-0209-0

Mo, J.-S. (2017). The role of extracellular biophysical cues in modulating the Hippo-YAP pathway. *BMB Rep.* 50, 71–78. doi: 10.5483/bmbrep.2017.50.2.199

Monroe, T. O., Hill, M. C., Morikawa, Y., Leach, J. P., Heallen, T., Cao, S., et al. (2019). YAP partially reprograms chromatin accessibility to directly induce adult cardiogenesis in vivo. *Dev. Cell* 48, 765–779.e7. doi: 10.1016/j.devcel.2019.01.017

Moon, S., Kim, W., Kim, S., Kim, Y., Song, Y., Bilousov, O., et al. (2017). Phosphorylation by NLK inhibits YAP-14-3-3-interactions and induces its nuclear localization. *EMBO Rep.* 18, 61–71. doi: 10.15252/embr.201642683

Morikawa, Y., Heallen, T., Each, J. L., Xiao, Y., and Martin, J. F. (2017). Dystrophin-glycoprotein complex sequesters Yap to inhibit cardiomyocyte proliferation. *Nature* 547, 227–231. doi: 10.1038/nature22979

Moroishi, T., Park, H. W., Qin, B., Chen, Q., Meng, Z., Plouffe, S. W., et al. (2015). A YAP/TAZ-induced feedback mechanism regulates Hippo pathway homeostasis. *Genes Dev.* 29, 1271–1284. doi: 10.1101/gad.262816.115

Moya, I. M., and Halder, G. (2018). Hippo-YAP/TAZ signalling in organ regeneration and regenerative medicine. *Nat. Rev. Mol. Cell Biol.* 20, 211–226. doi: 10.1038/s41580-018-0086-y

Nakamura, M., Zhai, P., Del Re, D. P., Maejima, Y., and Sadoshima, J. (2016). Mst1-mediated phosphorylation of Bcl-xL is required for myocardial reperfusion injury. *JCI Insight* 1:e86217.

Nasrollahi, S., and Pathak, A. (2017). Hydrogel-based microchannels to measure confinement- and stiffness-sensitive Yes-associated-protein activity in epithelial clusters. *Mrs Commun.* 7, 450–457. doi: 10.1557/mrc.2017.87

Negron-Perez, V. M., and Hansen, P. J. (2018). Role of yes-associated protein 1, angiomotin, and mitogen-activated kinase kinase 1/2 in development of the bovine blastocyst. *Biol. Reprod.* 98, 170–183. doi: 10.1093/biolre/iox172

Nishida, K., Yamaguchi, O., and Otsu, K. (2008). Crosstalk between autophagy and apoptosis in heart disease. *Circ. Res.* 103, 343–351. doi: 10.1161/CIRCRESAHA.108.175448

Odashima, M., Usui, S., Takagi, H., Hong, C., Liu, J., Yokota, M., et al. (2007). Inhibition of endogenous Mst1 prevents apoptosis and cardiac dysfunction without affecting cardiac hypertrophy after myocardial infarction. *Circ. Res.* 100, 1344–1352. doi: 10.1161/01.res.0000265846.23485.7a

Oudhoff, M. J., Freeman, S. A., Couzens, A. L., Antignano, F., Kuznetsova, E., Min, P. H., et al. (2013). Control of the Hippo pathway by Set7-dependent methylation of Yap. *Dev. Cell* 26, 188–194. doi: 10.1016/j.devcel.2013.05.025

Pan, D. (2007). Hippo signaling in organ size control. *Genes Dev.* 21, 886–897. doi: 10.1101/gad.1536007

Paramasivam, M., Sarkeshik, A., Yates, J. R. III, Fernandes, M. J., and Mccollum, D. (2011). Angiomotin family proteins are novel activators of the LATS2 kinase tumor suppressor. *Mol. Biol. Cell* 22, 3725–3733. doi: 10.1091/mbc.E11-04-0300

Peng, C., Zhu, Y., Zhang, W., Liao, Q., Chen, Y., Zhao, X., et al. (2017). Regulation of the Hippo-YAP pathway by glucose sensor O-GlcNAcylation. *Mol. Cell* 68:e595. doi: 10.1016/j.molcel.2017.10.010

Petroski, M. D., and Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nat. Rev. Mol. Cell Biol.* 6, 9–20. doi: 10.1038/nrm1547

Piccolo, S., Dupont, S., and Cordenonsi, M. (2014). The biology of Yap/Taz: Hippo signaling and beyond. *Physiol. Rev.* 94, 1287–1312. doi: 10.1152/physrev.00005.2014

Ponnusamy, M., Li, P.-F., and Wang, K. (2017). Understanding cardiomyocyte proliferation: an insight into cell cycle activity. *Cell. Mol. Life Sci.* 74, 1019–1034. doi: 10.1007/s00018-016-2375-y

Qin, Z., Xia, W., Fisher, G. J., Voorhees, J. J., and Quan, T. (2018). YAP/TAZ regulates TGF-beta/Smad3 signaling by induction of Smad7 via AP-1 in human skin dermal fibroblasts. *Cell Commun. Signal.* 16:18. doi: 10.1186/s12964-018-0232-3

Ragni, C. V., Diguet, N., Le Garrec, J.-F., Novotova, M., Resende, T. P., Pop, S., et al. (2017). Amotl1 mediates sequestration of the Hippo effector Yap1 downstream of Fat4 to restrict heart growth. *Nat. Commun.* 8:14582. doi: 10.1038/ncomms14582

Roos, W. P., and Kaina, B. (2013). DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. *Cancer Lett.* 332, 237–248. doi: 10.1016/j.canlet.2012.01.007

Rosenbluh, J., Nijhawan, D., Cox, A. G., Li, X., Neal, J. T., Schafer, E. J., et al. (2012). beta-Catenin-driven cancers require a YAP1 transcriptional complex for survival and tumorigenesis. *Cell* 151, 1457–1473. doi: 10.1016/j.cell.2012.11.026

Schindler, Y. L., Garske, K. M., Wang, J., Firulli, B. A., Firulli, A. B., Poss, K. D., et al. (2014). Hand2 elevates cardiomyocyte production during zebrafish heart development and regeneration. *Development* 141, 3112–3122. doi: 10.1242/dev.106336

Shao, D., Zhai, P., Del Re, D. P., Sciarretta, S., Yabuta, N., Nojima, H., et al. (2014). A functional interaction between Hippo-YAP signalling and FoxO1 mediates the oxidative stress response. *Nat. Commun.* 5:3315. doi: 10.1038/ncomms4315

Srivastava, D., and Yu, S. (2006). Stretching to meet needs: integrin-linked kinase and the cardiac pump. *Genes Dev.* 20, 2327–2331. doi: 10.1101/gad.1472506

Stelitano, D., Peche, L. Y., Dalla, E., Monte, M., Piazza, S., and Schneider, C. (2017). GTSE1: a novel TEAD4-E2F1 target gene involved in cell protrusions formation in triple-negative breast cancer cell models. *Oncotarget* 8, 67422–67438. doi: 10.18632/oncotarget.18691

Sudol, M. (1994). Yes-associated protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product. *Oncogene* 9, 2145–2152.

Tam, P. P., Parameswaran, M., Kinder, S. J., and Weinberger, R. P. (1997). The allocation of epiblast cells to the embryonic heart and other mesodermal lineages: the role of ingression and tissue movement during gastrulation. *Development* 124, 1631–1642.

Tamm, C., Bower, N., and Anneren, C. (2011). Regulation of mouse embryonic stem cell self-renewal by a Yes-YAP-TEAD2 signaling pathway downstream of LIF. *J. Cell Sci.* 124, 1136–1144. doi: 10.1242/jcs.075796

Tang, C., Takahashi-Kanemitsu, A., Kikuchi, I., Ben, C., and Hatakeyama, M. (2018). Transcriptional co-activator functions of YAP andTAZ are inversely regulated by tyrosine phosphorylation status of parafibromin. *iScience* 1, 1–15. doi: 10.1016/j.isci.2018.01.003

Tang, Z., Ma, Q., Wang, L., Liu, C., Gao, H., Yang, Z., et al. (2019). A brief review: some compounds targeting YAP against malignancies. *Future Oncol.* 15, 1535–1543. doi: 10.2217/fon-2019-0035

Taniguchi, K., Wu, L. W., Grivennikov, S. I., De Jong, P. R., Lian, I., Yu, F. X., et al. (2015). A gp130-Src-YAP module links inflammation to epithelial regeneration. *Nature* 519, 57–62. doi: 10.1038/nature14228

Tao, G., Kahr, P. C., Morikawa, Y., Zhang, M., Rahmani, M., Heallen, T. R., et al. (2016). Pitx2 promotes heart repair by activating the antioxidant response after cardiac injury. *Nature* 534, 119–123. doi: 10.1038/nature17959

Tian, Y., Liu, Y., Wang, T., Zhou, N., Kong, J., Chen, L., et al. (2015). A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. *Sci. Transl. Med.* 7:279ra38. doi: 10.1126/scitranslmed.3010841

Tu, K., Yang, W., Li, C., Zheng, X., Lu, Z., Guo, C., et al. (2014). Fbxw7 is an independent prognostic marker and induces apoptosis and growth arrest by regulating YAP abundance in hepatocellular carcinoma. *Mol. Cancer* 13:110. doi: 10.1186/1476-4598-13-110

Valis, K., Talacko, P., Grobarova, V., Cerny, J., and Novak, P. (2016). Shikonin regulates C-MYC and GLUT1 expression through the MST1-YAP1-TEAD1 axis. *Exp. Cell Res.* 349, 273–281. doi: 10.1016/j.yexcr.2016.10.018

Vassort, G. (2001). Adenosine 5'-triphosphate: a P2-purinergic agonist in the myocardium. *Physiol. Rev.* 81, 767–806. doi: 10.1152/physrev.2001.81.2.767

von Gise, A., Lin, Z., Schlegelmilch, K., Honor, L. B., Pan, G. M., Buck, J. N., et al. (2012). YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2394–2399. doi: 10.1073/pnas.1116136109

Wang, J., Ye, C., Chen, C., Xiong, H., Xie, B., Zhou, J., et al. (2017). Glucose transporter GLUT1 expression and clinical outcome in solid tumors: a systematic review and meta-analysis. *Oncotarget* 8, 16875–16886. doi: 10.18632/oncotarget.15171

Wang, P., Bai, Y., Song, B., Wang, Y., Liu, D., Lai, Y., et al. (2011). PP1A-mediated dephosphorylation positively regulates YAP2 activity. *PLoS One* 6:e24288. doi: 10.1371/journal.pone.0024288

Wang, X., Ha, T., Liu, L., Hu, Y., Kao, R., Kalbfleisch, J., et al. (2018). TLR3 mediates repair and regeneration of damaged neonatal heart through glycolysis dependent YAP1 regulated miR-152 expression. *Cell Death Differ.* 25, 966–982. doi: 10.1038/s41418-017-0036-9

Wang, Y., Hu, G., Liu, F., Wang, X., Wu, M., Schwarz, J. J., et al. (2014). Deletion of yes-associated protein (YAP) specifically in cardiac and vascular smooth muscle cells reveals a crucial role for YAP in mouse cardiovascular development. *Circ. Res.* 114, 957–965. doi: 10.1161/CIRCRESAHA.114.303411

Xiao, Y., Hill, M. C., Zhang, M., Martin, T. J., Morikawa, Y., Wang, S., et al. (2018). Hippo signaling plays an essential role in cell state transitions during cardiac fibroblast development. *Dev. Cell* 45, 153–169.e6. doi: 10.1016/j.devcel.2018.03.019

Xin, M., Kim, Y., Sutherland, L. B., Murakami, M., Qi, X., Mcanally, J., et al. (2013a). Hippo pathway effector Yap promotes cardiac regeneration. *Proc. Natl. Acad. Sci. U.S.A.* 110, 13839–13844. doi: 10.1073/pnas.1313192110

Xin, M., Kim, Y., Sutherland, L. B., Qi, X., Mcanally, J., Schwartz, R. J., et al. (2011). Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. *Sci. Signal.* 4:ra70. doi: 10.1126/scisignal.2002278

Xin, M., Olson, E. N., and Bassel-Duby, R. (2013b). Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. *Nat. Rev. Mol. Cell Biol.* 14, 529–541. doi: 10.1038/nrm3619

Yamamoto, S., Yang, G., Zablocki, D., Liu, J., Hong, C., Kim, S. J., et al. (2003). Activation of Mst1 causes dilated cardiomyopathy by stimulating apoptosis without compensatory ventricular myocyte hypertrophy. *J. Clin. Invest.* 111, 1463–1474. doi: 10.1172/jci17459

Yang, Y., Del Re, D. P., Nakano, N., Sciarretta, S., Zhai, P., Park, J., et al. (2015). miR-206 mediates YAP-induced cardiac hypertrophy and survival. *Circ. Res.* 117, 891–904. doi: 10.1161/CIRCRESAHA.115.306624

Yao, F., Zhou, Z., Kim, J., Hang, Q., Xiao, Z., Ton, B. N., et al. (2018). SKP2- and OTUD1-regulated non-proteolytic ubiquitination of YAP promotes YAP nuclear localization and activity. *Nat. Commun.* 9:2269. doi: 10.1038/s41467-018-04620-y

Zhang, Q., Han, X., Chen, J., Xie, X., Xu, J., Zhao, Y., et al. (2018a). Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) mediate cell density-dependent proinflammatory responses. *J. Biol. Chem.* 293, 18071–18085. doi: 10.1074/jbc.RA118.004251

Zhang, Q., Zhang, Y., Parsels, J. D., Lohse, I., Lawrence, T. S., Pasca Di Magliano, M., et al. (2016). Fbxw7 deletion accelerates kras(G12D)-driven pancreatic tumorigenesis via Yap accumulation. *Neoplasia* 18, 666–673. doi: 10.1016/j.neo.2016.08.009

Zhang, X., Li, Y., Ma, Y., Yang, L., Wang, T., Meng, X., et al. (2018b). Yes-associated protein (YAP) binds to HIF-1 alpha and sustains HIF-1 alpha protein stability to promote hepatocellular carcinoma cell glycolysis under hypoxic stress. *J. Exp. Clin. Cancer Res.* 37:216. doi: 10.1186/s13046-018-0892-2

Zhang, X., Qiao, Y., Wu, Q., Chen, Y., Zou, S., Liu, X., et al. (2017). The essential role of YAP O-GlcNAcylation in high-glucose-stimulated liver tumorigenesis. *Nat. Commun.* 8:15280. doi: 10.1038/ncomms15280

Zhao, B., Li, L., Tumaneng, K., Wang, C. Y., and Guan, K. L. (2010). A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). *Genes Dev.* 24, 72–85. doi: 10.1101/gad.1843810

Zhao, B., Wei, X., Li, W., Udan, R. S., Yang, Q., Kim, J., et al. (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev.* 21, 2747–2761. doi: 10.1101/gad.1602907

Zhao, W., Liu, H., Wang, J., Wang, M., and Shao, R. (2018). Cyclizing-berberine A35 induces G2/M arrest and apoptosis by activating YAP phosphorylation (Ser127). *J. Exp. Clin. Cancer Res.* 37:98. doi: 10.1186/s13046-018-0759-6

Zhao, W. B., Lu, Q., Nguyen, M. N., Su, Y., Ziemann, M., Wang, L. N., et al. (2019). Stimulation of beta-adrenoceptors up-regulates cardiac expression of galectin-3 and BIM through the Hippo signalling pathway. *Br. J. Pharmacol.* 176, 2465–2481. doi: 10.1111/bph.14674

Zhou, Q., Li, L., Zhao, B., and Guan, K. L. (2015). The hippo pathway in heart development, regeneration, and diseases. *Circ. Res.* 116, 1431–1447. doi: 10.1161/circresaha.116.303311

Zhou, Z., Zhang, Y., Lin, L., and Zhou, J. (2018). Apigenin suppresses the apoptosis of H9C2 rat cardiomyocytes subjected to myocardial ischemiareperfusion injury via upregulation of the PI3K/Akt pathway. *Mol. Med. Rep.* 18, 1560–1570. doi: 10.3892/mmr.2018.9115

Zou, J., Ma, W., Li, J., Littlejohn, R., Zhou, H., Kim, I.-M., et al. (2018). Neddylation mediates ventricular chamber maturation through repression of Hippo signaling. *Proc. Natl. Acad. Sci. U.S.A.* 115, E4101–E4110. doi: 10.1073/pnas.1719309115

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 06 May 2020
doi: 10.3389/fphys.2020.00427

Posttranslational Arginylation Enzyme Arginyltransferase1 Shows Genetic Interactions With Specific Cellular Pathways *in vivo*

*David J. Wiley[1], Gennaro D'Urso[1] and Fangliang Zhang[1,2]**

[1] Department of Molecular and Cellular Pharmacology, University of Miami Leonard M. Miller School of Medicine, Miami, FL, United States, [2] Sylvester Comprehensive Cancer Center, University of Miami Leonard M. Miller School of Medicine, Miami, FL, United States

Arginyltransferase1 (ATE1) is a conserved enzyme in eukaryotes mediating posttranslational arginylation, the addition of an extra arginine to an existing protein. In mammals, the dysregulations of the ATE1 gene (*ate1*) is shown to be involved in cardiovascular abnormalities, cancer, and aging-related diseases. Although biochemical evidence suggested that arginylation may be involved in stress response and/or protein degradation, the physiological role of ATE1 *in vivo* has never been systematically determined. This gap of knowledge leads to difficulties for interpreting the involvements of ATE1 in diseases pathogenesis. Since *ate1* is highly conserved between human and the unicellular organism *Schizosaccharomyces pombe* (*S. pombe*), we take advantage of the gene-knockout library of *S. pombe*, to investigate the genetic interactions between *ate1* and other genes in a systematic and unbiased manner. By this approach, we found that *ate1* has a surprisingly small and focused impact size. Among the 3659 tested genes, which covers nearly 75% of the genome of *S. pombe*, less than 5% of them displayed significant genetic interactions with *ate1*. Furthermore, these *ate1*-interacting partners can be grouped into a few discrete clustered categories based on their functions or their physical interactions. These categories include translation/transcription regulation, biosynthesis/metabolism of biomolecules (including histidine), cell morphology and cellular dynamics, response to oxidative or metabolic stress, ribosomal structure and function, and mitochondrial function. Unexpectedly, inconsistent to popular belief, very few genes in the global ubiquitination or degradation pathways showed interactions with *ate1*. Our results suggested that ATE1 specifically regulates a handful of cellular processes *in vivo*, which will provide critical mechanistic leads for studying the involvements of ATE1 in normal physiologies as well as in diseased conditions.

Keywords: posttranslational modification, arginylation, arginyltransferase1, double-knockout screening, genetic interactions

OPEN ACCESS

Edited by:
Natalia Polouliakh, Sony Computer Science Laboratories, Inc., Japan

Reviewed by:
Litao Sun, Sun Yat-sen University, China
Priyanka Baloni, Institute for Systems Biology (ISB), United States

****Correspondence:***
Fangliang Zhang
Fzhang2@miami.edu

Specialty section:
This article was submitted to Systems Biology, a section of the journal Frontiers in Physiology

Received: *04 February 2020*
Accepted: *07 April 2020*
Published: *06 May 2020*

Citation:
Wiley DJ, D'Urso G and Zhang F (2020) Posttranslational Arginylation Enzyme Arginyltransferase1 Shows Genetic Interactions With Specific Cellular Pathways in vivo. Front. Physiol. 11:427. doi: 10.3389/fphys.2020.00427

INTRODUCTION

Protein posttranslational modifications (PTM) change protein properties without requiring *de novo* synthesis. Thus, PTMs are frequently relied upon to respond to acute stress or cellular signaling, and are often used to activate lateral response factors such as transcription or epigenetic modulations.

For these reasons, dysregulation of PTMs are often indicated in cardiovascular diseases where stress response or cellular signaling play critical roles.

In eukaryotes, many proteins appear to be subjected to N-terminal arginylation (referred to as arginylation in this proposal), a ribosome-independent addition of one extra arginine on the N-terminus of a protein. Arginylation is catalyzed by the family of arginyltransferase (ATE). While plants contain ATE1 and ATE2, fungi and metazoans only contain ATE1, which is highly conserved across different species. Multiple lines of genetic studies have shown an important role of the ATE1 gene (*ate1*) in the cardiovascular and/or metabolic diseases in animals. For example, a genomic deletion of ATE1 was found to result in complete embryonic lethality in mice during the mid-gestation stage (E9-14) with severe defects in angiogenetic remodeling and cardiac development (Kwon et al., 2002). Moreover, organ/tissue-specific deletions of ATE1 in animals were found to cause a variety of abnormalities in morphology and function. Examples include a progressive dilated cardiomyopathy in mice when *ate1* knockout is driven by the cardiac myosin heavy chain promoter (Kurosaka et al., 2010; Kaji and Kaji, 2012; Wang et al., 2017b). Also, inducible systematic deletion of *ate1* appears to cause rapid weight loss, damaged spermatogenesis, neurological perturbations, and early lethality in adult mice (Brower and Varshavsky, 2009). In addition to these involvement in cardiovascular and metabolic abnormalities, a dysregulation of ATE1 is indicated in cancer as well. For example, reports from us and other groups showed that ATE1 is often downregulated in high grade cancer cases and is associated with poorer outcomes (Rai et al., 2015; Birnbaum et al., 2019), and that an inhibition of ATE1-mediated arginylation confers cancer cell resistance to apoptosis-induced by radiation (Masdehors et al., 2000). Furthermore, mounting evidence is also starting to indicate the involvement of ATE1 in aging-related conditions (Brower et al., 2013; Wang et al., 2017a). Unfortunately, the physiological role of ATE1 (and its arginylation activity) remains poorly understood, which adds to the difficulty of interpreting its involvements in normal conditions or diseases.

The studies about ATE1 and arginylation are still relatively scarce. Our understandings for these topics are being continuously reshaped with emerging new evidence. Arginylation has been found to take place on nearly a hundred eukaryotic proteins and the list is still expanding on a daily basis (Decca et al., 2006; Wong et al., 2007; Piatkov et al., 2012). Considering that a wide range of proteins are substrates of arginylation, it is reasonable to speculate that ATE1 may act as a root regulator of multiple cellular processes.

A popular theory about arginylation is its involvement in global protein degradation. Arginylation was shown to promote hyper-ubiquitination of the substrate proteins, which is then shown to be degraded by proteasome or autophagy (Saha and Kashina, 2011; Varshavsky, 2011). Based on mostly artificial substrates and *in vitro* data, arginylation was proposed to take place on proteins bearing certain amino acids on the 2nd residue on the N-terminus. These include the amino acids Asp, Glu, Asn, Gln (in fungi and mammals), and Cys (in mammal only) (Varshavsky, 2011). By this rule, in any given eukaryotic organism, at least 20–25% of its proteome would be estimated to be degraded by arginylation. Based on this assumption, arginylation was proposed as a central component in the generic protein degradation machinery (Varshavsky, 2011). However, the exact impact of arginylation on global homeostasis of proteins *in vivo* remain undetermined. In a more recent report, based on comparison of the sizes of protein spots on 2D-gels, the knockout of *ate1* in mouse cells appears to affect ∼20% of those spots on the steady-state levels. However, much of these effects appear to be derived from transcriptional changes. Also, proteasome inhibitors can only reverse the *ate1*-dependent reduction of less than 3% of the observed protein spots (Wong et al., 2007). Such a small impact size is inconsistent with the proposed role of arginylation as a global degradation machinery.

In addition to protein degradation, arginylation was also suggested to be involved in many other processes. These may include cellular response to various types of stressors such as those that are closely related to cardiovascular stresses. For example, the activity of arginylation in cells or animal tissues is altered during injury, high temperature, or exposures to high concentrations of oxidants or salt (Zanakis et al., 1984; Chakraborty et al., 1986; Shyne-Athwal et al., 1986, 1988; Luo et al., 1990; Jack et al., 1992; Chakraborty and Ingoglia, 1993; Xu et al., 1993; Wang and Ingoglia, 1997; Bongiovanni et al., 1999; Kumar et al., 2016). Furthermore, other lines of studies also suggested that ATE1 may regulate the dynamics of cytoskeleton (Karakozova et al., 2006; Saha et al., 2010, 2011; Kaji and Kaji, 2012).

While multiple lines of researches are starting to establish ATE1 as a root regulator for multiple processes in the cell, contradicting conclusions are very often seen among reports from different groups. While many of these discrepancies may arise from differences in test conditions, variations may also be generated because many past studies were focused on the role of individual arginylation substrates without considering the effects of arginylation on the other known or potential substrates. To further understand the function of ATE1 in normal or diseased conditions, it is desirable to investigate the physiological role of *ate1* in a systematic manner. Among the currently available tools for studying functional genomics, approaches based on a yeast gene-deletion library remain as one of the most robust and straight-forward methods (Boone et al., 2007; Giaever and Nislow, 2014). However, the role of ATE1 gene (*ate1*) has never been studied with such an unbiased manner, either as a specific query subject or as part of a comprehensive screening.

In this study, we took advantage of the *Schizosaccharomyces pombe* (*S. pombe*) single-gene knockout library. *S. pombe* is a commonly used test model for eukaryotic genes due to its similarity to metazoan organisms, while preserving its ease of genetic operation as a microbe. The meiosis and mating process of this organism also greatly facilitate the combination of different gene deletions to examine their synthetic effects, which can be quantitated by monitoring the growth rates of the resulting cells to deduce the genetic interactions between these two genes (Spirek et al., 2010; Wiley et al., 2014). The advantage of *S. pombe* as a model system is further demonstrated by the fact that nearly 70% of its gene have orthologs in the human genome, which is

higher than *S. cerevisiae*, the other commonly used yeast as test model (Yanagida, 2002; Hoffman et al., 2015). The conserved genes including *ate1*, which is nearly 70% homologous in amino acid sequence in the core domain (∼200 residues) compared to its counterpart in human. However, unlike mammalian cells, *S. pombe* allows > 75% of its genome to be individually knocked out for functional tests (Spirek et al., 2010). These facts made *S. pombe* a desirable test model to determine the physiological role of *ate1 in vivo*.

In this study, we examined the effects of combining *ate1*-deletion with the deletions of other genes in *S. pombe*. The library we employed covered 3659 genes in *S. pombe*, which account for nearly 75% of the predicted open reading frames (up to 4940) in this organism (Decottignies et al., 2003). Surprisingly, only 173 of these genes, which is less than 5% of the effective library size, showed significant genetic interactions with *ate1*. Furthermore, many of these genes can be clustered into a few discrete groups in relation to cellular pathways. These include ribosomal component and translation regulation, gene transcription, oxidative stress response, cytoskeletal/structural components, mitochondrial function, and synthesis/metabolism of organic molecules and amino acids (such as histidine). Also, unexpectedly no significant interactions were observed between *ate1* and genes involved in the ubiquitin/proteome system (UPS). Our results indicate that ATE1 may specifically regulate several physiological pathways *in vivo*. Many of these interactions can be used to provide satisfactory explanations for many observed involvements of ATE1-mediated arginylation in cardiovascular/metabolic abnormalities and other diseased conditions. These novel findings will also provide clues for designing approaches intervening ATE1-related phenotypes in various diseases including cancer and metabolic dysregulations.

RESULTS

The Arginyltransferase Gene *ate1* Showed Interactions With Only a Small Subset of Genes

While *ate1* is an essential gene for mammals, it can be knocked out in *S. pombe* without causing lethality. Although *S. pombe* usually exists in a haploid form and reproduces by symmetric division (fission), they can also be induced to perform mating, during which chromosome recombination proceeds in a relatively high rate. These unique properties allow the knockout of *ate1* to be easily combined with the knockout of other individual genes in the library, provided that different selection markers are being used to trace the knockout library and the query knockout (of *ate1*). Based on the growth of the products, compared to the parental strains, at least two types of interactions can be measured: (1) phenotype-enhancement, in which the crossing of the query results in a lower growth rate (also referred to as synthetic lethal); (2) phenotype-suppression, with a faster growth rate (also known as synthetic suppression).

By using the above-mentioned synthetic knockout approach, we examined the effects of combining *ate1*-knockout with 3721 individual knockouts in a *S. pombe* library, in which most of these genes were functionally annotated by either experimental evidence or prediction based on known orthologs. After excluding knockouts that lead to sterilization of the yeast, totally 3659 effective crossings were examined. Out of these crossings, we only found 173 of them resulted in significant phenotype enhancement (see **Table 1A**) or suppression (see **Table 1B**). This number is less than 5% of the library size.

Genes That Genetically Interact With *ate1* Can Be Clustered by Biological Function

Among the genes that showed significant interactions with *ate1*, we found specific enrichment of several types of functional relevancies. For example, simply by applying functional annotations such as gene ontology (GO), gene expression category, or Fission Yeast Phenotype Ontology (FYPO), we found that several terms are enriched or present in higher frequencies among genes interacting with *ate1* compared to the whole library (**Figure 1** and **Supplementary Table S1**). As a further validation of the functional interactions observed between *ate1* and these genes, many of them also have known physical interactions or associations of their products among each other (**Figure 2**).

The enriched or increased functional annotations can be grouped into several clustered categories. Based on the frequency of presence in the hit list, the most abundant genes (nearly 50% of the hits) are those related to biosynthesis of biological molecules or proteins (**Supplementary Table S1A**). These include many regulators of translation or transcription (17–39%). Interestingly, among these categories, those related to histidine/amidazole synthesis/metabolism appear to be particularly impacted by *ate1*, as most of the genes associated with these pathways (5 out of 7∼9) showed interaction with *ate1* (**Figure 1** and **Supplementary Table S1A**).

The second most abundant genre (up to 40%) is those related to cell morphology as well as cellular dynamics (fusion, conjugation, or division). These include many known regulators of cytoskeleton (**Figure 1** and **Supplementary Table S1B**).

The third type (10∼15%) are those related to oxidative stress response, which include many highly expressed genes coding for chaperones or redox regulators (**Figure 1** and **Supplementary Table S1C**).

The fourth are (10∼15%) ribosome components or ribosome-associated factors (**Figure 1** and **Supplementary Table S1A**).

The fifth are those related to nutrient or metabolite stresses, such as genes related to nitrogen starvation, and genes involved in responses to metabolism interferers (caffeine, rapamycin) or metabolite analogs of lipids or nucleotides (**Figure 1** and **Supplementary Tables S1B,C**).

In addition to the above analysis by functional annotations, the genes that have genetic interactions with *ate1* can also be grouped into a handful of clustered functional pathways or categories when being analyzed with PANTHER Classification

TABLE 1 | Genes whose knockout showing genetic interaction, either phenotype enhancement **(1A)** or suppression **(1B)** with *ate1* in *S. Pombe.*

A. Gene knockouts showing phenotype-enhancing effects (synthetic lethality) with *ate1*- knockout in *S. pombe*

Gene name	Systematic Gene ID	*p*-Value	*z*-Score	Product description	S. cerevisiae ortholog	Human ortholog
igo1	SPAC10F6.16	8.1414E-07	−4.93195	Endosulfine (ENSA) serine/threonine protein kinase Igo1	IGO2, IGO1	ARPP19, ENSA
gor2	SPBC1773.17c	0.000027474	−4.19346	Glyoxylate reductase (predicted)	GOR1	GRHPR
	SPBC19G7.04	0.000041554	−4.09867	HMG box protein	FYV8	GCNA
lsd90	SPBC16E9.16c	0.000079902	−3.94469	Lsd90 protein		
puc1	SPBC19F5.01c	0.000083432	−3.93432	cyclin Puc1		
pun1	SPAC15A10.09c	0.000109954	−3.86751	SUR7 family protein Pun1 (predicted)	PUN1	
mas5	SPBC1734.11	0.00013273	−3.82134	DNAJ domain protein Mas5 (predicted)	YDJ1	DNAJA2,DNAJA4,DNAJA1
tps2	SPAC3G6.09c	0.000147704	−3.7949	Trehalose-phosphate synthase Tps2 (predicted)	TPS2	
spn1	SPAC4F10.11	0.00028086	−3.63235	Mitotic septin Spn1	CDC3	SEPTIN7,SEPTIN1,SEPTIN2, SEPTIN5,SEPTIN4
sut1	SPAC2F3.08	0.00034674	−3.57762	Plasma membrane sucrose/maltose:proton symporter Sut1		SLC45A2,SLC45A3
plc1	SPAC22F8.11	0.00046324	−3.50116	Phosphoinositide phospholipase C Plc1	PLC1	PLCB1,PLCH2,PLCH1,PLCB2, PLCB3,PLCB4,PLCD1,PLCD3, PLCD4,PLCL1,PLCL2,PLCG1 PLCG2
gid5	SPAC26H5.04	0.00068454	−3.3957	GID complex armadillo repeat subunit Gid5 (predicted)	VID28	ARMC8
pcr1	SPAC21E11.03c	0.00083756	−3.34008	Transcription factor Pcr1		
est1	SPBC2D10.13	0.00090084	−3.3198	Telomerase regulator Est1	EST1	SMG6
puf4	SPAC6G9.14	0.00091582	−3.31519	Pumilio family RNA-binding protein Puf4 (predicted)	PUF4,MPT5	
msa1	SPAC13G7.13c	0.00092698	−3.3118	RNA-binding protein Msa1	RIM4	
med1	SPAC2F7.04	0.00099746	−3.29124	Mediator complex subunit Med1	MED1	MED1
	SPBCPT2R1.01c	0.00106888	−3.27174	*S. pombe* specific DUF999 protein family 9		
coq7	SPBC337.15c	0.0010739	−3.27041	Ubiquinone biosynthesis protein Coq7	CAT5	COQ7
spn4	SPAC9G1.11c	0.00109358	−3.26527	Mitotic septin Spn4	CDC12	
Mam3	SPAP11E10.02c	0.00146666	−3.1812	Cell surface adhesion protein for conjugation Mam3		
tif51	SPAC26H5.10c	0.0016832	−3.14109	Translation elongation and termination factor eIF5A (predicted)	HYP2,ANB1	EIF5A, EIF5A2
prp17	SPBC6B1.10	0.0021378	−3.07038	Prp19 complex WD repeat protein Prp17	CDC40	CDC40
scs7	SPAC19G12.08	0.002199	−3.06194	ER sphingosine hydroxylase Scs7	SCS7	FA2H
pet1	SPAC22F8.04	0.0022328	−3.05739	Golgi phosphoenolpyruvate transmembrane transporter Pet1		SLC35C1
vas2	SPAP27G11.06c	0.002345	−3.04265	AP-1 adaptor complex sigma subunit Aps1	APS1	AP1S3,AP1S1,AP1S2
rgs1	SPAC22F3.12c	0.002421	−3.03305	Regulator of G-protein signaling Rgs1	SST2	

(Continued)

TABLE 1 | Continued

Gene name	Systematic Gene ID	*p*-Value	*z*-Score	Product description	S. cerevisiae ortholog	Human ortholog
	SPAC186.08c	0.0038618	−2.88923	L-lactate dehydrogenase (predicted)		
ric1	SPAC1851.04c	0.0041792	−2.86431	Ypt/Rab-specific guanyl-nucleotide exchange factor (GEF) subunit Ric1	RIC1	RIC1
qcr8	SPAC1782.07	0.0043588	−2.85095	Ubiquinol-cytochrome-c reductase complex subunit 7	QCR8	UQCRQ
ubi5	SPAC589.10c	0.0051636	−2.79665	Ribosomal-ubiquitin fusion protein Ubi5 (predicted)	RPS31	RPS27A
mex67	SPBC1921.03c	0.006664	−2.71318	mRNA export receptor, Tap, nucleoporin Mex67	MEX67	NXF1, NXF3
rpl4302	SPBC83.02c	0.0073432	−2.68086	60S ribosomal protein L37a (predicted)	RPL43B, RPL43A	RPL37A
klp8	SPAC144.14	0.0073454	−2.68076	Kinesin-like protein Klp8		KIF13B,KIF13A,KIF16B, KIF14,KIF1C,KIF1A
rps1102	SPAC144.11	0.0077726	−2.66179	40S ribosomal protein S11 (predicted)	RPS11B,RPS11A	RPS11
	SPBC56F2.05c	0.007884	−2.657	Transcription factor (predicted)		
	SPAC17C9.11c	0.0080866	−2.64843	zf-C2H2 type zinc finger protein/UBA domain protein		UBXN1
ppr5	SPAC1093.01	0.0088172	−2.61906	Mitochondrial PPR repeat protein Ppr5		
rrp16	SPAC22F8.09	0.0092822	−2.60148	rRNA processing protein Rrp16 (predicted)	NOP53	NOP53
fsv1	SPAC6F12.03c	0.0098672	−2.58045	SNARE Fsv1	SYN8	STX8
	SPBC354.07c	0.010096	−2.57252	Sterol intermembrane transfer protein (predicted)	OSH7,OSH6,HES1,KES1	OSBPL9,OSBPL10,OSBPL11
	SPAC2F3.16	0.0101944	−2.56916	Ubiquitin-protein ligase E3, implicated in DNA repair (predicted)		RCHY1
msy1	SPCC1183.11	0.010212	−2.56857	MS calcium ion channel protein Msy1		
	SPAC22G7.03	0.0102284	−2.56801	Schizosaccharomyces specific protein		
alp13	SPAC23H4.12	0.0103838	−2.56278	MRG family Clr6 histone deacetylase complex subunit Alp13	EAF3	MORF4,MORF4L2, MORF4L1
cwf11	SPBC646.02	0.0109488	−2.54433	U2-type spliceosomal complex ATPase Cwf11		AQR
rpl26	SPBC29B5.03c	0.01175	−2.51956	60S ribosomal protein L26 (predicted)	RPL26B,RPL26A	RPL26,RPL26L1
rpl29	SPBC776.01	0.0128654	−2.48747	60S ribosomal protein L29	RPL29	RPL29
	SPAC144.01	0.0142774	−2.45021	Schizosaccharomyces specific protein		
rsn1	SPBC354.08c	0.0150762	−2.43054	Golgi to plasma membrane transport protein Rsn1 (predicted)	RSN1	TMEM63B,TMEM63C, TMEM63A
clr3	SPBC800.03	0.0176608	−2.37265	Histone deacetylase (class II) Clr3	HDA1	HDAC6,HDAC10
tef103	SPBC839.15c	0.0180524	−2.36454	Translation elongation factor EF-1 alpha Ef1a-c	TEF2,TEF1	EEF1A1,EEF1A2
hos2	SPAC3G9.07c	0.0188808	−2.34788	Histone deacetylase (class I) Hos2	HOS2	HDAC1,HDAC2
pab1	SPAC227.07c	0.0193724	−2.33829	Protein phosphatase PP2A regulatory subunit B-55 Pab1	CDC55	PPP2R2D,PPP2R2A,PPP2R2B, PPP2R2C
	SPCC553.12c	0.0195392	−2.33508	Transmembrane transporter (predicted)		
mug183	SPAC6G9.03c	0.0197084	−2.33185	Histone H3.3 H4 heterotetramer chaperone Rtt106-like (predicted)	RTT106	

(Continued)

TABLE 1 | Continued

Gene name	Systematic Gene ID	*p*-Value	*z*-Score	Product description	S. cerevisiae ortholog	Human ortholog
rpl1102	SPBC17G9.10	0.020086	−2.32475	60S ribosomal protein L11 (predicted)	RPL11B,RPL11A	RPL11
puf1	SPBC56F2.08c	0.020354	−2.31974	Pumilio family RNA-binding protein Puf1 (predicted)	JSN1,PUF2	
	SPBC1703.13c	0.021252	−2.30346	Mitochondrial carrier, inorganic phosphate (predicted)	PIC2,MIR1	SLC25A3
eaf7	SPBC16A3.19	0.021428	−2.30036	Histone acetyltransferase complex subunit Eaf7	EAF7	MRGBP
gcd1	SPCC794.01c	0.023688	−2.26214	Glucose dehydrogenase Gcd1	ZWF1	H6PD
sft1	SPAC31A2.13c	0.024076	−2.25592	SNARE Sft1 (predicted)	SFT1	BET1,BET1L
	SPAC14C4.01c	0.024618	−2.24736	DUF1770 family protein		
nod1	SPAC12B10.10	0.024672	−2.24651	Medial cortical node Gef2-related protein Nod1		
mad1	SPBC3D6.04c	0.025142	−2.23922	Mitotic spindle checkpoint protein Mad1	MAD1	MAD1L1
syp1	SPBC4C3.06	0.025222	−2.238	F-BAR domain protein Syp1 (predicted)	SYP1	FCHO2,SGIP1,FCHO1
rho2	SPAC16.01	0.025592	−2.23235	Rho family GTPase Rho2	RHO2	RHOA,RHOB,RHOC
tpp1	SPAC19G12.15c	0.026072	−2.22515	Trehalose-6-phosphate phosphatase Tpp1	TPS2	
	SPBC1711.15c	0.027244	−2.208	Schizosaccharomyces pombe specific protein		
rps101	SPAC13G6.02c	0.02798	−2.19757	40S ribosomal protein S3a	RPS1A,RPS1B	RPS3A
cuf1	SPAC31A2.11c	0.028144	−2.19529	Nutritional copper sensing transcription factor Cuf1	CUP2,MAC1, HAA1	
apl6	SPAC23H3.06	0.029952	−2.17073	AP-3 adaptor complex subunit Apl6 (predicted)	APL6	AP3B1,AP3B2
jmj1	SPAC25H1.02	0.02998	−2.17036	Histone demethylase Jmj1 (predicted)		JMJD4
nrl1	SPBC20F10.05	0.032478	−2.13847	RNAi-mediated silencing protein, human NRDE2 ortholog Nrl1		NRDE2
atp3	SPBC1734.13	0.032508	−2.13812	F1-FO ATP synthase gamma subunit (predicted)	ATP3	ATP5F1C
oma1	SPAP14E8.04	0.033356	−2.12778	Metallopeptidase Oma1 (predicted)	OMA1	OMA1
ace2	SPAC6G10.12c	0.034516	−2.11399	Transcription factor Ace2	ACE2	
	SPACUNK4.13c	0.036738	−2.08865	Mitochondrial NTPase Obg family, human OLA1 ortholog, implicated in mitochondrial translation, ribosome assembly, or tRNA metabolism (predicted)	YLF2	OLA1
rtn1	SPBC31A8.01c	0.037292	−2.08255	Reticulon Rtn1	RTN2,RTN1	RTN1,RTN2,RTN3,RTN4
mpn1	SPAC23C11.10	0.037338	−2.08206	poly(U)-specific exoribonuclease, producing 3' uridine cyclic phosphate ends Mpn1	USB1	USB1
rud3	SPBC119.12	0.03747	−2.08061	Golgi matrix protein Rud3 (predicted)	RUD3	TRIP11
laf1	SPAC14C4.12c	0.037782	−2.07722	Clr6 L associated factor 1 Laf1	FUN19, YOR338W	
mbx1	SPBC19G7.06	0.038252	−2.07215	MADS-box transcription factor Mbx1	ARG80,MCM1	MEF2A,MEF2B,MEF2C, MEF2D

(Continued)

TABLE 1 | Continued

Gene name	Systematic Gene ID	*p*-Value	*z*-Score	Product description	S. cerevisiae ortholog	Human ortholog
B. Gene knockouts showing phenotype-suppressing effects (synthetic rescue) with *ate1*- knockout in *S. pombe*						
cgr1	SPAC1556.05c	7.7134E-08	5.37371	Ribosome biogenesis CGR1 family (predicted)	CGR1	CCDC86
kap123	SPBC14F5.03c	3.4032E-07	5.09963	Karyopherin/importin beta family nuclear import signal receptor Kap123	KAP123	IPO4
rpl2802	SPCC5E4.07	3.7196E-07	5.08278	60S ribosomal protein L27/L28	RPL28	RPL27A
gcn1	SPAC18G6.05c	7.1728E-07	4.95663	Translation initiation regulator Gcn1	GCN1	GCN1
pap1	SPAC1783.07c	1.35728E-06	4.83118	Transcription factor Pap1/Caf3	YAP1	
	SPAC3F10.09	1.82054E-06	4.77239	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino) methylideneamino]imidazole-4-carboxamide isomerase (predicted)	HIS6	
mcl1	SPAPB1E7.02c	0.000002246	4.72993	DNA polymerase alpha accessory factor Mcl1	CTF4	WDHD1
mtq1	SPAC29B12.05c	4.4576E-06	4.58881	Mitochondrial N(5)-glutamine methyltransferase (predicted)	MTQ1	HEMK1
atg14	SPAC25A8.02	0.00002277	4.23584	Autophagy associated protein Atg14	ATG14	ATG14
byr3	SPAC13D6.02c	0.000032138	4.15776	Translational activator, zf-CCHC type zinc finger protein (predicted)	GIS2	CNBP, ZCCHC13
mms19	SPAC1071.02	0.000037518	4.12226	CIA machinery protein Mms19	MET18	MMS19
imt2	SPCC4F11.04c	0.000041484	4.09906	Mannosyltransferase Imt2	CSH1, SUR1	
atd1	SPAC9E9.09c	0.000043798	4.08647	Aldehyde dehydrogenase (predicted)	ALD5, ALD4, ALD6	ALDH1A2, ALDH1A1, ALDH2, ALDH1B1, ALDH1A3
crf1	SPAC22H10.11c	0.000048624	4.06214	Transcriptional corepressor for ribosomal proteins via TOR signaling pathway Crf1 (predicted)	CRF1, IFH1	
	SPCC61.05	0.000099978	3.89064	Schizosaccharomyces specific multicopy membrane protein family 1		
	SPAC29A4.09	0.00010776	3.87242	rRNA exonuclease Rrp17 (predicted)	RRP17	NOL12
his1	SPAC25G10.05c	0.000112986	3.86086	ATP phosphoribosyltransferase	HIS1	
brl1	SPCC1919.15	0.000122818	3.84043	Ubiquitin-protein ligase E3 Brl1	BRE1	RNF40
elf1	SPAC3C7.08c	0.000131224	3.82415	AAA family ATPase Elf1	NEW1	
met10	SPCC584.01c	0.000142772	3.80332	Sulfite reductase NADPH flavoprotein subunit (predicted)	MET10	
fil1	SPCC1393.08	0.000195024	3.72538	Transcription factor, zf-GATA type		
rps1802	SPCC1259.01c	0.000195418	3.72487	40S ribosomal protein S18 (predicted)	RPS18A,RPS18B	RPS18
atp1	SPAC14C4.14	0.000258	3.65419	F1-FO ATP synthase alpha subunit	ATP1	ATP5F1A
ctp1	SPCC338.08	0.00038412	3.55076	CtIP-related endonuclease	SAE2	RBBP8

(Continued)

TABLE 1 | Continued

Gene name	Systematic Gene ID	*p*-Value	*z*-Score	Product description	S. cerevisiae ortholog	Human ortholog
cox19	SPCC1672.04c	0.00050878	3.47609	Mitochondrial copper chaperone for cytochrome c oxidase Cox19 (predicted)	COX19	COX19
rep2	SPBC2F12.11c	0.0007297	3.37817	MBF transcription factor activator Rep2		
rpl3602	SPBC405.07	0.00082778	3.34334	60S ribosomal protein L36	RPL36A,RPL36B	RPL36
ght8	SPCC548.06c	0.00086908	3.3298	Plasma membrane hexose:proton symporter, unknown specificity Ght8 (predicted)	HXT15,HXT7,HXT6,STL1, HXT13, MAL11,HXT4, HXT1,HXT5,HXT8,HXT9, HXT16, GAL2,HXT2,HXT14, HXT17,HXT11	
rpl35b	SPBC1921.01c	0.00110496	3.26234	60S ribosomal protein L35a (predicted)	RPL33B,RPL33A	RPL35A
rpl901	SPAC4G9.16c	0.00138818	3.1971	60S ribosomal protein L9	RPL9A,RPL9B	RPL9
rpl1801	SPBC11C11.07	0.00140148	3.19435	60S ribosomal protein L18	RPL18B,RPL18A	RPL18
rpl3702	SPCC1223.05c	0.00161788	3.15266	60S ribosomal protein L37 (predicted)	RPL37B,RPL37A	RPL37
	SPAC17G8.06c	0.00198848	3.09195	Dihydroxy-acid dehydratase (predicted)	ILV3	
his7	SPBC29A3.02c	0.0021838	3.06403	Phosphoribosyl-AMP cyclohydrolase/phosphoribosyl- ATP pyrophosphohydrolase His7	HIS4	
lys9	SPBC3B8.03	0.0023364	3.04376	Saccharopine dehydrogenase	LYS9	AASS
his5	SPBC21H7.07c	0.002481	3.02565	Imidazoleglycerol-phosphate dehydratase His5	HIS3	
rpl15	SPCC576.11	0.0028468	2.98382	60S ribosomal protein L15 (predicted)	RPL15A,RPL15B	RPL15
dhm1	SPCP1E11.10	0.0029186	2.97618	Ankyrin repeat protein, unknown biological role	YCR051W	
zfs1	SPBC1718.07c	0.0033112	2.93727	zf-CCCH tandem zinc finger protein, human Tristetraprolin homolog Zfs1, involved in mRNA catabolism	CTH1,TIS11	ZFP36L1, ZFP36L2, ZFP36
trm112	SPAC31A2.02	0.0035714	2.91373	eRF1 methyltransferase complex and tRNA (m2G10) methyltransferase complex regulatory subunit Trm112 (predicted)	TRM112	TRMT112
his2	SPBC1711.13	0.006125	2.74102	Histidinol dehydrogenase His2 (predicted)	HIS4	
hmt2	SPBC2G5.06c	0.0065568	2.71855	Sulfide-quinone oxidoreductase		SQOR
rps1201	SPCC962.04	0.0066316	2.7148	40S ribosomal protein S12 (predicted)	RPS12	RPS12
mms1	SPAC3H8.05c	0.0075438	2.67184	Cul8-RING ubiquitin ligase complex subunit Mms1 (predicted)	MMS1	0
rpl902	SPCC613.06	0.0100146	2.57533	60S ribosomal protein L9	RPL9A,RPL9B	RPL9
pnk1	SPAC23C11.04c	0.0101678	2.57007	DNA kinase/phosphatase Pnk1	TPP1	PNKP
dml1	SPAC30C2.06c	0.0109928	2.54293	Mitochondrial inheritance GTPase, tubulin-like (predicted)	DML1	MSTO1
	SPAC732.02c	0.0127802	2.48984	Fructose-2,6-bisphosphate 2-phosphatase activity (predicted)	FBP26	PFKFB1, PFKFB2, PFKFB3, PFKFB4
ser2	SPBC3H7.07c	0.0131908	2.47858	Phosphoserine phosphatase Ser2 (predicted)	SER2	PSPH
ifa38	SPAC4G9.15	0.0135804	2.46818	Ketoreductase involved in fatty acid elongation (predicted)	IFA38	HSDL1, HSD17B12, HSD17B3

(Continued)

TABLE 1 | Continued

Gene name	Systematic Gene ID	*p*-Value	*z*-Score	Product description	S. cerevisiae ortholog	Human ortholog
rps2801	SPAC25G10.06	0.0137516	2.46369	40S ribosomal protein S28 (predicted)	RPS28B,RPS28A	RPS28
lip2	SPAC4F10.05c	0.0162584	2.40306	Mitochondrial lipoate-protein ligase Lip2	LIP2	LIPT2
clg1	SPBC1D7.03	0.0179078	2.36752	Cyclin-like protein involved in autophagy Clg1 (predicted)	CLG1	
arp5	SPBC365.10	0.018288	2.35974	Ino80 complex actin-like protein Arp5	ARP5	ACTR5
mre11	SPAC13C5.07	0.018601	2.35343	Mre11 nuclease	MRE11	MRE11
met14	SPAC1782.11	0.0203	2.32077	Adenylyl-sulfate kinase (predicted)	MET14	PAPSS1, PAPSS2
rps1502	SPAC1071.07c	0.022698	2.27847	40S ribosomal protein S15 (predicted)	RPS15	RPS15
	SPAC3C7.04	0.024088	2.25573	Transcription factor (predicted)		
rps2802	SPCC285.15c	0.026352	2.22097	40S ribosomal protein S28, Rps2802	RPS28B,RPS28A	RPS28
git1	SPBC21C3.20c	0.027796	2.20016	C2 domain protein Git1		
rpa12	SPCC1259.03	0.028074	2.19625	DNA-directed RNA polymerase complex I subunit Rpa12	RPA12	ZNRD1
cys2	SPBC106.17c	0.028446	2.19109	Homoserine O-acetyltransferase (predicted)		
	SPBC1A4.04	0.030716	2.16072	Schizosaccharomyces specific protein		
lys7	SPAC17C9.02c	0.03157	2.14981	Alpha-aminoadipate reductase phosphopantetheinyl transferase Lys7	LYS5	AASDHPPT
ade10	SPCPB16A4.03c	0.033428	2.1269	Bifunctional IMP cyclohydrolase/ phosphoribosylaminoimidazole- carboxamide formyltransferase	ADE16,ADE17	ATIC
rpl3001	SPAC9G1.03c	0.036378	2.09268	60S ribosomal protein L30 (predicted)	RPL30	RPL30
	SPBC1271.14	0.037576	2.07945	Acetyl-CoA:L-glutamate N-acetyltransferase (predicted)	ARG7	
ftp105	SPAC17A5.16	0.04206	2.03293	Golgi localized protein, human HID1 ortholog 3, implicated in vesicle-mediated transport	ECM30	HID1
ppa2	SPBC16H5.07c	0.044676	2.00769	Serine/threonine protein phosphatase Ppa2	PPH21,PPH22	PPP2CA,PPP2CB
sod2	SPAC1486.01	0.044806	2.00647	Mitochondrial superoxide dismutase Sod2	SOD2	SOD2
gpd1	SPBC215.05	0.046392	1.99181	Glycerol-3-phosphate dehydrogenase Gpd1	GPD1	GPD1L,GPD1

The genetic interaction was determined by the effects of combing two gene knockouts on the growth rate of the resulting yeast colony compared to the individual knockouts. Sexual mating of S. pombe was used to generate the crossings between the ate1-knockout and the other gene-knockouts in a library. While the original library contains 3721 different knockout strains, some of these deletion strains appear to be sterile and cannot be mated. Thus, the effective library size was 3659 in our tests. Four replicates were used to determine the effects on growth of the crossed, double-knockout colony compared to the parental strain with a single knockout (without ate1 deletion). Based on the comparison, a result of slower growth is considered as phenotype-enhancement (in ***Table 1A****). Vice versa, those that grow faster than the parental strain is considered phenotype-suppression (****Table 1B****). A confidence value (p-values) of 0.05 is the minimum for significance.*

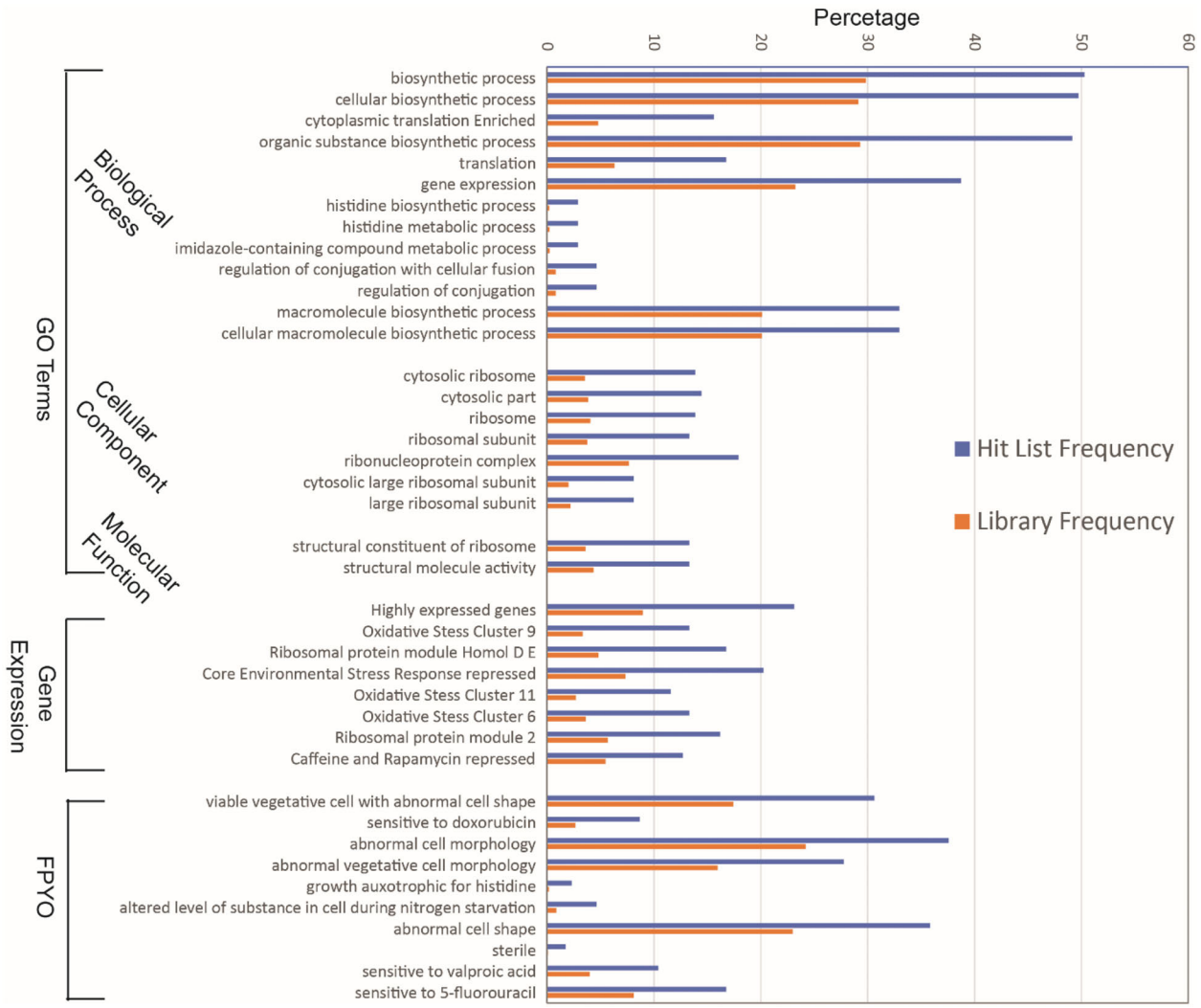


FIGURE 1 | Gene features that are enriched in the *ate1*-interacting hit list compared to the library pool. The features of genes were determined by either gene ontology (GO) terms, expression category, or fission yeast phenotype ontology (FYPO).

System[1] (Mi et al., 2013, 2019), as shown in **Supplementary Table S2**.

As an example to validate the observations reached in the above non-biased study, we examined the sensitivity of *S. pombe* to exogenous histidine, which is not an essential amino acid for this organism. While a lower amount of exogenous histidine usually promotes the growth of yeasts, at high concentrations it is known to generate cytotoxicity and requires the actions of the histidine/amidazole synthesis/metabolism pathway for mitigation (Winkler and Ramos-Montanez, 2009; Watanabe et al., 2014; Duncan et al., 2018). When we compared the growth rates of the yeasts hosting the *ate1*-deletion to the control cells, we found that, although there is no apparent difference in their proliferation in the absence of exogenous histidine (**Figure 3A**), *ate1*-deleted yeast grow much slower than the control in high concentrations of histidine (**Figures 3B–E**). To ensure these observations are not specific to the colony of yeast used in the test, we repeated these experiments with two additional clones of the *ate1*-deleted *S. pombe* and reached similar conclusions (**Figure 3F**). Therefore, these data suggested that *ate1*-deletion indeed possesses an interaction with the histidine/amidazole synthesis/metabolism pathway.

Many Genes Related to Mitochondria or Energy Production Genetically Interact With *ate1*

Compared to the relative abundance of literatures showing the effects of arginylation in protein degradation or cytoskeletal regulations, the potential involvements of

[1]www.pantherdb.org

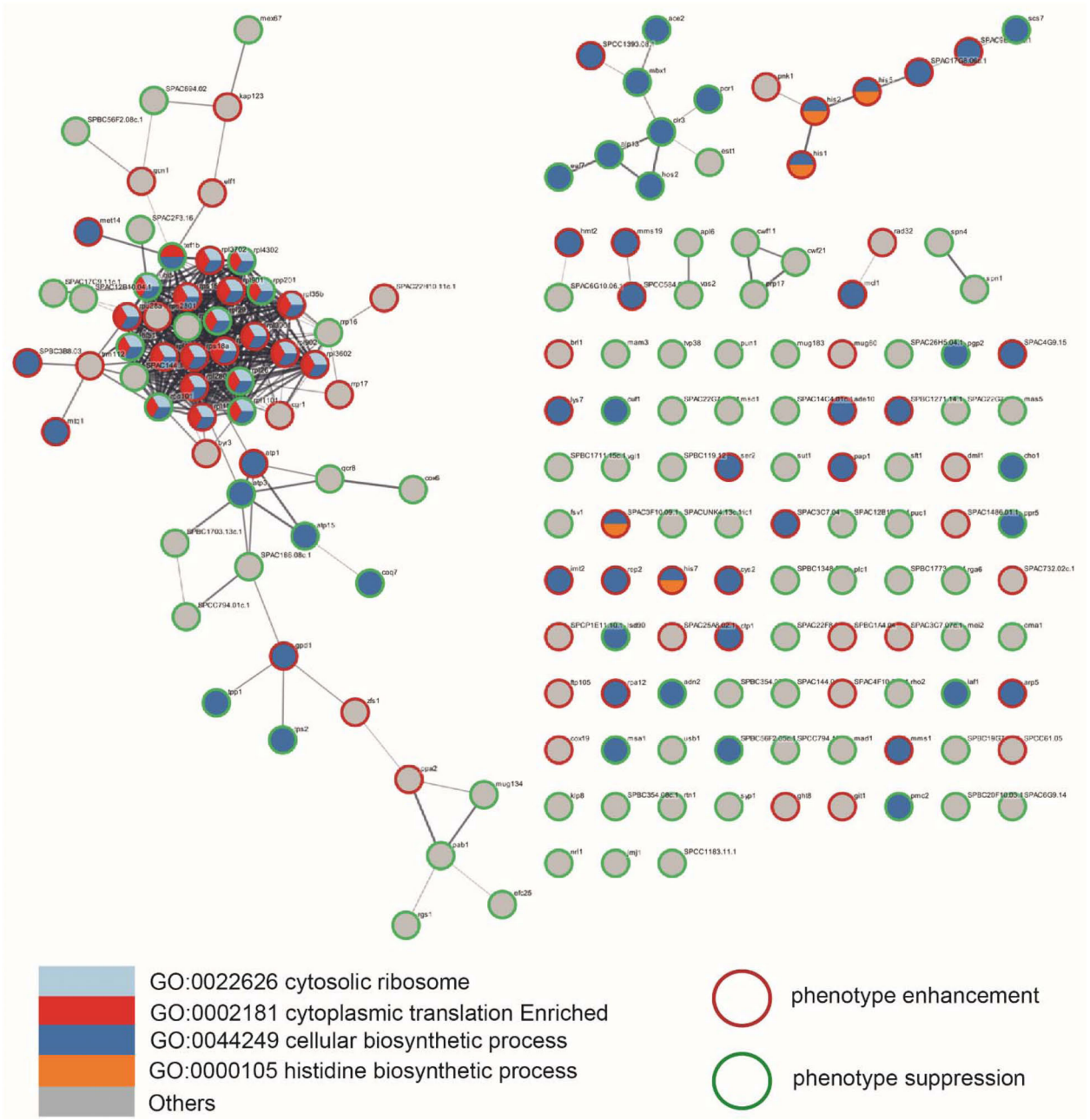


FIGURE 2 | Known protein–protein interactions (PPI) between products of genes that have genetic interactions with *ate1* in *S. pombe*. The PPI database being utilized is Version 11 of STRING (https://string-db.org). The interaction map was generated with Cytoscape using the STRING App for creating the PPI information and enrichment mapping. The thickness of the connecting line represents the confidence of the experimental protein-protein interaction. A thicker line represents a higher confidence (a minimum of 0.4% confidence was used). Each rounded shape represent the product of a gene (with gene name labeled on the side). The assigned color of the rounded shape represents gene category (by GO terms), while the color of the ring (red or green) represent the direction of the genetic interaction (phenotype enhancing or suppressing).

arginylation or ATE1 in other cellular processes are less known. Interestingly, many of the observed phenotypes in animals resulted from ate1 dysregulation may be at least partly attributed to a disruption of mitochondrial function, which supplies the majority of energy molecules (ATP, NADH, and NADPH, etc.) required for many biosynthesis processes, and also constitute a major source of oxidative stressors in the cell. For example, postnatal systemic knockout of *ate1* appears to lead to drastic loss of fat and infertility, which are common consequences expected from a compromise of mitochondrial function. Consistent to this possibility, we observed a significant

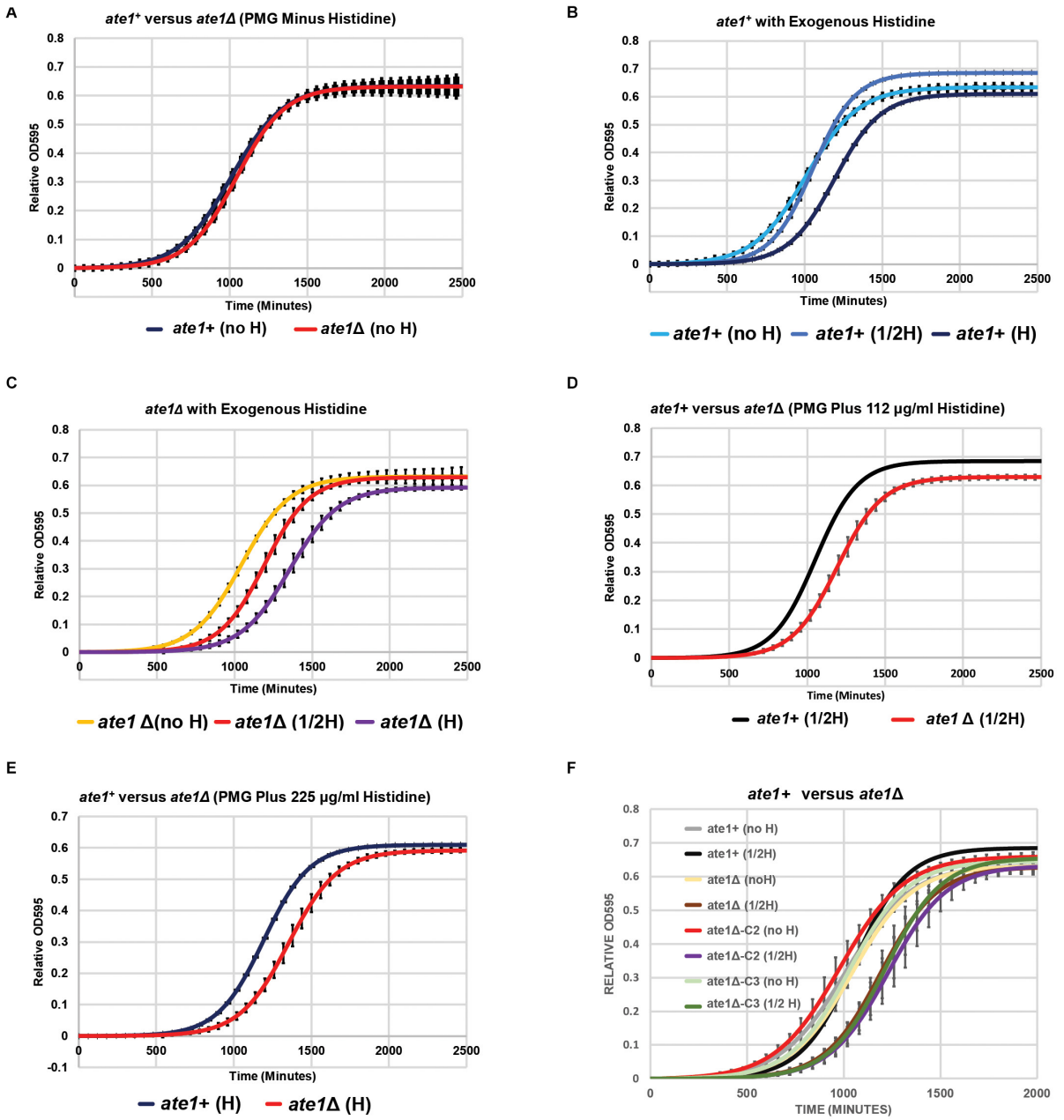


FIGURE 3 | The deletion of *ate1* leads to lower tolerance of exogenous histidine in the media. The yeast strains were grown in PMG liquid media supplemented with adenine, leucine, and uracil plus histidine as indicated (no H = no histidine; 1/2H = 112 µg/ml histidine; H = 225 µg/ml histidine), while the culture density was monitor by absorbance (OD 595 nm). In the absence of histidine (referred as to "no H"), both the *ate1*Δ and the control (*ate1* +) strains grow in a similar rate (see **A**). The addition of moderate amount of histidine (112 µg/ml; "1/2H") increases the growth of control strain (*ate1* +), while a higher concentration (225 µg/ml; "H") exhibits cytotoxic effect as anticipated (see **B**). However, in *ate1*Δ strain, the addition of either 112 or 225 mg/ml of exogenous histidine both lead to slower growth in a dose-dependent manner (see **C**). Direct comparisons of the growth of *ate1*Δ and the control strain (*ate1* +) at different concentrations of histidine are presented at panel **(D)** and **(E)**. To exclude the possibility of clone-specific, two different clones of *ate1*Δ strain (*ate1*Δ −C2, and −C3) isolated from the same knockout process were subjected to the challenge of 112 µg/ml exogenous histidine, compared to when no histidine was added (see **F**). The curves were generated with non-linear regression. Error Bars represent standard deviation from 6 replicates.

number (at least 19) of genes related to mitochondria or energy production showing interactions with *ate1* (**Table 2**). These genes represent more than 10% of the total hits, although they were not present as "enriched" because the frequency of them in the hit-list is not higher than that in the library.

TABLE 2 | Mitochondria-related genes, as determined by GO terms, which are genetically interacting with *ate1*.

Gene description	GO_Term	# of non-sterile genes in library	# of genes in the hits	Gene name/systematic ID in the hits list
Mitochondrion (mitochondria)	GO:0005739	428	19	mpn1, SPACUNK4.13c, lip2, gor2, hmt2, sod2, SPAC14C4.01c, qcr8, SPAC17G8.06c, cox6, oma1, cys2, SPBC1271.14, SPBC1703.13c, atp3, atp15, coq7, rps1802, ppr5
Mitochondria inner membrane	GO:0005743	71	4	oma1, SPBC1703.13c, coq7, atp15

The numbers of those genes in the effective library (non-sterile) and the hit list are being shown. The names (or systematic ID) of the genes in the hit list are also shown.

Few Genes in Global Protein Degradation Pathways Showed Genetic Interactions With *ate1*

Another surprising finding in the interacting partners of *ate1* is the rarity of genes involved in global protein ubiquitination or degradation machineries.

In principle, the uncertainties about the role of arginylation in proteome homeostasis can at least be partly addressed by the genetic interaction screening. If *ate1* is involved in global protein ubiquitination or degradation, it would be expected to have genetic interactions with the other components in these pathways. However, while many genes with known roles in global ubiquitination or degradation are present in the *S. pombe* knockout library we employed (**Supplementary Table S3**), only two (*ubi5* and *atg14*) showed significant interactions (either enhancing or suppressing) with *ate1* (**Table 3**). Among these two genes, *ubi5* is a fusion gene of ubiquitin and ribosomal component. As such, its genetic interaction with *ate1* may derive from the ribosomal component and not necessarily the ubiquitin, since no interaction was observed between *ate1* and other genes coding for ubiquitin. The rarity of interactions between *ate1* and degradation-related genes is actually consistent with the selectivity of the interacting partners of *ate1* as described above (**Figure 2** and **Tables 1**–**3**). If arginylation is a generic degradation pathway for up to 20% proteins as predicted, then the expected interactions between *ate1* and other genes should be much larger than the observed number (< 5%), because many components in the pathways regulated by these arginylation-target proteins would be expected to show genetic interaction with *ate1*. Interestingly, the small impact size (< 5%) of *ate1* in genetic interactions is well consistent with the impact size (∼3%) of arginylation on the degradation of endogenous proteins as estimated in 2D-gels (Wong et al., 2007). These evidences suggest that ATE1-mediated arginylation may not be a major degradation pathway *in vivo* in the experimental condition we employed.

TABLE 3 | Genes related to global ubiquitination and degradation, as determined by GO terms, that are in the screen library or genetically interacting with *ate1*.

Gene description	GO_term	# of genes in library (non-sterile)	# of genes in the hits
Protein ubiquitination	GO:0016567	19	1 (ubi5)
Ubiquitin binding	GO:0043130	25	0
Ubiquitin (protein tag)	GO:0031386	7	1 (ubi5)
Ubiquitin ligase complex	GO:0000151	20	0
Proteasome complex	GO:0000502	1	0
Lysosome	GO:0005764	5	0
Autophagy	GO:0006914	18	0
Autophagosome	GO:0005776	4	1 (atg14)
Protein catabolic process	GO:0030163	5	0

The numbers of those genes in the effective library (non-sterile) and the hit list are being shown. The names of the genes in the hit list are also shown.

DISCUSSION

Despite that *ate1* gene has been identified for 30 years, it's *in vivo* role has remained poorly defined. For the first time, by using a systematic approach, our results showed that *ate1* possesses significant genetic interactions with a small and focused subset of genes concerning multiple critical cellular processes. The results from this study can also provide important leads for mechanistic investigations about the role of *ate1* or arginylation in normal or diseased conditions.

The power of the systematic approach employed in our study is demonstrated by the fact that many results from this unbiased investigation are highly consistent or complementing to data from past reports. For example, the observed genetic interactions between *ate1* and regulators of cell morphology are well consistent with previous reports showing the impact of arginylation on many cytoskeletal proteins (Wong et al., 2007) and cytoskeletal dynamics (Karakozova et al., 2006; Rai et al., 2008; Saha et al., 2010; Zhang et al., 2010, 2012; Kurosaka et al., 2012). The interactions between *ate1* and genes involved in oxidative stress response also support the proposed role of arginylation in stress response process (Zanakis et al., 1984; Chakraborty et al., 1986; Shyne-Athwal et al., 1986, 1988; Luo et al., 1990; Jack et al., 1992; Chakraborty and Ingoglia, 1993; Xu et al., 1993; Wang and Ingoglia, 1997; Bongiovanni et al., 1999; Kumar et al., 2016). Overall, it appears that our non-biased screening study was able to recapitulate many past observations performed in cell or animal.

Many results of this study will provide new clues for investigate the role of arginylation in physiological processes. Arginylation was shown to be involved in stress response, but the exact mechanism still awaits clarification and may benefit from the genetic interactions revealed in our study. For example, while several redox regulators, including manganese

superoxide dismutase (SOD) appear to have phenotype-enhancing relationship with *ate1*, many genes related to mitochondria, the main source of reactive oxygen species (ROS) in the cell, display synthetic suppression relationship. These differences imply that ATE1 may act as a scavenger of oxidatively damaged proteins in stress response. Also, the broad interactions between *ate1* and many transcription regulators and histone modulators may help to elucidate the observed but unexplained effects of ATE1 on global transcriptional landscape (Lee et al., 2012; Eisenach et al., 2014; Deka et al., 2016). Particularly, many genes of histone modulators appear to have a phenotype-suppression relationship with *ate1*. This is highly intriguing considering that previous studies have found that histone proteins are subjected to arginylation modification, which may also affect the other PTMs on histone (Wong et al., 2007; Saha et al., 2011). The interactions between *ate1* and genes related to biomolecule synthesis/metabolism are also of high interest because emerging evidence indicated a potential involvement of ATE1 in metabolism. As an example, the enrichment of the genes in histidine synthesis/metabolism pathways in principle is consistent with one of our previous findings about arginylation of phosphoribosyl pyrophosphate synthetase (PRPPS). This is because PRPPS is responsible for production of Phosphoribosyl Diphosphate, which serves as a precursor substrate for biosynthesis of histidine (Zhang et al., 2015). Due to the conserved nature of ATE1-mediated arginylation in eukaryotes (McGary et al., 2010), many of these findings may provide mechanistic insights for the role of ATE1/arginylation in cardiovascular diseases, metabolic dysregulations, and cancer in human. These exciting possibilities will become important directions for future studies.

The lack of interactions between ate1 and genes related to global ubiquitination or protein degradation, as revealed in our study, is quite surprising. Based on the loose consensus on amino acid sequence of known substrates, arginylation was long hypothesized as a signal for the ubiquitination and degradation of at least 20–25% members of the proteome in yeast or metazoan. Such a broad range of substrates would predict a very large impact size of genetic interactions of *ate1*. However, less than 5% of the tested genes, which cover nearly 75% of the genome in *S. pombe* yeast, showed interactions with *ate1*. Consistently, we found that *ate1* has very few interactions with components of the ubiquitination and degradation pathways. While these findings appear to be at odds with popular theories, it is worthy pointing out that the exact nature of arginylation in the degradation of endogenous proteins has not been decisively determined and the current theories about arginylation were mainly built on studies with artificial substrates (Bachmair et al., 1986; Varshavsky, 2011). It is possible that the substrate preference of ATE1 is more complexed than originally expected. For example, recent evidence suggested that the efficiency of arginylation may be affected by at least 11 residues on the N-terminus of a peptide, and the substrate preference of ATE1 may also be influenced by additional *in vivo* factors (Wang et al., 2011, 2018). Also, since the majority of arginylation takes place on the N-terminus of a protein, it must compete with N-terminally acetylation *in vivo*, which is a dominant PTM in most eukaryotes (Wang et al., 2011). Furthermore, while arginylation in many cases indeed promotes ubiquitination and degradation, exceptions are also abundant on endogenous arginylated proteins (Karakozova et al., 2006; Zhang et al., 2010, 2012, 2015). Maybe not coincidentally, past experimental attempts examining the impact of arginylation on the whole proteome also revealed a relatively small size. In one of such reports, the usage of 2D gels showed that less than 3% of individual proteins appear to be affected by arginylation on proteasome-dependent degradation (Karakozova et al., 2006; Wong et al., 2007). As such, alternative interpretation for the role of arginylation in protein ubiquitination/degradation may be needed and our study may provide clues for that. It is possible that arginylation is not activated under resting state but is specifically utilized for protein degradation during certain conditions such as stress response or nutrient deprivation. This possibility is at least indirectly supported by existing evidence showing the activation of arginylation during stress response and that arginylation preferentially takes place on oxidatively damaged proteins (Zhang et al., 1998; Kumar et al., 2016). It is also supported by the extensive genetic interactions between *ate1* and genes related to oxidative and metabolic stress as revealed in our study. However, this current study was conducted in a non-stressed condition and therefore cannot directly test this possibility, which will require further investigations for validation.

The other unexpected finding is the extensive interaction between *ate1* and ribosome-related genes. While the biological meaning of this interaction is still unclear, existing literatures may provide a few clues. Particularly, the ATE1 protein was found to at least partially co-localize with ribosome during cellular fractionation (Wang et al., 2011). Also, many ribosome-associated proteins were found to be arginylated (Wong et al., 2007; Wang et al., 2011). Based on this evidence, it is likely ATE1 may regulate the function of ribosome. In addition, current evidence suggest that arginylation may take place during co-translational stage and the outcome in arginylation-mediated degradation may be dependent on the dynamic of co-translational folding (Zhang et al., 2010). As such, it is also likely that arginylation is part of the mechanism for quality check of nascent peptide synthesis. This unexpected connection between arginylation and ribosome, as well as many other newly information about arginylation uncovered in this study, constitute intriguing directions for future research endeavors.

CONCLUSION

By using a systematic approach, we found that the gene for arginyltransferase1 has specific interactions with a small subset of genes in the eukaryotic genome, which fall into a few clustered functional categories. Our data suggest that ATE1 may specifically regulate a few cellular pathways *in vivo*. These results will provide novel mechanistic clues to understand the role of protein arginylation in the development of cardiovascular system and the pathogenesis of related diseases.

MATERIALS AND METHODS

Query Yeast Strain Creation

Growth conditions and genetic manipulations of *S. pombe* were performed as previously described (Moreno et al., 1991). The query strain containing *ate1*-deletion (*ate1*Δ) was prepared by targeted deletion with a linear DNA containing a nourseothricin-resistance gene flanked with sequence derived from the *ate1* gene (systemic ID: SPAC3C7.07c) loci. The 5′-region (394 bp) flanking the resistance gene was cloned with these primers, with underline to indicate the regions matching the genome sequence while the rest primes to the resistance cassette:

5′-FWD: TAGAACTTGGTGGATGGTATCGTGG
5′-REV: GGGGATCCGTCGACCTGCAGCGTACGAAACTAT TGTTTGAAAATTTCCCTGTTTAAT

The 3′-region (385 bp) flanking the resistance gene was cloned with these primers:

3′-FWD: GTTTAAACGAGCTCGAATTCATCTTATATTATC TGTCTACGTGTTTTATTTGC
3′-REV: TCCTTTCTCACCTACTATGCACTGTTTTG

The knockout was performed in an *h- leu1-32 ura4-D18 Ade6-M210 S. pombe* strain (PN572) as the parental strain with published protocols (Krawchuk and Wahls, 1999). The success of knockout is confirmed with this primer specific for the *ate1* gene: TCTTTGGATTGACAAGTTGAGAGTTG.

Yeast Synthetic Genetic Array

The *ate1*Δ strain was grown to exponential phase in liquid PMG media (Sunrise Scientific Cat. #2060) supplemented with adenine, leucine and uracil. It was then pinned to agar plates in a 384-matrix format using a robotic platform RoToR HDA (Singer Instruments). The query strain was then crossed with individual strains in the *S. pombe* haploid deletion library containing individual gene deletions marked by a kanamycin resistance gene (Bioneer, version 4.0 equivalent) utilizing a modified SGA procedure (Dixon et al., 2008). This procedure was described in detailed in our previous publication (Wiley et al., 2014). In brief, the crossing was induced on a SPAS mating media (1% glucose, 7.3mM KH_2PO_4, with 45mg/L supplements of adenine, histidine, leucine, uracil and lysine-HCl, and vitamin supplement for pantothenic acid, nicotinic acid, inositol, and biotin; see this website for detailed recipe: https://dornsife.usc.edu/pombenet/media/). For germination, four replicates of each crossing product were pinned to a 1536 format on selective PAU + G418 media, which is the PMG media containing adenine (225 mg/L, Sigma Cat. #A8751), leucine (225 mg/L, Sigma Cat. #L8912), uracil (225 mg/L, Sigma Cat. #U0750), and the antibiotics G418 and nourseothricin. Colony growth was monitored for 3 days utilizing a flatbed scanner, and then quantified and compared using ScreenMill according to published protocols (Dittmar et al., 2010). Double mutants with a significant growth rate difference compared to the corresponding library gene deletion alone were scored as either slowed (referred to as "phenotype-enhancement") or accelerated (referred to as "phenotype-suppression") growth.

Hit Analysis and Bio-Informatics

The gene feature enrichments (over-representation) in the hit list versus the library were examined by AnGeLi[2] (Bitton et al., 2015). The hit list was compared directly to the genes screened in our assay using the false discovery rate setting for multiple testing with a *p*-value setting of 0.01. In addition, for this enrichment we performed pairwise interaction enrichment with 1000 permutations and allowed for the *p*-value to adjust. As a negative control, we also looked for underrepresented terms and we cannot find significant underrepresented entries based on inputs, which suggesting that our analysis did not create artifacts.

The PANTHER Classification System[3] used in this study is Version 14.1, released 2019-03-12 (Mi et al., 2019).

The image of protein-protein interaction was created using Cytoscape with the STRING App (Szklarczyk et al., 2019). The PPI database being utilized is Version 11 of STRING[4].

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

DW performed most of the experiments and the analysis of data. GD'U was involved in supervising the performance of the experiments. FZ conceptualized and designed the project and also supervised the analysis of the data.

FUNDING

This project was partly supported by a pilot funding from the Florida Breast Cancer Foundation, and an American Cancer Society Institutional Research Grant 98-277-13, to FZ.

ACKNOWLEDGMENTS

We thank Drs. Daniel Isom and Fulvia Verde (University of Miami) for providing critical reading of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00427/full#supplementary-material

[2]http://bahlerweb.cs.ucl.ac.uk/cgi-bin/GLA/GLA_input
[3]www.pantherdb.org
[4]https://string-db.org

REFERENCES

Bachmair, A., Finley, D., and Varshavsky, A. (1986). In vivo half-life of a protein is a function of its amino-terminal residue. *Science* 234, 179–186. doi: 10.1126/science.3018930

Birnbaum, M. D., Zhao, N., Moorthy, B. T., Patel, D. M., Kryvenko, O. N., Heidman, L., et al. (2019). Reduced Arginyltransferase 1 is a driver and a potential prognostic indicator of prostate cancer metastasis. *Oncogene* 38, 838–851. doi: 10.1038/s41388-018-0462-2

Bitton, D. A., Schubert, F., Dey, S., Okoniewski, M., Smith, G. C., Khadayate, S., et al. (2015). AnGeLi: a tool for the analysis of gene lists from fission yeast. *Front. Genet.* 6:330. doi: 10.3389/fgene.2015.00330

Bongiovanni, G., Fissolo, S., Barra, H. S., and Hallak, M. E. (1999). Posttranslational arginylation of soluble rat brain proteins after whole body hyperthermia. *J. Neurosci. Res.* 56, 85–92. doi: 10.1002/(SICI)1097-4547(19990401)56:1<85::AID-JNR11>3.0.CO;2-T

Boone, C., Bussey, H., and Andrews, B. J. (2007). Exploring genetic interactions and networks with yeast. *Nat. Rev. Genet.* 8, 437–449. doi: 10.1038/nrg2085

Brower, C. S., Piatkov, K. I., and Varshavsky, A. (2013). Neurodegeneration-associated protein fragments as short-lived substrates of the N-end rule pathway. *Mol. Cell.* 50, 161–171. doi: 10.1016/j.molcel.2013.02.009

Brower, C. S., and Varshavsky, A. (2009). Ablation of arginylation in the mouse N-end rule pathway: loss of fat, higher metabolic rate, damaged spermatogenesis, and neurological perturbations. *PLoS One* 4:e7757. doi: 10.1371/journal.pone.0007757

Chakraborty, G., and Ingoglia, N. A. (1993). N-terminal arginylation and ubiquitin-mediated proteolysis in nerve regeneration. *Brain Res. Bull.* 30, 439–445. doi: 10.1016/0361-9230(93)90276-h

Chakraborty, G., Leach, T., Zanakis, M. F., and Ingoglia, N. A. (1986). Posttranslational protein modification by amino acid addition in regenerating optic nerves of goldfish. *J. Neurochem.* 46, 726–732. doi: 10.1111/j.1471-4159.1986.tb13032.x

Decca, M. B., Bosc, C., Luche, S., Brugiere, S., Job, D., Rabilloud, T., et al. (2006). Protein arginylation in rat brain cytosol: a proteomic analysis. *Neurochem. Res.* 31, 401–409. doi: 10.1007/s11064-005-9037-z

Decottignies, A., Sanchez-Perez, I., and Nurse, P. (2003). Schizosaccharomyces pombe essential genes: a pilot study. *Genome Res.* 13, 399–406. doi: 10.1101/gr.636103

Deka, K., Singh, A., Chakraborty, S., Mukhopadhyay, R., and Saha, S. (2016). Protein arginylation regulates cellular stress response by stabilizing HSP70 and HSP40 transcripts. *Cell Death Discov.* 2:16074. doi: 10.1038/cddiscovery.2016.74

Dittmar, J. C., Reid, R. J. D., and Rothstein, R. (2010). ScreenMill: a freely available software suite for growth measurement, analysis and visualization of high-throughput screen data. *BMC Bioinformatics* 11:353. doi: 10.1186/1471-2105-11-353

Dixon, S. J., Fedyshyn, Y., Koh, J. L., Prasad, T. S., Chahwan, C., Chua, G., et al. (2008). Significant conservation of synthetic lethal genetic interaction networks between distantly related eukaryotes. *Proc. Natl. Acad. Sci. U.S.A.* 105, 16653–16658. doi: 10.1073/pnas.0806261105

Duncan, C. D. S., Rodriguez-Lopez, M., Ruis, P., Bahler, J., and Mata, J. (2018). General amino acid control in fission yeast is regulated by a nonconserved transcription factor, with functions analogous to Gcn4/Atf4. *Proc. Natl. Acad. Sci. U.S.A.* 115, E1829–E1838. doi: 10.1073/pnas.1713991115

Eisenach, P. A., Schikora, F., and Posern, G. (2014). Inhibition of arginyltransferase 1 induces transcriptional activity of myocardin-related transcription factor A (MRTF-A) and promotes directional migration. *J. Biol. Chem.* 289, 35376–35387. doi: 10.1074/jbc.M114.578674

Giaever, G., and Nislow, C. (2014). The yeast deletion collection: a decade of functional genomics. *Genetics* 197, 451–465. doi: 10.1534/genetics.114.161620

Hoffman, C. S., Wood, V., and Fantes, P. A. (2015). An ancient yeast for young geneticists: a primer on the *Schizosaccharomyces pombe* model system. *Genetics* 201, 403–423. doi: 10.1534/genetics.116.187088

Jack, D. L., Chakraborty, G., and Ingoglia, N. A. (1992). Ubiquitin is associated with aggregates of arginine modified proteins in injured nerves. *Neuroreport* 3, 47–50. doi: 10.1097/00001756-199201000-00012

Kaji, H., and Kaji, A. (2012). Global cellular regulation including cardiac function by post-translational protein arginylation. *J. Mol. Cell Cardiol.* 53, 314–316. doi: 10.1016/j.yjmcc.2012.06.009

Karakozova, M., Kozak, M., Wong, C. C., Bailey, A. O., Yates, J. R. III, Mogilner, A., et al. (2006). Arginylation of beta-actin regulates actin cytoskeleton and cell motility. *Science* 313, 192–196. doi: 10.1126/science.1129344

Krawchuk, M. D., and Wahls, W. P. (1999). High-efficiency gene targeting in *Schizosaccharomyces Pombe* using a modular, PCR-based approach with long tracts of flanking homology. *Yeast* 15, 1419–1427. doi: 10.1002/(SICI)1097-0061(19990930)15:13<1419::AID-YEA466>3.0.CO;2-Q.

Kumar, A., Birnbaum, M. D., Patel, D. M., Morgan, W. M., Singh, J., Barrientos, A., et al. (2016). Posttranslational arginylation enzyme Ate1 affects DNA mutagenesis by regulating stress response. *Cell Death Dis.* 7:e2378. doi: 10.1038/cddis.2016.284

Kurosaka, S., Leu, N. A., Pavlov, I., Han, X., Ribeiro, P. A., Xu, T., et al. (2012). Arginylation regulates myofibrils to maintain heart function and prevent dilated cardiomyopathy. *J. Mol. Cell Cardiol.* 53, 333–341. doi: 10.1016/j.yjmcc.2012.05.007

Kurosaka, S., Leu, N. A., Zhang, F., Bunte, R., Saha, S., Wang, J., et al. (2010). Arginylation-dependent neural crest cell migration is essential for mouse development. *PLoS Genet.* 6:e1000878. doi: 10.1371/journal.pgen.1000878

Kwon, Y. T., Kashina, A. S., Davydov, I. V., Hu, R. G., An, J. Y., et al. (2002). An essential role of N-terminal arginylation in cardiovascular development. *Science* 297, 96–99. doi: 10.1126/science.1069531

Lee, M. J., Kim, D. E., Zakrzewska, A., Yoo, Y. D., Kim, S. H., et al. (2012). Characterization of arginylation branch of N-end rule pathway in G-protein-mediated proliferation and signaling of cardiomyocytes. *J. Biol. Chem.* 287, 24043–24052. doi: 10.1074/jbc.M112.364117

Luo, D., Chakraborty, G., and Ingoglia, N. A. (1990). Post-translational modification of proteins by arginine and lysine following crush injury and during regeneration of rat sciatic nerves. *Restor. Neurol. Neurosci.* 2, 53–61. doi: 10.3233/RNN-1990-2201

Masdehors, P., Glaisner, S., Maciorowski, Z., Magdelenat, H., and Delic, J. (2000). Ubiquitin-dependent protein processing controls radiation-induced apoptosis through the N-end rule pathway. *Exp. Cell Res.* 257, 48–57. doi: 10.1006/excr.2000.4870

McGary, K. L., Park, T. J., Woods, J. O., Cha, H. J., Wallingford, J. B., and Marcotte, E. M. (2010). Systematic discovery of nonobvious human disease models through orthologous phenotypes. *Proc. Natl. Acad. Sci. U.S.A.* 107, 6544–6549. doi: 10.1073/pnas.0910200107

Mi, H., Muruganujan, A., Casagrande, J. T., and Thomas, P. D. (2013). Large-scale gene function analysis with the PANTHER classification system. *Nat. Protoc.* 8, 1551–1566. doi: 10.1038/s41596-019-0128-8

Mi, H., Muruganujan, A., Huang, X., Ebert, D., Mills, C., Guo, X., et al. (2019). Protocol Update for large-scale genome and gene function analysis with the PANTHER classification system (v.14.0). *Nat. Protoc.* 14, 703–721. doi: 10.1038/s41596-019-0128-8

Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic-analysis of fission yeast *Schizosaccharomyces-pombe*. *Methods Enzymol.* 194, 795–823.

Piatkov, K. I., Brower, C. S., and Varshavsky, A. (2012). The N-end rule pathway counteracts cell death by destroying proapoptotic protein fragments. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1839–E1847. doi: 10.1073/pnas.1207786109

Rai, R., Wong, C. C., Xu, T., Leu, N. A., Dong, D. W., Guo, C., et al. (2008). Arginyltransferase regulates alpha cardiac actin function, myofibril formation and contractility during heart development. *Development* 135, 3881–3889. doi: 10.1242/dev.022723

Rai, R., Zhang, F., Colavita, K., Leu, N. A., Kurosaka, S., Kumar, A., et al. (2015). Arginyltransferase suppresses cell tumorigenic potential and inversely correlates with metastases in human cancers. *Oncogene* 35, 4058–4068. doi: 10.1038/onc.2015.473

Saha, S., and Kashina, A. (2011). Posttranslational arginylation as a global biological regulator. *Dev. Biol.* 358, 1–8. doi: 10.1016/j.ydbio.2011.06.043

Saha, S., Mundia, M. M., Zhang, F., Demers, R. W., Korobova, F., Svitkina, T., et al. (2010). Arginylation regulates intracellular actin polymer level by modulating actin properties and binding of capping and severing proteins. *Mol. Biol. Cell* 21, 1350–1361. doi: 10.1091/mbc.e09-09-0829

Saha, S., Wong, C. C., Xu, T., Namgoong, S., Zebroski, H., Yates, J. R. III, et al. (2011). Arginylation and methylation double up to regulate nuclear proteins

and nuclear architecture in vivo. *Chem. Biol.* 18, 1369–1378. doi: 10.1016/j.chembiol.2011.08.019

Shyne-Athwal, S., Chakraborty, G., Gage, E., and Ingoglia, N. A. (1988). Comparison of posttranslational protein modification by amino acid addition after crush injury to sciatic and optic nerves of rats. *Exp. Neurol.* 99, 281–295. doi: 10.1016/0014-4886(88)90148-3

Shyne-Athwal, S., Riccio, R. V., Chakraborty, G., and Ingoglia, N. A. (1986). Protein modification by amino acid addition is increased in crushed sciatic but not optic nerves. *Science* 231, 603–605. doi: 10.1126/science.3080804

Spirek, M., Benko, Z., Carnecka, M., Rumpf, C., Cipak, L., Batova, M., et al. (2010). S-pombe genome deletion project An update. *Cell Cycle* 9, 2399–2402.

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., et al. (2019). STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 47, D607–D613. doi: 10.1093/nar/gky1131

Varshavsky, A. (2011). The N-end rule pathway and regulation by proteolysis. *Protein Sci.* 20, 1298–1345.

Wang, J., Han, X., Leu, N. A., Sterling, S., Kurosaka, S., Fina, M., et al. (2017a). Protein arginylation targets alpha synuclein, facilitates normal brain health, and prevents neurodegeneration. *Sci. Rep.* 7:11323. doi: 10.1038/s41598-017-11713-z

Wang, J., Pavlyk, I., Vedula, P., Sterling, S., Leu, N. A., Dong, D. W., et al. (2017b). Arginyltransferase ATE1 is targeted to the neuronal growth cones and regulates neurite outgrowth during brain development. *Dev. Biol.* 430, 41–51. doi: 10.1016/j.ydbio.2017.08.027

Wang, J., Han, X., Saha, S., Xu, T., Rai, R., Zhang, F., et al. (2011). Arginyltransferase is an ATP-independent self-regulating enzyme that forms distinct functional complexes in vivo. *Chem. Biol.* 18, 121–130. doi: 10.1016/j.chembiol.2010.10.016

Wang, J., Pejaver, V. R., Dann, G. P., Wolf, M. Y., Kellis, M., Huang, Y., et al. (2018). Target site specificity and in vivo complexity of the mammalian arginylome. *Sci. Rep.* 8:16177. doi: 10.1038/s41598-018-34639-6

Wang, Y. M., and Ingoglia, N. A. (1997). N-terminal arginylation of sciatic nerve and brain proteins following injury. *Neurochem. Res.* 22, 1453–1459. doi: 10.1023/a:1021998227237

Watanabe, D., Kikushima, R., Aitoku, M., Nishimura, A., Ohtsu, I., Nasuno, R., et al. (2014). Exogenous addition of histidine reduces copper availability in the yeast *Saccharomyces cerevisiae*. *Microb. Cell* 1, 241–246. doi: 10.15698/mic2014.07.154

Wiley, D. J., Juan, I., Le, H., Cai, X., Baumbach, L., Beattie, C., et al. (2014). Yeast Augmented Network Analysis (YANA): a new systems approach to identify therapeutic targets for human genetic diseases. *F1000Research* 3:121. doi: 10.12688/f1000research.4188.1

Winkler, M. E., and Ramos-Montanez, S. (2009). Biosynthesis of Histidine. *EcoSal Plus* 3:19.

Wong, C. C., Xu, T., Rai, R., Bailey, A. O., Yates, J. R. III, Wolf, Y. I., et al. (2007). Global analysis of posttranslational protein arginylation. *PLoS Biol.* 5:e258. doi: 10.1371/journal.pbio.0050258

Xu, N. S., Chakraborty, G., Hassankhani, A., and Ingoglia, N. A. (1993). N-terminal arginylation of proteins in explants of injured sciatic nerves and embryonic brains of rats. *Neurochem. Res.* 18, 1117–1123. doi: 10.1007/bf00978361

Yanagida, M. (2002). The model unicellular eukaryote, *Schizosaccharomyces pombe*. *Genome Biol.* 3, comment2003.1–comment2003.4.

Zanakis, M. F., Chakraborty, G., Sturman, J. A., and Ingoglia, N. A. (1984). Posttranslational protein modification by amino acid addition in intact and regenerating axons of the rat sciatic nerve. *J. Neurochem.* 43, 1286–1294. doi: 10.1111/j.1471-4159.1984.tb05385.x

Zhang, F., Patel, D. M., Colavita, K., Rodionova, I., Buckley, B., Scott, D. A., et al. (2015). Arginylation regulates purine nucleotide biosynthesis by enhancing the activity of phosphoribosyl pyrophosphate synthase. *Nat. Commun.* 6:7517. doi: 10.1038/ncomms8517

Zhang, F., Saha, S., and Kashina, A. (2012). Arginylation-dependent regulation of a proteolytic product of talin is essential for cell-cell adhesion. *J. Cell Biol.* 197, 819–836. doi: 10.1083/jcb.201112129

Zhang, F., Saha, S., Shabalina, S. A., and Kashina, A. (2010). Differential arginylation of actin isoforms is regulated by coding sequence-dependent degradation. *Science* 329, 1534–1537. doi: 10.1126/science.1191701

Zhang, N., Donnelly, R., and Ingoglia, N. A. (1998). Evidence that oxidized proteins are substrates for N-terminal arginylation. *Neurochem. Res.* 23, 1411–1420.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 25 May 2020
doi: 10.3389/fphys.2020.00515

Mitochondrial Quality Control and Cellular Proteostasis: Two Sides of the Same Coin

*Justin M. Quiles and Åsa B. Gustafsson**

Department of Pharmacology, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA, United States

Mitochondrial dysfunction is a hallmark of cardiac pathophysiology. Defects in mitochondrial performance disrupt contractile function, overwhelm myocytes with reactive oxygen species (ROS), and transform these cellular powerhouses into pro-death organelles. Thus, quality control (QC) pathways aimed at identifying and removing damaged mitochondrial proteins, components, or entire mitochondria are crucial processes in post-mitotic cells such as cardiac myocytes. Almost all of the mitochondrial proteins are encoded by the nuclear genome and the trafficking of these nuclear-encoded proteins necessitates significant cross-talk with the cytosolic protein QC machinery to ensure that only functional proteins are delivered to the mitochondria. Within the organelle, mitochondria contain their own protein QC system consisting of chaperones and proteases. This system represents another level of QC to promote mitochondrial protein folding and prevent aggregation. If this system is overwhelmed, a conserved transcriptional response known as the mitochondrial unfolded protein response is activated to increase the expression of proteins involved in restoring mitochondrial proteostasis. If the mitochondrion is beyond repair, the entire organelle must be removed before it becomes cytotoxic and causes cellular damage. Recent evidence has also uncovered mitochondria as participants in cytosolic protein QC where misfolded cytosolic proteins can be imported and degraded inside mitochondria. However, this process also places increased pressure on mitochondrial QC pathways to ensure that the imported proteins do not cause mitochondrial dysfunction. This review is focused on discussing the pathways involved in regulating mitochondrial QC and their relationship to cellular proteostasis and mitochondrial health in the heart.

Keywords: mitochondria, UPS, proteasome, UPR, proteotoxicity, import, mitophagy, Parkin

OPEN ACCESS

Edited by:
Huabo Su,
Medical College of Georgia, United States

Reviewed by:
Sergio Lavandero,
University of Chile, Chile
Elena N. Dedkova,
University of California, Davis, United States

***Correspondence:**
Åsa B. Gustafsson
asag@ucsd.edu

Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *18 February 2020*
Accepted: *27 April 2020*
Published: *25 May 2020*

Citation:
Quiles JM and Gustafsson ÅB (2020) Mitochondrial Quality Control and Cellular Proteostasis: Two Sides of the Same Coin. Front. Physiol. 11:515. doi: 10.3389/fphys.2020.00515

INTRODUCTION

Mitochondrial dysfunction is a hallmark of cardiac pathophysiology. Defects in mitochondrial performance disrupt contractile function, overwhelm myocytes with reactive oxygen species (ROS), and transform these cellular powerhouses into pro-death organelles (Zhou and Tian, 2018). Accordingly, quality control (QC) pathways aimed at identifying and removing damaged

mitochondrial proteins, components, or entire mitochondria represent crucial adaptive responses for cardiac myocytes. Because the majority of the mitochondrial proteins are encoded by the nucleus and translated in the cytosol, cytosolic protein QC mechanisms are intimately linked with mitochondrial fidelity. The cytosolic ubiquitin proteasome system (UPS) ensures that functional proteins are delivered to the mitochondria (Bragoszewski et al., 2013; Wang and Chen, 2015). Thus, it is not surprising that perturbations to protein homeostasis or proteostasis during cardiac pathophysiology (Hofmann et al., 2019) also antagonize mitochondrial function and activate cell death pathways in cardiac myocytes (Zhou and Tian, 2018).

Mitochondria contain their own protein QC system to prevent damaged or misfolded proteins from accumulating. There are chaperones that assist with folding the newly imported proteins, as well as proteases that cleave misfolded or non-functional proteins (Szczepanowska et al., 2016; Weinhäupl et al., 2018). However, if levels of misfolded proteins exceed the folding and degradative capacity of the resident chaperones and proteases, activation of the mitochondrial unfolded protein response (UPR^{mt}) will ensue. This conserved response involves retrograde signaling to the nucleus to activate a transcriptional program aimed at restoring mitochondrial proteostasis (Münch and Harper, 2016). Intriguingly, recent reports suggest that mitochondria themselves can contribute to cytosolic protein QC through the import and degradation of misfolded proteins in the matrix (Burman et al., 2017; Ruan et al., 2017; Li et al., 2019). Thus, while mitochondria require cytosolic protein QC mechanisms for proper structure, these organelles directly promote cytosolic proteostasis during proteotoxic stress. However, at a certain threshold, the damage may become too great for protein repair and the entire mitochondrion must be eliminated from the cell. The primary mechanism by which entire mitochondria are eliminated is via autophagy and involves engulfment of the organelle into autophagosomes. In this review, we discuss the pathways involved in regulating mitochondrial QC and their relationship to cellular proteostasis and mitochondrial health in the heart.

REGULATION OF MITOCHONDRIAL QUALITY BY THE UBIQUITIN PROTEASOME SYSTEM (UPS)

The UPS is essential for routine protein turnover as well as the degradation of misfolded and unfolded proteins. Even modest decreases in proteasomal efficiency sensitize mice to cardiac pathogenesis (Ranek et al., 2015). Ventricular biopsies from human patients have been shown to exhibit decreased proteasomal activity and increased levels of protein ubiquitination (Predmore et al., 2010). The UPS is also involved in degrading accessible proteins in the outer mitochondrial membrane (OMM) with downstream effects on mitochondrial morphology and apoptosis. For example, UPS-mediated degradation of the anti-apoptotic protein MCL-1 allows for activation of pro-death proteins Bax/Bak (Zhong et al., 2005), while degradation of the mitochondrial fusion protein Mitofusin 2 leads to a shift toward fragmented mitochondria and disconnection from the endoplasmic reticulum (McLelland et al., 2018). After ubiquitination, OMM proteins are extracted from the membrane and delivered to the proteasome, a pathway analogous to the degradation of endoplasmic reticulum cargo during protein misfolding. The AAA-ATPase p97 is required for the extraction of specific substrates such as MCL-1 (Xu et al., 2011) and the general degradation of oxidized OMM proteins (Hemion et al., 2014). Studies employing a dominant negative p97 indicate that this QC pathway is critical to maintain a mitochondrial membrane potential (Fang et al., 2015). In addition to the outer membrane, proteins within the intermembrane space can also be exported through the translocase of the outer mitochondrial membrane (TOM) complex for proteasomal degradation. These QC mechanisms provide a means for the turnover of specific mitochondrial resident proteins and have been reviewed elsewhere (Karbowski and Youle, 2011).

More recently, the UPS has emerged as an important mechanism in maintaining mitochondrial QC through the turnover of nuclear-encoded mitochondrial proteins prior to their import. All but 13 of the >1000 mitochondrial proteins are nuclear-encoded and need to be transported to the mitochondrion where they are subsequently imported through the TOM/TIM complexes in an unfolded state. As such, the UPS provides the first line of mitochondrial QC through its consistent surveilling of mitochondrial proteins during their translation (**Figure 1A**). Increases in subunits of respiratory complexes I, II, and IV, as well as the F1-F0-ATPase upon acute proteasomal inhibition indicate that many of these proteins are quickly degraded by the UPS before they even reach their subcellular localization in the mitochondrial matrix (Margineantu et al., 2007). Similar increases in endonuclease G (Radke et al., 2008) and UCP2 (Azzu and Brand, 2010), proteins known to reside in the intermembrane space and inner membrane, respectively, upon UPS inhibition suggest that most precursor proteins routed to the inner mitochondrial sub-compartments are subject to this level of QC. The ubiquillin family of proteins have recently emerged as a key link between mitochondrial precursor protein targeting and UPS monitoring. In the cytosol, ubiquillins specifically interact with mitochondrial transmembrane proteins to facilitate their processing and membrane insertion (Itakura et al., 2016). In the event of failed insertion, ubiquillins recruit an E3 ligase for the polyubiquitination and proteasomal degradation of transmembrane domain-containing mitochondrial proteins. Mammals possess four ubquillin proteins with seemingly redundant functions in mitochondrial protein QC (Itakura et al., 2016). Interestingly, ubiquillin1 was recently shown to be necessary for myocardial proteostasis. Cardiac-restricted ubiquillin1 knockout (KO) mice develop cardiac dysfunction by 5 months of age and die prematurely (Hu et al., 2018). Moreover, at 10 weeks of age, ubiquillin1-deficient mice subjected to ischemia-reperfusion (I/R) injury have significantly greater cardiac dysfunction and infarct size compared to age-matched wild type mice, suggesting that ubiquillin1 plays an important role in the adaptation to myocardial stress (Hu et al., 2018). However, whether loss of ubiquillin1 in myocytes leads to

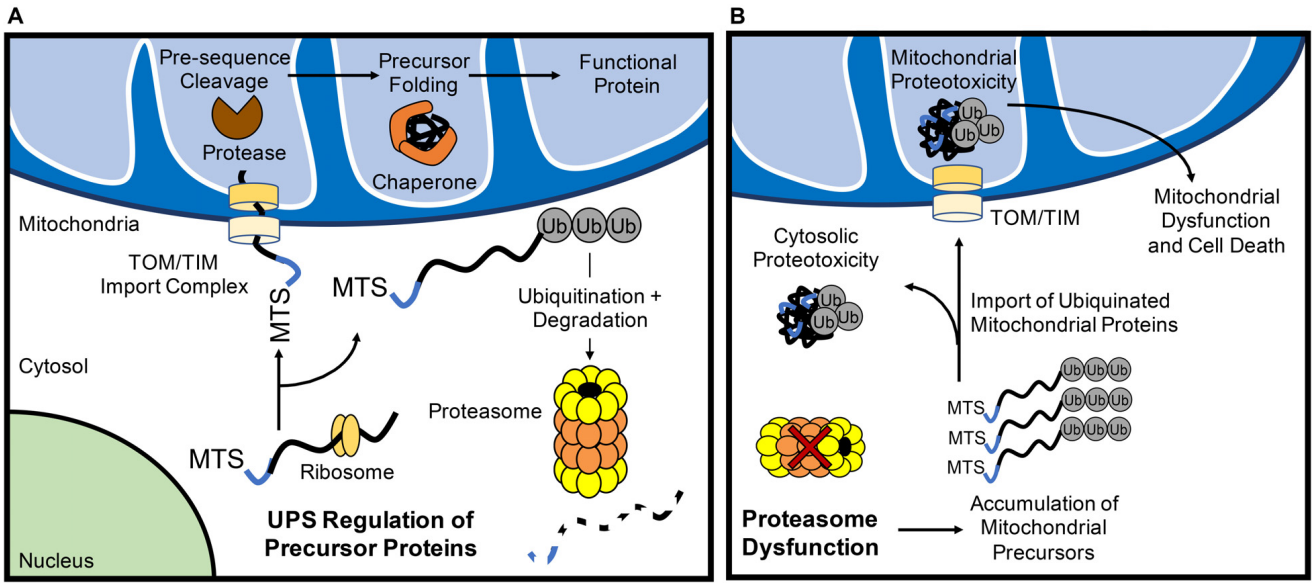


FIGURE 1 | The ubiquitin proteasome system (UPS) regulates mitochondrial protein turnover. **(A)** The UPS monitors mitochondrial precursor proteins before they are imported into mitochondria. After import via the TOM/TIM complex in an unfolded state, the mitochondrial targeting sequence (MTS) is cleaved off by a protease and chaperones ensure proposer folding of the protein. **(B)** Inhibition of UPS results in the accumulation of polyubiquinated mitochondrial precursor proteins in the cytosol. Some of the ubiquitinated proteins are still imported into the mitochondrion which can contribute to mitochondrial dysfunction and activation of cell death.

accumulation of mitochondrial transmembrane proteins in the cytosol and its effect on mitochondrial function still need to be investigated.

If the UPS continuously monitors mitochondrial precursor protein localization, what are the fates of these proteins when proteasomal activity is inhibited? Investigations into this question have yielded varying results that likely relate to the structure and import efficiency of particular mitochondrial proteins. For example, pharmacological inhibition of the proteasome in HeLa cells leads to a significant increase in the levels of ubiquitinated proteins in the inner mitochondrial membrane (Lavie et al., 2018), suggesting that ubiquitinated proteins are imported into mitochondria (**Figure 1B**). Li et al. (2019) also found that inhibition of the proteasome leads to increased import of proteins into mitochondria. In contrast, a recent study in Neuro-2a cells revealed that inhibiting the UPS leads to the irreversible aggregation of respiratory complex subunits in the cytosol (Rawat et al., 2019) (**Figure 1B**). In this report, several electron transport chain proteins were found to accumulate in insoluble protein fractions which leads to increased oxidative stress and respiratory defects (Rawat et al., 2019). Similarly, disruption in various mitochondrial functions including inhibited ATP/ADP exchange across the inner membrane (Wang and Chen, 2015) and impaired mitochondrial translation (Fakruddin et al., 2018) leads to mitochondrial protein aggregation in the cytosol. Cytosolic aggregation of mitochondrial proteins can occur downstream of import defects, resulting in a compensatory attenuation of cytosolic translation and concomitant increase in proteasomal activity to process mistargeted precursor proteins (Wrobel et al., 2015). Although these responses transiently increase protein QC (Wrobel et al., 2015), the sustained accumulation of the precursor proteins leads to cell death (Wang and Chen, 2015). Thus, it is clear that a dysfunctional UPS results in accumulation of ubiquinated mitochondrial protein aggregates in both the cytosolic and mitochondrial compartments of the cell. Taken together, this illustrates the importance of proper trafficking and import of nuclear-encoded mitochondrial proteins for cytosolic proteostasis.

MITOCHONDRIAL UNFOLDED PROTEIN RESPONSE (UPR^{MT})

Because the majority of mitochondrial proteins are nuclear-encoded and imported in an unfolded state, mitochondria contain resident chaperones and proteases to ensure proper protein folding and degradation of aberrant precursor molecules. The proteases in the matrix are also responsible for the normal turnover of proteins inside the mitochondria (Bulteau et al., 2017). LonP1 is the most abundant protease in cardiac mitochondria (Bota and Davies, 2002; Lau et al., 2012) and is responsible for degrading various misfolded and oxidatively damaged matrix and inner membrane proteins, thereby preventing their deleterious accumulation (Bota and Davies, 2002). When the levels of misfolded proteins exceed the folding and degradative capacity of the resident chaperones and proteases, a conserved mitochondrial unfolded protein response (UPR^{mt}) is activated (**Figure 2**). This involves retrograde signaling to the nucleus to promote a transcriptional program aimed at restoring mitochondrial proteostasis through the induction of chaperones and proteases (Münch and Harper, 2016). Similar to the endoplasmic reticulum UPR (UPR^{ER}), the

UPR^{mt} is activated by an accumulation of misfolded proteins and results in nuclear transcription of proteostatic genes. However, the UPR^{mt} can be distinguished from the UPR^{ER} in the downstream transcriptional targets. For example, the UPR^{ER} induces the expression of resident ER chaperones Calreticulin (*CALR*), GRP78/BiP (*HSPA5*), GRP94 (*HSP90B1*), which harbor the ER-targeting peptide sequence KDEL, to promote protein folding in the ER lumen (Yamamoto et al., 2003). For a more comprehensive understanding of ER-stress signaling and its relationship to cardiovascular disease, readers are referred to a recent review (Amen et al., 2019).

The UPR specific to the mitochondria was initially discovered in cultured mammalian cells through overexpression of the truncated mitochondrial matrix protein ornithine transcarbamylase (OTC-Δ) which is prone to aggregation. This study found that overexpression of OTC-Δ and the resulting mitochondrial stress trigger increased expression of the mitochondrial chaperones HSP60 (*HSPD1*) and mtDNAJ (*DNAJ3*), as well as the matrix protease CLPP via the transcription factor CHOP (*DDIT3*) (Zhao et al., 2002). The UPR^{mt} also upregulates glycolysis while downregulating subunits in the respiratory chain and mitochondrial translation to unburden the mitochondria while proteostasis is restored (Nargund et al., 2012, 2015). In parallel, the UPR^{mt} promotes the expression of various genes responsible for mitochondrial protein import, OXPHOS assembly, and mitochondrial ROS detoxification (Shpilka and Haynes, 2018) (**Figure 2**). In addition to intraorganellar proteotoxicity, the UPR^{mt} has emerged as a multifaceted response to several mitochondrial stressors including mito-nuclear protein imbalance, import defects, and mtDNA depletion. As such, the transcriptional regulators and downstream effectors are currently under intense investigation. In mammalian cells, activating transcription factor 5 (ATF5) is a major regulator of UPR^{mt} activation and possesses both a mitochondrial translocation sequence and a nuclear localization signal (Fiorese et al., 2016). In the basal state, ATF5 is imported into mitochondria and subsequently degraded by resident proteases. UPR^{mt} activation disrupts the mitochondrial import of ATF5 enabling its nuclear translocation and activation of downstream effector genes. ATF5 has been reported to induce a variety of genes involved in the UPR^{mt}, including HSP60, mtHSP70, and the matrix protease LonP1 (Fiorese et al., 2016). In addition to ATF5, CHOP (Aldridge et al., 2007) and ATF4 (Quirós et al., 2017), two known transcriptional regulators of the UPR^{ER} (Amen et al., 2019) have been identified as mediators of the UPR^{mt}, although it is currently unclear how these transcription factors can orchestrate distinct proteostatic responses in the ER and mitochondria.

Activation of the UPR^{mt} has been reported to be important in myocytes. Inhibition of the mitochondrial chaperone HSP90 or OXPHOS Complex I in neonatal rat myocytes leads to activation of a canonical UPR^{mt} transcriptional response including the induction of ATF5, CHOP, HSP60, mtDNAJ, LonP1, and ClpP (Smyrnias et al., 2019). The authors confirmed that overexpression of the aggregate prone mitochondrial OTC-Δ in myocytes leads to a similar transcriptional profile. These genes are also increased in a small cohort of human patients undergoing valve replacement for aortic stenosis. Interestingly, a subset of patients with strong induction of UPR^{mt} exhibit reduced levels of serum markers for myocardial damage, as well as fewer apoptotic myocytes and fibrosis in tissue sections relative to patients with low induction of UPR^{mt} (Smyrnias et al., 2019). These clinical observations indicate that a robust UPR^{mt} response in cardiac myocytes might protect the heart against pathological remodeling and dysfunction. Indeed, pharmacological activation of the UPR^{mt} preserves ejection fraction and reduces infarct size in murine models of pressure overload (Smyrnias et al., 2019) and I/R injury (Wang et al., 2019), respectively. Importantly, ATF5 is required for the cardioprotective effects of the UPR^{mt} in these studies (Smyrnias et al., 2019; Wang et al., 2019). This is in contrast to the UPR^{ER} which is primarily regulated by ATF6 in the heart (Jin et al., 2017; Blackwood et al., 2019). Consistent with the need for transcriptional activation, acute activation of the UPR^{mt} is insufficient to protect against I/R injury (Wang et al., 2019). Furthermore, while UPR^{mt} activation preserves cardiac and mitochondrial functions, the hypertrophy response is not abrogated suggesting that early responses in cardiac growth occur independent of mitochondrial impairment (Smyrnias et al., 2019).

Current knowledge of the molecular mechanism of UPR^{mt}-mediated protection during cardiac pathophysiology is limited. In addition to ATF5, Hsp60 and LonP1 that function downstream also appear to be essential in cardiac myocytes for protection against stress. Deletion of Hsp60 in adult cardiomyocytes produces a lethal cardiomyopathy within weeks of tamoxifen-mediated gene deletion. Interestingly, although a UPR^{mt} response occurred following the loss of Hsp60, it is not sufficient to restore mitochondrial homeostasis suggesting a key role for Hsp60 in this response (Fan et al., 2019). Similarly, the LonP1 protease is critical for OXPHOS turnover during myocardial ischemia (Sepuri et al., 2017) and limits myocyte damage during reoxygenation (Venkatesh et al., 2019). Failing mouse hearts display reduced proteolytic capacity due in large part to oxidative modifications of LonP1 which diminish its activity (Hoshino et al., 2014). The detrimental effects of prolonged UPR^{mt} induction (Gitschlag et al., 2016; Lin et al., 2016) and its paracrine effects on distal tissues (Berendzen et al., 2016; Shao et al., 2016) highlight the complexity of this mitochondrial QC mechanism and the need for additional studies.

ROLE OF MITOCHONDRIA IN CYTOSOLIC PROTEIN QUALITY CONTROL

Recent studies demonstrate that mitochondria can also directly contribute to cytosolic protein QC. For instance, induction of the UPR^{mt} is observed in various models of Alzheimer's disease (AD) and is involved in reducing amyloid-β proteotoxicity (Sorrentino et al., 2017). Because the UPR^{mt} promotes the expression of mitochondrial chaperones and proteases, these observations indicate that this pathway might also support cytosolic proteostasis through import and degradation of

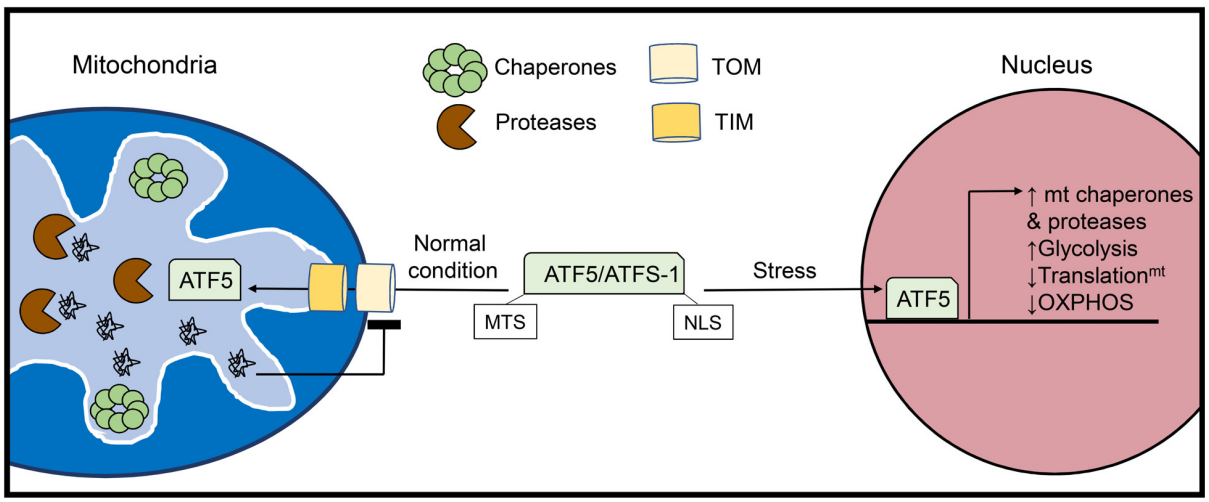


FIGURE 2 | Activation of the mitochondrial unfolded protein response (UPR^{mt}). The UPR^{mt} is activated when there is an excess of misfolded proteins within the organelle. The UPR^{mt} transcriptional regulator ATF5 (ATFS-1 in yeast) contains both a mitochondrial targeting sequence (MTS) and a nuclear localization signal (NLS). Under normal conditions, ATF5 is imported into mitochondria where it is degraded. However, increased levels of misfolded proteins inside mitochondria lead to abrogation of ATF5 import. Instead, ATF5 translocates to the nucleus where it activates a transcriptional response aimed at restoring mitochondrial health and proteostasis.

pathogenic amyloid-β peptides. However, whether amyloid-β is imported into mitochondria is controversial (Petersen et al., 2008; Cenini et al., 2016). Petersen et al. (2008) reported that amyloid-β is transported into the mitochondrial matrix via the TOM complex, while Cenini et al. (2016) found that amyloid-β interferes with mitochondrial import of nuclear-encoded mitochondrial proteins. It is possible that amyloid-β can be imported into mitochondria before it forms aggregates; however, large proteotoxic aggregates are likely to interfere with various mitochondrial functions such as import. In addition, the UPR^{mt} has also been shown to promote a cytosolic chaperone response through the transcriptional regulator heat shock factor 1 that blunts the polyglutamine repeat protein (PolyQ) aggregation accompanying Huntington's disease (Kim et al., 2016; Labbadia et al., 2017). Thus, the recent reports of UPR^{mt}-mediated cardioprotection during pressure overload and ischemia may be applicable to protetotoxic cardiac diseases such as desmin-related cardiomyopathy and transthyretin amyloidosis.

A study by Ruan et al. (2017) demonstrated that in yeast, cytosolic proteins prone to aggregation are indeed imported into mitochondria via the TOM complex where they are subsequently degraded by the LonP1 protease. Although import of misfolded proteins at baseline is minimal, proteotoxic stress by heat-shock significantly increases mitochondrial import of cytosolic proteins. Importantly, loss of mitochondrial membrane potential abrogates this adaptive response, exacerbating cytosolic aggregation. This suggests that this potential novel QC pathway can compensate and reduce the load of aggregated proteins in the cytosol when cytosolic degradation is compromised (**Figure 3**). This study also noted that mitochondrial uptake of an aggregate-prone protein also occurs in human epithelial cells, but they did not examine effects on cell viability and proteostasis.

The uptake of aggregate-prone proteins by mitochondria in mammalian cells was recently confirmed by another study. This group reported that proteosomal inhibition leads to the import of misfolded proteins where they are degraded in the matrix by LonP1 (Li et al., 2019). Interestingly, the OMM protein FUNDC1 was found to facilitate the mitochondrial translocation and import of UPS substrates through its interaction with the cytosolic chaperone HSC70 (Li et al., 2019) (**Figure 3**). As discussed below, FUNDC1 is also a known regulator of mitophagy. The authors also found that excessive uptake of misfolded proteins interferes with mitochondrial function and contributes to cellular senescence (Li et al., 2019). Excessive import of misfolded cytosolic proteins into the matrix is clearly toxic to mitochondria, but whether this led to activation of the UPR^{mt} still needs to be investigated. Mitochondrial import of cytosolic proteins might function as an important QC mechanism to ensure cellular proteostasis. However, whether this pathway is relevant in metabolically active tissues such as the heart remains to be investigated. It will be important to thoroughly evaluate the relevance of these QC mechanisms in cardiac myocytes considering the differences between their mitochondrial network, energetic demands, and regenerative potential relative to the various systems employed in the aforementioned investigations.

SELECTIVE ELIMINATION OF MITOCHONDRIA

Mitochondrial autophagy (mitophagy) serves as a QC mechanism to remove entire damaged organelles that cannot be repaired by smaller scale stress responses such as the UPR^{mt} or UPS. Mitophagy targets dysfunctional mitochondria for

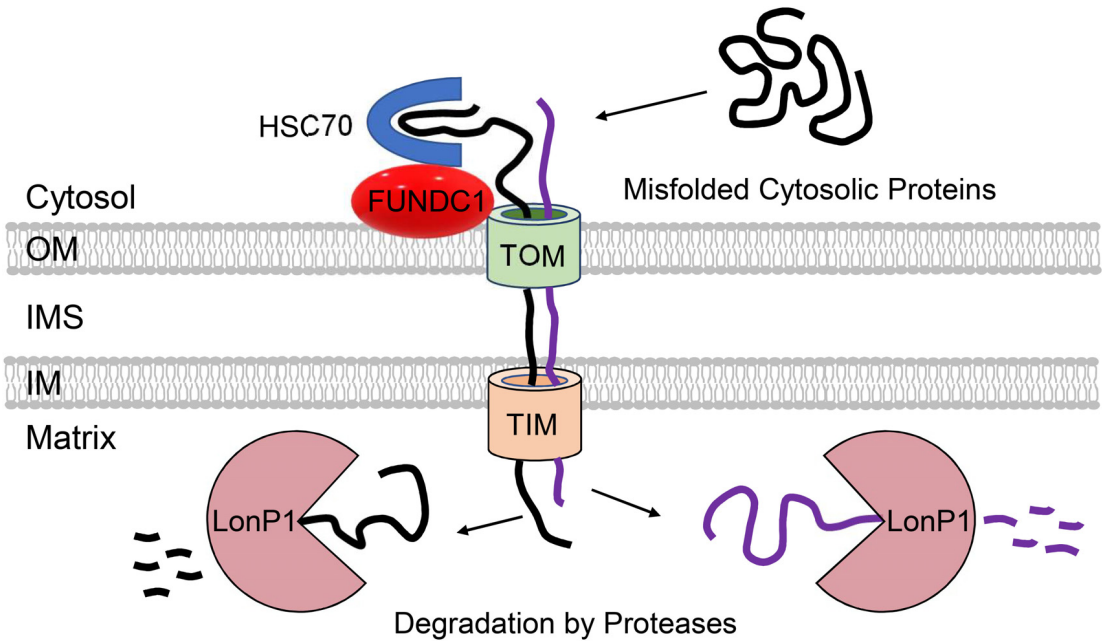


FIGURE 3 | Participation of mitochondria in regulation of cytosolic proteostasis. Mitochondria contribute to cytosolic protein quality control through the uptake of aggregate-prone misfolded proteins via the TOM and TIM import machinery. FUNDC1 and HSC70 can facilitate the import of proteins into mitochondrial matrix where they are degraded by LonP1 protease.

autophagosomal engulfment and lysosomal degradation to ensure their elimination prior to cytotoxicity. The primary pathways involved in eliminating mitochondria are the PTEN-induced putative kinase 1 (PINK1)/Parkin pathway and mitophagy receptors in the OMM. The detailed molecular mechanisms of PINK1/Parkin- and mitophagy receptor-mediated mitochondrial elimination have previously been reviewed in detail (Gustafsson and Dorn, 2018; Pickles et al., 2018). The conditions that activate these different pathways are under intense investigation and the two pathways appear to function under both distinct and overlapping conditions, making it a challenging area of research.

Most studies to date in the heart have focused on the PINK1/Parkin pathway, which is involved in the selective removal of damaged mitochondria during stress. In this pathway, the serine/threonine kinase PINK1 is constitutively imported into healthy mitochondria, where it is cleaved by proteases in the intermembrane space and then returned to the cytosol for proteasomal degradation (Jin et al., 2010; Greene et al., 2012; Yamano and Youle, 2013). When damaged or dysfunctional mitochondria lose their membrane potential, import and degradation of PINK1 are abrogated, resulting in its accumulation on the OMM (Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010). Interestingly, although loss of mitochondrial membrane potential has been reported to be the primary mechanism for activation of this pathway, one study reported that excessive protein misfolding in the matrix of respiring mitochondria with intact membrane potential also leads to inhibition of PINK import and activation of mitophagy (Jin and Youle, 2013). At the OMM, PINK1 recruits the E3 ubiquitin ligase Parkin (Narendra et al., 2010), which in turn, proceeds to ubiquitinate numerous proteins in the OMM (Sarraf et al., 2013). This leads to recruitment of the autophagosome machinery and sequestration of the mitochondria (**Figure 4A**). The PINK1/Parkin pathway plays an important role in the heart and many studies have reported on its importance in repair and adaptation to stress. For instance, PINK1/Parkin-mediated mitophagy is activated during pressure-overload (Billia et al., 2011; Shirakabe et al., 2016), following myocardial infarction (MI) (Kubli et al., 2013), and in I/R (Siddall et al., 2013). A recent study also discovered that mitophagy is increased in hearts of mice fed a high-fat diet and that Parkin deficiency results in increased cardiac dysfunction (Tong et al., 2019). Overall, these studies have confirmed a key role of PINK1/Parkin-mediated mitophagy in the adult heart where dysfunctional mitochondrial must be eliminated to prevent loss of myocytes and cardiac dysfunction.

Voltage-dependent anion channels (VDACs), which regulate metabolite and ion transport across the OMM, are also involved in regulating PINK1/Parkin-dependent mitophagy. It has been reported that the VDACs are responsible for recruiting Parkin to depolarized mitochondria (Sun et al., 2012). There are three VDAC genes (VDAC1, VADC2, and VDAC3) and only simultaneous knockdown or deletion of all three VDACs abrogates mitochondrial Parkin translocation (Sun et al., 2012). In addition, the 18 kDa outer mitochondrial translocator protein TSPO disrupts mitophagy through its interaction with VDAC1 (Gatliff et al., 2014). This study found that TSPO does not affect Parkin translocation to mitochondria, but prevents Parkin from ubiquitinating its substrates. How TSPO inhibits Parkin activity

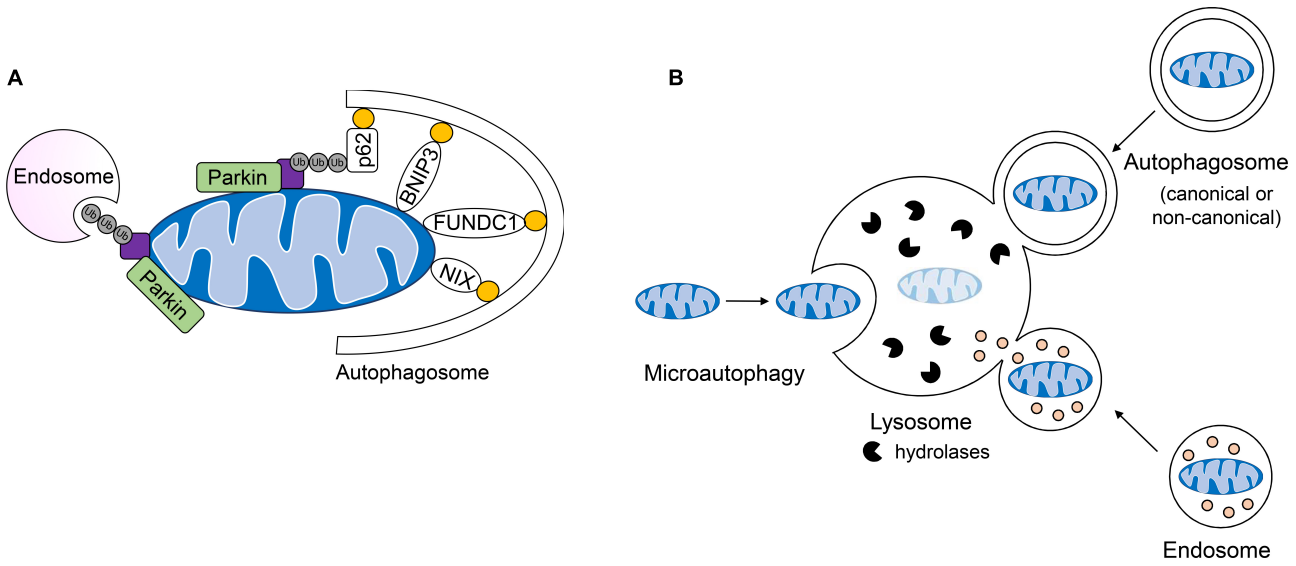


FIGURE 4 | Lysosomal-mediated degradation of mitochondria. **(A)** Mitochondria are labeled by the E3 ubiquitin ligase Parkin for mitochondrial autophagy (mitophagy) or removal by endosomes. Engulfment of ubiquinated cargo involves autophagy adaptors such as p62/SQSTM1 which link ubiquinated cargo to the autophagosome through LC3/GABARAP interaction. Outer mitochondrial membrane mitophagy receptors BNIP3, BNIPL3L/NIX, and FUNDC1 facilitate the degradation of whole organelles through direct interaction with LC3/GABARAP. **(B)** Mitochondria can be directly taken up by the lysosome, engulfed by single-membraned endosomes or double-membraned autophagosomes which fuse with the lysosome for the degradation of cargo.

is currently unknown. Interestingly, heart failure is associated with increased TSPO expression and cardiac-specific TSPO KO mice are protected from pressure-overload-induced cardiac remodeling and dysfunction (Thai et al., 2018). This study also found that while mitophagy is impaired in wild-type myocytes after 8 weeks of pressure overload, mitophagy is preserved in TSPO-deficient myocytes (Thai et al., 2018). Collectively, these findings point to important functions for TSPO and VDAC in regulating Parkin-dependent mitophagy in cardiac myocytes.

Interestingly, PINK1/Parkin-mediated clearance of damaged mitochondria in tissues also limits inflammation by preventing overactivation of the inflammasome. These cytosolic multimeric protein complexes function as sensors of infectious microbes and molecules derived from pathogens. Once activated, the inflammasome activates caspase-1 and induces inflammation in tissues (Malik and Kanneganti, 2017). Unfortunately, overactivation of the inflammasome can lead to excessive inflammation and tissue damage. Because mitochondria are descendants of bacteria, release of mtDNA into cytosol from damaged mitochondria will also activate the inflammasome. Induction of mitophagy to eliminate these mitochondria prior to release of mtDNA prevents inflammasome activation (Zhong et al., 2016). Early studies on Parkin in the heart led to the observation that Parkin-deficient mice are more sensitive to endotoxins and develop increased mitochondrial damage and contractile dysfunction after endotoxin exposure compared with wild-type mice (Piquereau et al., 2013). Subsequent studies have reported that mice with enhanced PINK1/Parkin-mediated mitophagy in hearts render these mice more resistant to cardiac dysfunction and alleviate inflammation and fibrosis after endotoxin exposure (Sun et al., 2018; Essandoh et al., 2019). Interestingly, another study focused on investigating why heart failure is accelerated after an MI in patients with Type 2 diabetes mellitus discovered a defect in myocardial mitophagy in these patients that correlates with increased release of mitochondrial DNA and hyperactivation of the NLRC4 inflammasome (Devi et al., 2017). Overall, these studies demonstrate an important link between mitophagy and inflammation in the heart.

Receptor-mediated mitophagy is also important in the heart and differs from the PINK1/Parkin pathway in that ubiquitination and adaptor proteins are not needed for recognition of cargo by the autophagosome (Gustafsson and Dorn, 2018). Rather, OMM-anchored mitophagy receptors such as BNIP3, BNIP3L/NIX, and FUNDC1 directly tether the mitochondrion to the autophagosome membrane via their interaction with LC3 and GABARAP to facilitate its clearance (Gustafsson and Dorn, 2018) (**Figure 4A**). The conditions that induce activation of receptor-mediated mitophagy are less clear. However, there is strong evidence that while PINK1/Parkin-dependent mitophagy requires mitochondrial membrane depolarization, receptor-mediated mitophagy is often induced by hypoxia. Many of the mitophagy receptors are induced during hypoxic conditions (Chen et al., 2017; Esteban-Martínez and Boya, 2018). For instance, BNIP3 and NIX are hypoxia-inducible pro-apoptotic members of the BH3-only BCL-2 family with 56% sequence homology (Zhang and Ney, 2009). Both BNIP3 and NIX have dual roles in inducing apoptosis and mitophagy, which has made it challenging to dissect their functions in cells. Overexpression of BNIP3 and NIX in cells, including myocytes, leads to activation of both mitophagy and cell death (Regula et al., 2002; Chen et al., 2010). However, studies indicate that their primary function is to induce mitophagy

but that during overwhelming stress and mitochondrial damage, they turn on their pro-death function. This is supported by the fact that BNIP3-deficiency in mice leads to attenuated myocyte apoptosis and preserves contractile performance in models of I/R injury (Diwan et al., 2007) and doxorubicin cardiotoxicty (Dhingra et al., 2014). This suggests that BNIP3 contributes to cell death and cardiac injury under these conditions. However, simultaneous deletion of NIX and BNIP3 in hearts leads to accelerated accumulation of dysfunctional mitochondria in myocytes with age (Dorn, 2010), implicating these proteins as key regulators of normal mitochondrial turnover in hearts in the absence of stress.

FUNDC1 is another mitophagy receptor that facilitates hypoxia-induced mitochondrial clearance (Chen et al., 2017). FUNDC1 activity is regulated by phosphorylation through several different kinases. Dephosphorylation by the mitochondrial phosphatase PGAM5 is required for its activation and interaction with LC3 to induce mitophagy (Chen et al., 2014). Inhibition of FUNDC1 seems to be a major factor underlying myocardial I/R injury. Several kinases, including MST1 (Yu et al., 2019), CK2α (Zhou et al., 2018), and RIPK3 (Zhou et al., 2017), have been reported to inhibit FUNDC1-mediated mitophagy during I/R. For instance, Zhou et al. (2018) reported that upregulation of CK2α during myocardial I/R inhibits FUNDC1-mediated mitophagy and results in accumulation of dysfunctional mitochondria, opening of the mitochondrial permeability transition pore, and activation of cell death. What happens to other mitophagy receptors under these conditions and their relationship with FUNDC1 activity remain to be investigated.

Recent observations of mitochondrial clearance in cells lacking key canonical autophagy regulators *Atg5* and *Atg7* have led to the identification of alternative mechanisms of mitochondrial degradation in cells. For instance, a non-canonical autophagy pathway have been identified where Rab9-positive double-membrane vesicles derived from the trans-Golgi can engulf mitochondria which are then delivered to lysosomes for degradation (Nishida et al., 2009). This alternative autophagy pathway plays a role in clearing mitochondria during starvation and in myocardial ischemia (Hirota et al., 2015; Saito et al., 2019). It has also been reported that dysfunctional mitochondria can be sequestered in Rab5-positive early endosomes for subsequent delivery to the lysosome for degradation (Hammerling et al., 2017a,b). The uptake of depolarized mitochondria into early endosomes is dependent on Parkin-mediated ubiquitination of mitochondrial proteins which are then recognized by the ESCRT machinery (**Figure 4A**). These ESCRT complexes facilitate capture and delivery of mitochondria into the endosomal lumen. Interestingly, the mitophagy receptor BNIP3 can also utilize the endosomal pathway for mitochondrial elimination (Hammerling et al., 2017b), but whether this requires ubiquitination and the ESCRT machinery is currently unclear. Although BNIP3-mediated sequestration of mitochondria into Rab5-positive endosomes is independent of Parkin, it is possible that BNIP3 still utilizes a resident E3 ubiquitin ligase to facilitate uptake into endosomes. In addition to the endosome pathway, microautophagy represents another form of non-canonical autophagy that involves the direct uptake of cargo into lysosomes; however, this process is not well characterized (Gustafsson and Dorn, 2018). It has been reported that mitochondria can be directly delivered to lysosomes in mammalian cells and that GAPDH regulates their engulfment (Hwang et al., 2015). This mode of mitochondrial degradation has been reported to be inhibited in myocardial I/R injury (Yogalingam et al., 2013) and in Huntington's disease (Hwang et al., 2015). Thus, it is likely that GAPDH-mediated mitochondrial engulfment by the lysosome represents another major mechanism of mitochondrial QC, in particular when formation of autophagosomes is reduced or compromised. In sum, whole mitochondria are degraded by the lysosome through direct fusion, or engulfment in double and single-membraned autophagosomes and endosomes, respectively (**Figure 4B**).

COORDINATION BETWEEN MITOCHONDRIAL ELIMINATION AND BIOGENESIS

Mitophagy is tightly coordinated with mitochondrial biogenesis to appropriately balance the degradation and formation of new organelles. Baseline mitophagy involving removal of dysfunctional and aged organelles is always accompanied by biogenesis for replacement of mitochondria that were degraded. This continuous balance between degradation and synthesis is particularly important in highly energetic cells such as cardiac myocytes and neurons that rely on mitochondria for function. Similarly, during stress, when a larger portion of mitochondria are eliminated, there is a coordinated activation of mitochondrial biogenesis. In addition to inducing mitophagy, Parkin simultaneously induces mitochondrial biogenesis by indirectly activating the PPARG coactivator 1 alpha (PGC-1α), a master regulator of mitochondrial biogenesis in the heart (Lehman et al., 2000; Arany et al., 2005). Parkin promotes the ubiquitination and proteasomal degradation of PARIS, a transcriptional repressor of PGC-1α (Shin et al., 2011). Parkin deficiency leads to increased levels of PARIS and reduced mitochondrial mass in dopaminergic neurons (Stevens et al., 2015). In skeletal muscle injury (Vainshtein et al., 2015) and exercise (Erlich et al., 2018) models, PGC-1α exerts a positive feedback loop to enhance mitophagy through the induction of TFEB, a transcriptional regulator of the lysosomal machinery. Similarly, the metabolic sensor and autophagy-promoting kinase AMPK has been shown to phosphorylate PGC-1α (Jäger et al., 2007) and induce downstream PGC-1α expression (Cantó et al., 2009). Apart from PGC-1α and AMPK, emerging evidence suggests that the transcriptional regulator of antioxidant gene expression nuclear factor, erythroid 2 like 2 (Nfe2l2/Nrf2) coordinates both mitophagy and mitochondrial biogenesis. Nrf2 promotes the expression of biogenesis genes Nrf-1 (Merry and Ristow, 2016) and Tfam (Wu et al., 2016) as well as mitophagy factors p62/SQSTM1 (Jain et al., 2010) and DCT-1 (BNIP3 ortholog) (Palikaras et al., 2015). However, while Nrf2 protects the

heart from ischemic injury (Zhang et al., 2010) and pressure overload (Li et al., 2009), constitutive myocardial Nrf2 activation paradoxically promotes pathophysiology through proteotoxicity (Rajasekaran et al., 2011; Shanmugam et al., 2020). Thus, a closer examination into Nrf2-mediated mitochondrial QC in cardiac myocytes is warranted. Mitochondrial uptake of misfolded proteins was recently linked to increased mitophagy and biogenesis in a human cell model of ribosomal mistranslation (Shcherbakov et al., 2019). Therefore, mitochondrial import, clearance, and biogenesis may integrate as a coordinated stress response against proteotoxicity; however, more studies are needed to examine the potential for this cross-talk in cardiac myocytes.

In contrast, proper erythrocyte maturation necessitates bulk degradation of mitochondria in the absence of compensatory biogenesis, and this is achieved through the mitophagy receptor NIX (Sandoval et al., 2008). However, how mitochondrial biogenesis is suppressed in these cells during mitophagy is unclear, but could be due to the mechanism by which mitochondria are cleared (Parkin vs. NIX). Although a clear link exists between Parkin and activation of mitochondrial biogenesis, a similar link between NIX and mitochondrial biogenesis has not been reported. Clearly, coordination between mitochondrial degradation and biogenesis varies according to cell-type and physiological context.

MITOCHONDRIAL-DERIVED VESICLES

To avoid eliminating the entire mitochondrion, damaged proteins and lipids can be selectively incorporated into vesicles that bud off from the OMM. These mitochondrial-derived vesicles (MDVs) have been found to be enriched with oxidized protein cargo suggesting that their formation is an additional mitochondrial QC mechanism (Soubannier et al., 2012b). Interestingly, ROS-induced MDV formation precedes mitophagy, with vesicles forming as early as 2 h following antimycin A treatment to inhibit mitochondrial respiration (McLelland et al., 2014). The MDVs are 70–150 nM in diameter and ultrastructural analysis has confirmed that they can be either double-membrane vesicles containing matrix cargo or single-membraned containing outer membrane proteins (Soubannier et al., 2012b). Incorporation of MDV cargo appears to be highly specific as the origin of ROS (e.g., intra vs. extraorganellar) dictates whether MDVs contain oxidized cargo from the inner compartments or OMM (Soubannier et al., 2012b). A subset of MDVs is targeted for lysosomal degradation through the endosomal system (Soubannier et al., 2012a) (**Figure 5**). The formation of these MDVs during mitochondrial stress requires PINK1 and Parkin, and Parkinson's disease-associated Parkin mutants are defective in MDV formation (McLelland et al., 2014, 2016). Their formation is independent of mitochondrial fission (Soubannier et al., 2012a) and does not require Atg5 and Beclin1 (McLelland et al., 2014) suggesting an upstream mechanism that is distinct from general autophagy. Also, the SNARE protein syntaxin-17 (STX17) is recruited to the MDVs where it forms a complex with SNAP29 and VAMP7 to facilitate their trafficking and endolysosomal fusion in a homotypic fusion and vacuole protein sorting (HOPS) tethering complex-dependent manner (McLelland et al., 2016).

Interestingly, Matheoud et al. identified a distinct set of MDVs that function in mitochondrial antigen presentation (**Figure 5**). Antigen presentation plays a role in establishing immune tolerance and is important in limiting autoimmunity (Matheoud et al., 2016). This study found that exposure to heat stress or LPS leads to transportation of mitochondrial proteins in MDVs to lysosomes for subsequent presentation on MHC class I molecules at the cell surface. The formation of these MDVs requires Rab7, Rab9, and sorting nexin 9 (SNX9) and is negatively regulated by PINK1/Parkin (Matheoud et al., 2016). Moreover, the fact that MDV-mediated mitochondrial antigen presentation requires Rab9 (Matheoud et al., 2016) while oxidative stress-induced lysosomal degradation of MDVs is Rab-9-independent (McLelland et al., 2014) indicates that distinct MDV transport mechanisms exist for diverse cellular stimuli. Indeed, peroxisomal delivery represents an alternative fate for MDV cargo and this process appears to uniquely require the vacuolar sorting protein VPS35 (Braschi et al., 2010). Continued investigation is needed to elucidate the key regulators of MDV formation, trafficking and degradation as a QC mechanism, and to differentiate this process from other homeostatic routes of MDV transport.

Little is still known about the tissue-specific role for MDVs and their relevance in human disease; however, this level of mitochondrial QC is likely to be critical for cell types with a high energetic demand that cannot afford to clear large portions of the mitochondrial network via mitophagy. Indeed, MDV formation appears to be an active process in the heart. Cadete et al. demonstrated that MDVs are formed in the H9c2 myoblast cell-line and murine hearts at baseline. MDVs carrying OMM and matrix proteins are observed in H9c2 cells under baseline conditions and vesicle biogenesis is significantly increased by ROS-inducing stimuli. Transmission electron microscopy of mouse hearts after exposure to antimycin A show formation of both single and double-membraned MDVs. Importantly, while hearts from control mice show MDV budding in the basal state, doxorubicin-treated mice depict a significant increase in myocardial MDV accumulation indicating that MDV formation contributes to mitochondrial quality during physiologic and pathological conditions *in vivo* (Cadete et al., 2016). The identification of MDVs adds yet another layer of mitochondrial QC in cardiac myocytes. It is likely that this process represents the first level of defense against mitochondrial damage; however, myocyte-specific effectors remain elusive as these studies employed wild-type mice without individual silencing of previously identified MDV regulators. As Cadete and colleagues utilized a doxorubicin model, future studies should aim to assess the temporal contribution of lysosomal MDV degradation in myocardial ischemia and reperfusion injury, and elucidate cross-talk with other QC pathways discussed above.

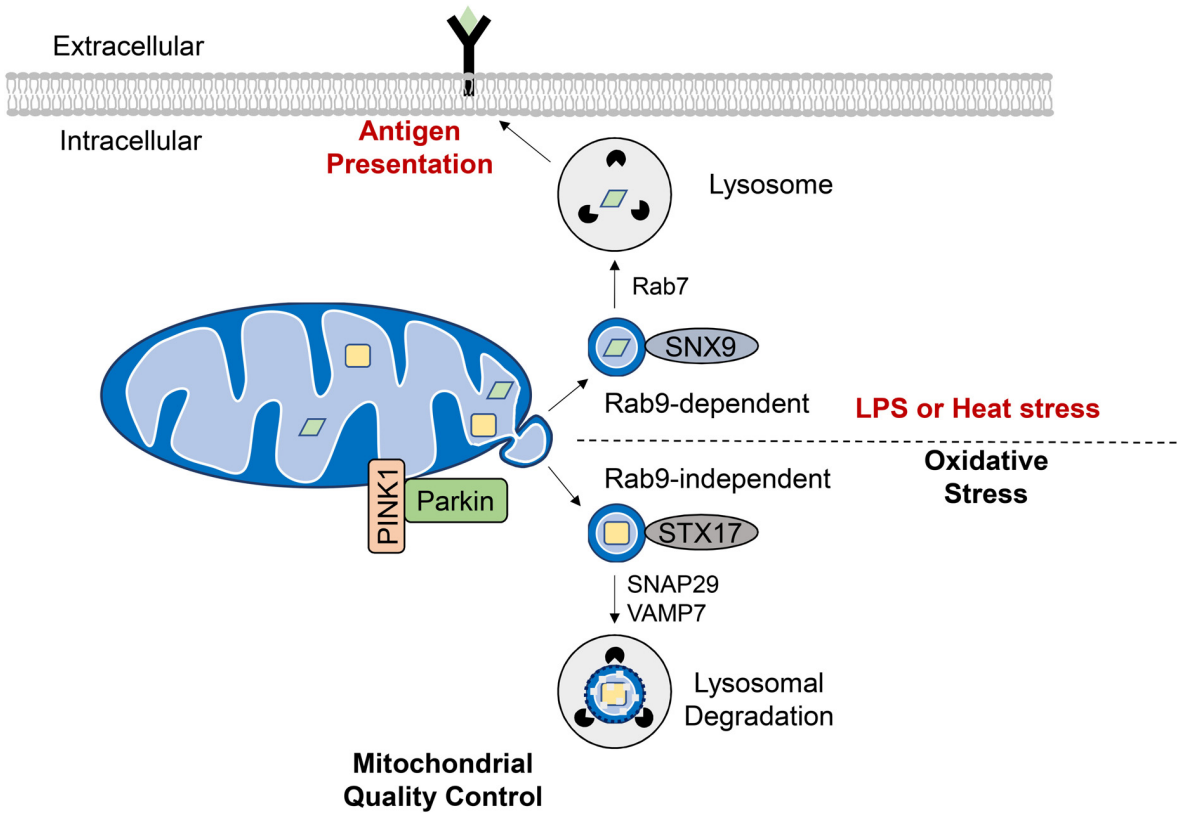


FIGURE 5 | Formation of mitochondrial derived vesicles (MDVs) as a quality control mechanism or antigen presentation. Generation of MDVs in response to oxidative stress requires PINK1 and Parkin, while STX17, SNAP29, and VAMP7 are required for their fusion with the lysosome. These vesicles contain oxidized cargo that are degraded in the lysosomes. MDVs are also formed in response to LPS exposure or heat stress and require the Rab7, Rab9, and SNX9. The mitochondrial antigens are processed in the lysosome for subsequent presentation on MHC class I molecules at the cell surface.

THERAPEUTIC POTENTIAL OF ENHANCING PROTEOSTASIS AND MITOCHONDRIAL QUALITY CONTROL

The fact that damaged mitochondria are consistently reported to accumulate in the diseased myocardium suggests that strategies designed to promote mitochondrial QC may hold therapeutic potential. Both general and mitochondrial autophagy transiently increase during pathological cardiac stress, but these compensatory mechanisms are ultimately exhausted at the point of overt heart failure (Shirakabe et al., 2016). Moreover, cardiac aging has been linked to diminished autophagy and mitophagy. The aged myocardium is often burdened with excessive reactive oxygen and nitrogen species which exert an inhibitory effect on the function of autophagy related proteins (Frudd et al., 2018) and Parkin (Meng et al., 2011). Proteotoxic aggregates also promote oxidative stress (Tanase et al., 2016) and can overwhelm UPS and lysosomal machinery in cardiac myocytes (Pan et al., 2017; Xu et al., 2020). Thus, enhancing lysosomal degradation of proteotoxic species and dysfunctional mitochondria promotes cardiac longevity.

Spermidine and urolithin A are two naturally occurring compounds recently reported to induce autophagy and mitophagy in pre-clinical models. Interestingly, "late-in-life" spermidine supplementation to 18-month old mice attenuates the pathological cardiac remodeling and dysfunction normally seen at 23 months in rodents (Eisenberg et al., 2016). Spermidine is also cardioprotective in high salt diets suggesting that this bioavailable compound may translate to human aging and congestive heart failure (Eisenberg et al., 2016). While spermidine-mediated protection is Atg5-dependent (Eisenberg et al., 2016), urolithin A appears to be a more selective activator of mitophagy as urolithin A-mediated increases in aged nematode mobility and lifespan require PINK1 (Ryu et al., 2016). Urolithin A administration to 24-month old mice increases skeletal muscle strength and exercise performance (Ryu et al., 2016). While this study did not examine the cardiac-specific effects, a different group recently reported diminished myocardial I/R injury in adult mice pre-treated with urolithin A (Tang et al., 2017). In addition to spermidine and urolithin A, the mTOR inhibitor rapamycin has been tested as a cardioprotective agent for the age-associated decline in myocardial autophagy. A 3-month rapamycin regimen in 24-month old mice reverses age-induced cardiac hypertrophy and dysfunction (Flynn et al., 2013). At the molecular level, the cardioprotective effects of rapamycin have been attributed to

decreased protein oxidation and ubiquitination (Dai et al., 2014) as well as enhanced mitochondrial biogenesis (Chiao et al., 2016). Taken together, these observations highlight the importance of targeting proteostasis and mitochondrial QC in therapeutic interventions for cardiac aging.

Caloric restriction is also a well-documented inducer of autophagy in mammalian cells (Escobar et al., 2019), and has been reported to reduce age-associated cardiovascular disease (Colman et al., 2009) and increase the lifespan (Pifferi et al., 2018) of primates. The heart is sensitive to nutrient availability as a single overnight fast is sufficient to stimulate autophagy in adult mouse hearts (Andres et al., 2016). Moreover, long-term gradual caloric reductions (e.g., 20–60% deficit for 4 weeks) also promote myocardial autophagic flux (Finckenberg et al., 2012; Chen et al., 2013). Considering the cost, availability, and multifaceted benefits of dietary intervention on human health, caloric restriction may provide the greatest translational value of the aforementioned therapies. Indeed, a recent randomized control trial of alternate day fasting in humans reports improved heart rate, blood pressure, and Framingham Risk Score, suggesting enhanced cardiovascular health (Stekovic et al., 2019). Although caloric intake was reduced in this study (Stekovic et al., 2019), time-restricted feeding, often referred to as intermittent fasting, elicits cardiometabolic benefits even when total caloric intake is maintained (Hatori et al., 2012). The fact that intermittent fasting modulates the expression of electron transport chain and protein folding machinery in a manner that reduces cardiac aging suggests that feeding rhythms are tightly linked to mitochondrial QC and proteostasis (Gill et al., 2015). This is further supported by recent data demonstrating that an intermittent fasting regimen during advanced protein aggregation cardiomyopathy restores autophagic flux and cardiac function in mice (Ma et al., 2019). In summary, caloric restriction or intermittent fasting holds therapeutic promise in part through protein and mitochondrial QC downstream of enhanced autophagy.

Intermittent fasting increases circulating ketone levels in humans (Jamshed et al., 2019). As an alternative fuel substrate, ketone bodies have recently received significant attention for their protective affects in a variety of neurological disorders such as AD (Kashiwaya et al., 2013), epilepsy (Kovács et al., 2019), and anxiety (Ari et al., 2017). During heart failure, diminished fatty acid oxidation results in a compensatory increase in myocardial ketone oxidation (Aubert et al., 2016). This response appears to be adaptive as genetically modified mice incapable of catabolizing 3-hydroxybutyrate (3OHB) display worsened contractile dysfunction and cardiac remodeling following transverse aortic constriction combined with a small apical MI (Horton et al., 2019). Importantly, exogenous 3OHB infusion in an established canine model of congestive heart failure preserves cardiac output and ejection fraction while attenuating left ventricular hypertrophy and chamber dilation (Horton et al., 2019). While the effects of ketone metabolism on mitochondrial QC are limited, Thai et al. (2019) recently demonstrated that 24 h of ketone treatment *in vitro* promotes Parkin-dependent mitophagy in young (2.5-month) and aged (2.5-year) rabbit ventricular myocytes. However, aged cardiac myocytes subjected to heart failure are not protected by β-hydroxybutyrate. Rather, ketone administration in these cells aggravates the incidence of Parkin aggregation at depolarized mitochondria (Thai et al., 2019). Interestingly, combining β-hydroxybutyrate with the mitochondrial fusion promoting peptide TAT-MP1^{Gly} (Franco et al., 2016) promotes mitochondrial QC in aged and failing myocytes, suggesting that a sufficient level of mitochondrial dynamics is required for the protective effects of ketone supplementation in heart failure (Thai et al., 2019). Combined activation of fusion and mitophagy is also cardioprotective in a model of angiotensin-II-induced cardiomyocyte injury (Xiong et al., 2019). These recent observations indicate that ketones exert a beneficial effect on cardiac myocytes through bioenergetic efficiency (Horton et al., 2019) and mitochondrial QC (Thai et al., 2019). However, more studies are needed to differentiate the effects from fasting-induced ketosis and exogenous ketone supplementation. Because a long-term ketogenic diet increases mitochondrial ROS and reduces respiratory control ratio in the skeletal muscle (Kephart et al., 2017), it will be important to optimize dose and duration before translating to the clinic.

While it is clear that promoting autophagy through caloric restriction or intermittent fasting is beneficial for cardiac aging, more studies are needed to address mitochondria-specific effects of these therapeutic strategies. The removal of mitochondria represents only one aspect of the autophagy pathway. Furthermore, little is known about the relative contributions of various mitophagy receptors and the endosomal-mediated pathway across various cardiac disease settings. As lysosomal degradation likely represents the last stage of QC, a deeper understanding of lower scale responses such as the mitochondrial UPR and MDV's will be critical for more personalized approaches. Although much of the mechanistic underpinnings remain elusive, the pharmacological UPR^{mt} activator nicotinamide riboside (NR) has been shown to protect against pressure overload (Smyrnias et al., 2019) and dilated cardiomyopathy (Diguet et al., 2018) in mice, thereby justifying an early phase clinical trial in human heart failure patients (NCT03727646). However, reports leading up to this trial have focused on the reversal of mitochondrial protein hyperacteylation (Lee et al., 2016; Airhart et al., 2017; Walker and Tian, 2018), rather than UPR^{mt} induction. Moving forward, it will be important to elucidate the relationship between these processes as it relates to the efficacy of NR. There is also evidence that excessive autophagy induction (Gao et al., 2018) and Parkin expression (Woodall et al., 2019) are maladaptive in the heart. As such, fine-tuning safe thresholds for bulk degradation, and continued investigation into novel regulators of alternative pathways is warranted.

CONCLUSION

Pathophysiological stress often damages mitochondria in myocytes which are vital for the heart's contractile activity.

Therefore, continuous monitoring and repair of mitochondria are needed to maintain a healthy mitochondrial population in cells. Multiple levels of mitochondrial QC exist both at the protein and organelle level. Here, we have reviewed the intricate pathways that coordinate mitochondrial quality in cells and how they are altered in the diseased heart. First, because the majority of mitochondrial proteins are encoded in the nucleus, significant monitoring of mitochondrial precursor proteins is needed during their cytosolic translation and import. The UPS shapes the mitochondrial proteome through steady-state turnover of mitochondrial precursors to ensure an appropriate stoichiometry between nuclear and mitochondrially encoded proteins and their proper localization (Margineantu et al., 2007; Radke et al., 2008; Azzu and Brand, 2010; Bragoszewski et al., 2013, 2015). Second, mitochondria contain resident chaperones and proteases to ensure QC within the mitochondria (Lau et al., 2012; Bulteau et al., 2017). Third, excessive levels of misfolded proteins in the mitochondrial matrix or a mito-nuclear protein imbalance activates a conserved UPR^{mt} which functions to selectively induce a transcriptional response aimed at restoring mitochondrial proteostasis (Zhao et al., 2002; Shpilka and Haynes, 2018). A closer examination into these processes reveals an inextricable link between mitochondrial QC and cytosolic proteostasis. More recently, mitochondria themselves have been found to participate in general protein QC through the import and degradation of misfolded cytosolic proteins (Ruan et al., 2017; Li et al., 2019). In the event that the mitochondria cannot be repaired, myocytes have the option of either eliminating damaged mitochondrial components via MDVs (McLelland et al., 2014, 2016), or by removing the entire organelle through mitophagy (Gustafsson and Dorn, 2018). Elimination of the entire mitochondria is likely a last resort response because it requires the cell to replace the mitochondrion. Continued investigations into the molecular drivers of mitochondrial quality have the potential to elucidate novel interventions for general the proteostatic stress seen during myocardial ischemia, pressure overload, and protein aggregation cardiomyopathies (Hofmann et al., 2019). Collectively, these mitochondrial QC pathways represent essential adaptive responses in cardiac myocytes, and fruitful avenues for the development of novel therapies against cardiovascular diseases. Once a better understanding of the regulators and relationships between the various QC pathways is gained, we will hopefully be able to translate this knowledge into improved treatments for disease.

AUTHOR CONTRIBUTIONS

Both authors contributed to the content of this article and approved of its submission.

FUNDING

ÅG was supported by grants from NIH (R01HL138560 and R01HL132300), and TRDRP (27IR-0013 and 28IP-0025). JQ was supported by NIH T32HL007444.

REFERENCES

Airhart, S. E., Shireman, L. M., Risler, L. J., Anderson, G. D., Gowda, G. N., Raftery, D., et al. (2017). An open-label, non-randomized study of the pharmacokinetics of the nutritional supplement nicotinamide riboside (NR) and its effects on blood NAD+ levels in healthy volunteers. *PLoS One* 12:e0186459. doi: 10.1371/journal.pone.0186459

Aldridge, J. E., Horibe, T., and Hoogenraad, N. J. (2007). Discovery of genes activated by the mitochondrial unfolded protein response (mtUPR) and cognate promoter elements. *PLoS One* 2:e874. doi: 10.1371/journal.pone.0000874

Amen, O. M., Sarker, S. D., Ghildyal, R., and Arya, A. (2019). Endoplasmic reticulum stress activates UPR signaling and mediates inflammation, obesity and cardiac dysfunction: therapeutic and molecular approach. *Front. Pharmacol.* 10:977. doi: 10.3389/fphar.2019.00977

Andres, A. M., Kooren, J. A., Parker, S. J., Tucker, K. C., Ravindran, N., Ito, B. R., et al. (2016). Discordant signaling and autophagy response to fasting in hearts of obese mice: implications for ischemia tolerance. *Am. J. Physiol. Heart Circ. Physiol.* 311, H219–H228. doi: 10.1152/ajpheart.00041.2016

Arany, Z., He, H., Lin, J., Hoyer, K., Handschin, C., Toka, O., et al. (2005). Transcriptional coactivator PGC-1α controls the energy state and contractile function of cardiac muscle. *Cell Metab.* 1, 259–271. doi: 10.1016/j.cmet.2005.03.002

Ari, C., Kovács, Z., Juhasz, G., Murdun, C., Goldhagen, C. R., Koutnik, A. P., et al. (2017). Exogenous ketone supplements reduce anxiety-related behavior in Sprague-Dawley and Wistar Albino Glaxo/Rijswijk rats. *Front. Mol. Neurosci.* 9:137. doi: 10.3389/fnmol.2016.00137

Aubert, G., Martin, O. J., Horton, J. L., Lai, L., Vega, R. B., Leone, T. C., et al. (2016). The failing heart relies on ketone bodies as a fuel. *Circulation* 133, 698–705. doi: 10.1161/CIRCULATIONAHA.115.017355

Azzu, V., and Brand, M. D. (2010). Degradation of an intramitochondrial protein by the cytosolic proteasome. *J. Cell Sci.* 123(Pt 4), 578–585. doi: 10.1242/jcs.060004

Berendzen, K. M., Durieux, J., Shao, L.-W., Tian, Y., Kim, H.-E., Wolff, S., et al. (2016). Neuroendocrine coordination of mitochondrial stress signaling and proteostasis. *Cell* 166, 1553–1563.e16. doi: 10.1016/j.cell.2016.08.042

Billia, F., Hauck, L., Konecny, F., Rao, V., Shen, J., and Mak, T. W. (2011). PTEN-inducible kinase 1 (PINK1)/Park6 is indispensable for normal heart function. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9572–9577. doi: 10.1073/pnas.1106291108

Blackwood, E. A., Azizi, K., Thuerauf, D. J., Paxman, R. J., Plate, L., Kelly, J. W., et al. (2019). Pharmacologic ATF6 activation confers global protection in widespread disease models by reprograming cellular proteostasis. *Nat. Commun.* 10:187. doi: 10.1038/s41467-018-08129-2

Bota, D. A., and Davies, K. J. (2002). Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nat. Cell Biol.* 4, 674–680. doi: 10.1038/ncb836

Bragoszewski, P., Gornicka, A., Sztolsztener, M. E., and Chacinska, A. (2013). The ubiquitin-proteasome system regulates mitochondrial intermembrane space proteins. *Mol. Cell. Biol.* 33, 2136–2148. doi: 10.1128/MCB.01579-12

Bragoszewski, P., Wasilewski, M., Sakowska, P., Gornicka, A., Böttinger, L., Qiu, J., et al. (2015). Retro-translocation of mitochondrial intermembrane space proteins. *Proc. Natl. Acad. Sci. U.S.A.* 112, 7713–7718. doi: 10.1073/pnas.1504615112

Braschi, E., Goyon, V., Zunino, R., Mohanty, A., Xu, L., and Mcbride, H. M. (2010). Vps35 mediates vesicle transport between the mitochondria and peroxisomes. *Curr. Biol.* 20, 1310–1315. doi: 10.1016/j.cub.2010.05.066

Bulteau, A.-L., Mena, N. P., Auchère, F., Lee, I., Prigent, A., Lobsiger, C. S., et al. (2017). Dysfunction of mitochondrial Lon protease and identification of oxidized protein in mouse brain following exposure to MPTP: implications for Parkinson disease. *Free Radic. Biol. Med.* 108, 236–246. doi: 10.1016/j.freeradbiomed.2017.03.036

Burman, J. L., Pickles, S., Wang, C., Sekine, S., Vargas, J. N. S., Zhang, Z., et al. (2017). Mitochondrial fission facilitates the selective mitophagy of protein aggregates. *J. Cell Biol.* 216, 3231–3247. doi: 10.1083/jcb.201612106

Cadete, V. J., Deschênes, S., Cuillerier, A., Brisebois, F., Sugiura, A., Vincent, A., et al. (2016). Formation of mitochondrial−derived vesicles is an active and physiologically relevant mitochondrial quality control process in the cardiac system. *J. Physiol.* 594, 5343–5362. doi: 10.1113/JP272703

Cantó, C., Gerhart-Hines, Z., Feige, J. N., Lagouge, M., Noriega, L., Milne, J. C., et al. (2009). AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* 458, 1056–1060. doi: 10.1038/nature07813

Cenini, G., Rüb, C., Bruderek, M., and Voos, W. (2016). Amyloid β-peptides interfere with mitochondrial preprotein import competence by a coaggregation process. *Mol. Biol. Cell* 27, 3257–3272. doi: 10.1091/mbc.E16-05-0313

Chen, G., Han, Z., Feng, D., Chen, Y., Chen, L., Wu, H., et al. (2014). A regulatory signaling loop comprising the PGAM5 phosphatase and CK2 controls receptor-mediated mitophagy. *Mol. Cell* 54, 362–377. doi: 10.1016/j.molcel.2014.02.034

Chen, K., Kobayashi, S., Xu, X., Viollet, B., and Liang, Q. (2013). AMP activated protein kinase is indispensable for myocardial adaptation to caloric restriction in mice. *PLoS One* 8:e59682. doi: 10.1371/journal.pone.0059682

Chen, Y., Lewis, W., Diwan, A., Cheng, E. H.-Y., Matkovich, S. J., and Dorn, G. W. (2010). Dual autonomous mitochondrial cell death pathways are activated by Nix/BNip3L and induce cardiomyopathy. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9035–9042. doi: 10.1073/pnas.0914013107

Chen, Z., Siraj, S., Liu, L., and Chen, Q. (2017). MARCH5-FUNDC1 axis fine-tunes hypoxia-induced mitophagy. *Autophagy* 13, 1244–1245. doi: 10.1080/15548627.2017.1310789

Chiao, Y. A., Kolwicz, S. C., Basisty, N., Gagnidze, A., Zhang, J., Gu, H., et al. (2016). Rapamycin transiently induces mitochondrial remodeling to reprogram energy metabolism in old hearts. *Aging (Albany NY)* 8, 314. doi: 10.18632/aging.100881

Colman, R. J., Anderson, R. M., Johnson, S. C., Kastman, E. K., Kosmatka, K. J., Beasley, T. M., et al. (2009). Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science* 325, 201–204. doi: 10.1126/science.1173635

Dai, D. F., Karunadharma, P. P., Chiao, Y. A., Basisty, N., Crispin, D., Hsieh, E. J., et al. (2014). Altered proteome turnover and remodeling by short−term caloric restriction or rapamycin rejuvenate the aging heart. *Aging Cell* 13, 529–539. doi: 10.1111/acel.12203

Devi, T. D., Babu, M., Mäkinen, P., Kaikkonen, M. U., Heinaniemi, M., Laakso, H., et al. (2017). Aggravated postinfarct heart failure in type 2 diabetes is associated with impaired mitophagy and exaggerated inflammasome activation. *Am. J. Pathol.* 187, 2659–2673. doi: 10.1016/j.ajpath.2017.08.023

Dhingra, R., Margulets, V., Chowdhury, S. R., Thliveris, J., Jassal, D., Fernyhough, P., et al. (2014). Bnip3 mediates doxorubicin-induced cardiac myocyte necrosis and mortality through changes in mitochondrial signaling. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5537–E5544. doi: 10.1073/pnas.1414665111

Diguet, N., Trammell, S. A., Tannous, C., Deloux, R., Piquereau, J., Mougenot, N., et al. (2018). Nicotinamide riboside preserves cardiac function in a mouse model of dilated cardiomyopathy. *Circulation* 137, 2256–2273. doi: 10.1161/CIR.0000000000000581

Diwan, A., Krenz, M., Syed, F. M., Wansapura, J., Ren, X., Koesters, A. G., et al. (2007). Inhibition of ischemic cardiomyocyte apoptosis through targeted ablation of Bnip3 restrains postinfarction remodeling in mice. *J. Clin. Invest.* 117, 2825–2833. doi: 10.1172/JCI32490

Dorn, G. W. (2010). Mitochondrial pruning by Nix and BNip3: an essential function for cardiac-expressed death factors. *J. Cardiovasc. Transl. Res.* 3, 374–383. doi: 10.1007/s12265-010-9174-x

Eisenberg, T., Abdellatif, M., Schroeder, S., Primessnig, U., Stekovic, S., Pendl, T., et al. (2016). Cardioprotection and lifespan extension by the natural polyamine spermidine. *Nat. Med.* 22, 1428–1438. doi: 10.1038/nm.4222

Erlich, A. T., Brownlee, D. M., Beyfuss, K., and Hood, D. A. (2018). Exercise induces TFEB expression and activity in skeletal muscle in a PGC-1α-dependent manner. *Am. J. Physiol. Cell Physiol.* 314, C62–C72. doi: 10.1152/ajpcell.00162.2017

Escobar, K. A., Cole, N. H., Mermier, C. M., and Vandusseldorp, T. A. (2019). Autophagy and aging: maintaining the proteome through exercise and caloric restriction. *Aging Cell* 18:e12876. doi: 10.1111/acel.12876

Essandoh, K., Wang, X., Huang, W., Deng, S., Gardner, G., Mu, X., et al. (2019). Tumor susceptibility gene 101 ameliorates endotoxin-induced cardiac dysfunction by enhancing Parkin-mediated mitophagy. *J. Biol. Chem.* 294, 18057–18068. doi: 10.1074/jbc.RA119.008925

Esteban-Martínez, L., and Boya, P. (2018). BNIP3L/NIX-dependent mitophagy regulates cell differentiation via metabolic reprogramming. *Autophagy* 14, 915–917. doi: 10.1080/15548627.2017.1332567

Fakruddin, M., Wei, F.-Y., Suzuki, T., Asano, K., Kaieda, T., Omori, A., et al. (2018). Defective mitochondrial tRNA taurine modification activates global proteostress and leads to mitochondrial disease. *Cell Rep.* 22, 482–496. doi: 10.1016/j.celrep.2017.12.051

Fan, F., Duan, Y., Yang, F., Trexler, C., Wang, H., Huang, L., et al. (2019). Deletion of heat shock protein 60 in adult mouse cardiomyocytes perturbs mitochondrial protein homeostasis and causes heart failure. *Cell Death Differ.* 27, 587–600. doi: 10.1038/s41418-019-0374-x

Fang, L., Hemion, C., Pinho Ferreira Bento, A. C., Bippes, C. C., Flammer, J., and Neutzner, A. (2015). Mitochondrial function in neuronal cells depends on p97/VCP/Cdc48-mediated quality control. *Front. Cell. Neurosci.* 9:16. doi: 10.3389/fncel.2015.00016

Finckenberg, P., Eriksson, O., Baumann, M., Merasto, S., Lalowski, M. M., Levijoki, J., et al. (2012). Caloric restriction ameliorates angiotensin II–induced mitochondrial remodeling and cardiac hypertrophy. *Hypertension* 59, 76–84. doi: 10.1161/HYPERTENSIONAHA.111.179457

Fiorese, C. J., Schulz, A. M., Lin, Y.-F., Rosin, N., Pellegrino, M. W., and Haynes, C. M. (2016). The transcription factor ATF5 mediates a mammalian mitochondrial UPR. *Curr. Biol.* 26, 2037–2043. doi: 10.1016/j.cub.2016.06.002

Flynn, J. M., O'leary, M. N., Zambataro, C. A., Academia, E. C., Presley, M. P., Garrett, B. J., et al. (2013). Late−life rapamycin treatment reverses age−related heart dysfunction. *Aging Cell* 12, 851–862. doi: 10.1111/acel.12109

Franco, A., Kitsis, R. N., Fleischer, J. A., Gavathiotis, E., Kornfeld, O. S., Gong, G., et al. (2016). Correcting mitochondrial fusion by manipulating mitofusin conformations. *Nature* 540, 74–79. doi: 10.1038/nature20156

Frudd, K., Burgoyne, T., and Burgoyne, J. R. (2018). Oxidation of Atg3 and Atg7 mediates inhibition of autophagy. *Nat. Commun.* 9:95. doi: 10.1038/s41467-017-02352-z

Gao, W., Zhou, Z., Liang, B., Huang, Y., Yang, Z., Chen, Y., et al. (2018). Inhibiting receptor of advanced glycation end products attenuates pressure overload-induced cardiac dysfunction by preventing excessive autophagy. *Front. Physiol.* 9:1333. doi: 10.3389/fphys.2018.01333

Gatliff, J., East, D., Crosby, J., Abeti, R., Harvey, R., Craigen, W., et al. (2014). TSPO interacts with VDAC1 and triggers a ROS-mediated inhibition of mitochondrial quality control. *Autophagy* 10, 2279–2296. doi: 10.4161/15548627.2014.991665

Gill, S., Le, H. D., Melkani, G. C., and Panda, S. (2015). Time-restricted feeding attenuates age-related cardiac decline in *Drosophila*. *Science* 347, 1265–1269. doi: 10.1126/science.1256682

Gitschlag, B. L., Kirby, C. S., Samuels, D. C., Gangula, R. D., Mallal, S. A., and Patel, M. R. (2016). Homeostatic responses regulate selfish mitochondrial genome dynamics in C. elegans. *Cell Metab.* 24, 91–103. doi: 10.1016/j.cmet.2016.06.008

Greene, A. W., Grenier, K., Aguileta, M. A., Muise, S., Farazifard, R., Haque, M. E., et al. (2012). Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. *EMBO Rep.* 13, 378–385. doi: 10.1038/embor.2012.14

Gustafsson, Å. B., and Dorn, G. W. (2018). Evolving and expanding the roles of mitophagy as a homeostatic and pathogenic process. *Physiol. Rev.* 99, 853–892. doi: 10.1152/physrev.00005.2018

Hammerling, B. C., Najor, R. H., Cortez, M. Q., Shires, S. E., Leon, L. J., Gonzalez, E. R., et al. (2017a). A Rab5 endosomal pathway mediates Parkin-dependent mitochondrial clearance. *Nat. Commun.* 8:14050. doi: 10.1038/ncomms14050

Hammerling, B. C., Shires, S. E., Leon, L. J., Cortez, M. Q., and Gustafsson, A. B. (2017b). Isolation of Rab5-positive endosomes reveals a new mitochondrial degradation pathway utilized by BNIP3 and Parkin. *Small GTPases* 11, 69–76. doi: 10.1080/21541248.2017.1342749

Hatori, M., Vollmers, C., Zarrinpar, A., Ditacchio, L., Bushong, E. A., Gill, S., et al. (2012). Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. *Cell Metab.* 15, 848–860. doi: 10.1016/j.cmet.2012.04.019

Hemion, C., Flammer, J., and Neutzner, A. (2014). Quality control of oxidatively damaged mitochondrial proteins is mediated by p97 and the proteasome. *Free Radic. Biol. Med.* 75, 121–128. doi: 10.1016/j.freeradbiomed.2014.07.016

Hirota, Y., Yamashita, S., Kurihara, Y., Jin, X., Aihara, M., Saigusa, T., et al. (2015). Mitophagy is primarily due to alternative autophagy and requires the MAPK1 and MAPK14 signaling pathways. *Autophagy* 11, 332–343. doi: 10.1080/15548627.2015.1023047

Hofmann, C., Katus, H. A., and Doroudgar, S. (2019). Protein misfolding in cardiac disease. *Circulation* 139, 2085–2088.

Horton, J. L., Davidson, M. T., Kurishima, C., Vega, R. B., Powers, J. C., Matsuura, T. R., et al. (2019). The failing heart utilizes 3-hydroxybutyrate as a metabolic stress defense. *JCI Insight* 4:e124079. doi: 10.1172/jci.insight.124079

Hoshino, A., Okawa, Y., Ariyoshi, M., Kaimoto, S., Uchihashi, M., Fukai, K., et al. (2014). Oxidative post-translational modifications develop LONP1 dysfunction in pressure overload heart failure. *Circulation Heart Fail.* 7, 500–509. doi: 10.1161/CIRCHEARTFAILURE.113.001062

Hu, C., Tian, Y., Xu, H., Pan, B., Terpstra, E. M., Wu, P., et al. (2018). Inadequate ubiquitination-proteasome coupling contributes to myocardial ischemia-reperfusion injury. *J. Clin. Invest.* 128, 5294–5306. doi: 10.1172/JCI98287

Hwang, S., Disatnik, M. H., and Mochly-Rosen, D. (2015). Impaired GAPDH-induced mitophagy contributes to the pathology of Huntington's disease. *EMBO Mol. Med.* 7, 1307–1326. doi: 10.15252/emmm.201505256

Itakura, E., Zavodszky, E., Shao, S., Wohlever, M. L., Keenan, R. J., and Hegde, R. S. (2016). Ubiquilins chaperone and triage mitochondrial membrane proteins for degradation. *Mol. Cell* 63, 21–33. doi: 10.1016/j.molcel.2016.05.020

Jäger, S., Handschin, C., Pierre, J. S., and Spiegelman, B. M. (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α. *Proc. Natl. Acad. Sci. U.S.A.* 104, 12017–12022. doi: 10.1073/pnas.0705070104

Jain, A., Lamark, T., Sjøttem, E., Larsen, K. B., Awuh, J. A., Øvervatn, A., et al. (2010). p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *J. Biol. Chem.* 285, 22576–22591. doi: 10.1074/jbc.M110.118976

Jamshed, H., Beyl, R. A., Della Manna, D. L., Yang, E. S., Ravussin, E., and Peterson, C. M. (2019). Early time-restricted feeding improves 24-hour glucose levels and affects markers of the circadian clock, aging, and autophagy in humans. *Nutrients* 11:1234. doi: 10.3390/nu11061234

Jin, J.-K., Blackwood, E. A., Azizi, K., Thuerauf, D. J., Fahem, A. G., Hofmann, C., et al. (2017). ATF6 decreases myocardial ischemia/reperfusion damage and links ER stress and oxidative stress signaling pathways in the heart. *Circ. Res.* 120, 862–875. doi: 10.1161/CIRCRESAHA.116.310266

Jin, S. M., Lazarou, M., Wang, C., Kane, L. A., Narendra, D. P., and Youle, R. J. (2010). Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. *J. Cell Biol.* 191, 933–942. doi: 10.1083/jcb.201008084

Jin, S. M., and Youle, R. J. (2013). The accumulation of misfolded proteins in the mitochondrial matrix is sensed by PINK1 to induce PARK2/Parkin-mediated mitophagy of polarized mitochondria. *Autophagy* 9, 1750–1757. doi: 10.4161/auto.26122

Karbowski, M., and Youle, R. J. (2011). Regulating mitochondrial outer membrane proteins by ubiquitination and proteasomal degradation. *Curr. Opin. Cell Biol.* 23, 476–482. doi: 10.1016/j.ceb.2011.05.007

Kashiwaya, Y., Bergman, C., Lee, J.-H., Wan, R., King, M. T., Mughal, M. R., et al. (2013). A ketone ester diet exhibits anxiolytic and cognition-sparing properties, and lessens amyloid and tau pathologies in a mouse model of Alzheimer's disease. *Neurobiol. Aging* 34, 1530–1539. doi: 10.1016/j.neurobiolaging.2012.11.023

Kephart, W. C., Mumford, P. W., Mao, X., Romero, M. A., Hyatt, H. W., Zhang, Y., et al. (2017). The 1-week and 8-month effects of a ketogenic diet or ketone salt supplementation on multi-organ markers of oxidative stress and mitochondrial function in rats. *Nutrients* 9:1019. doi: 10.3390/nu9091019

Kim, H.-E., Grant, A. R., Simic, M. S., Kohnz, R. A., Nomura, D. K., Durieux, J., et al. (2016). Lipid biosynthesis coordinates a mitochondrial-to-cytosolic stress response. *Cell* 166, 1539–1552.e16. doi: 10.1016/j.cell.2016.08.027

Kovács, Z., D'agostino, D. P., Diamond, D. M., and Ari, C. (2019). Exogenous ketone supplementation decreased the lipopolysaccharide-induced increase in absence epileptic activity in Wistar Albino Glaxo Rijswijk rats. *Front. Mol. Neurosci.* 12:45. doi: 10.3389/fnmol.2019.00045

Kubli, D. A., Zhang, X., Lee, Y., Hanna, R. A., Quinsay, M. N., Nguyen, C. K., et al. (2013). Parkin protein deficiency exacerbates cardiac injury and reduces survival following myocardial infarction. *J. Biol. Chem.* 288, 915–926. doi: 10.1074/jbc.M112.411363

Labbadia, J., Brielmann, R. M., Neto, M. F., Lin, Y.-F., Haynes, C. M., and Morimoto, R. I. (2017). Mitochondrial stress restores the heat shock response and prevents proteostasis collapse during aging. *Cell Rep.* 21, 1481–1494. doi: 10.1016/j.celrep.2017.10.038

Lau, E., Wang, D., Zhang, J., Yu, H., Lam, M. P., Liang, X., et al. (2012). Substrate- and isoform-specific proteome stability in normal and stressed cardiac mitochondria. *Circ. Res.* 110, 1174–1178. doi: 10.1161/CIRCRESAHA.112.268359

Lavie, J., De Belvalet, H., Sonon, S., Ion, A. M., Dumon, E., Melser, S., et al. (2018). Ubiquitin-dependent degradation of mitochondrial proteins regulates energy metabolism. *Cell Rep.* 23, 2852–2863. doi: 10.1016/j.celrep.2018.05.013

Lee, C. F., Chavez, J. D., Garcia-Menendez, L., Choi, Y., Roe, N. D., Chiao, Y. A., et al. (2016). Normalization of NAD+ redox balance as a therapy for heart failure. *Circulation* 134, 883–894. doi: 10.1161/CIRCULATIONAHA.116.022495

Lehman, J. J., Barger, P. M., Kovacs, A., Saffitz, J. E., Medeiros, D. M., and Kelly, D. P. (2000). Peroxisome proliferator–activated receptor γ coactivator-1 promotes cardiac mitochondrial biogenesis. *J. Clin. Invest.* 106, 847–856. doi: 10.1172/JCI10268

Li, J., Ichikawa, T., Villacorta, L., Janicki, J. S., Brower, G. L., Yamamoto, M., et al. (2009). Nrf2 protects against maladaptive cardiac responses to hemodynamic stress. *Arterioscler. Thromb. Vasc. Biol.* 29, 1843–1850. doi: 10.1161/ATVBAHA.109.189480

Li, Y., Xue, Y., Xu, X., Wang, G., Liu, Y., Wu, H., et al. (2019). A mitochondrial FUNDC1/HSC70 interaction organizes the proteostatic stress response at the risk of cell morbidity. *EMBO J.* 38:e98786. doi: 10.15252/embj.201798786

Lin, Y.-F., Schulz, A. M., Pellegrino, M. W., Lu, Y., Shaham, S., and Haynes, C. M. (2016). Maintenance and propagation of a deleterious mitochondrial genome by the mitochondrial unfolded protein response. *Nature* 533, 416–419. doi: 10.1038/nature17989

Ma, X., Mani, K., Liu, H., Kovacs, A., Murphy, J. T., Foroughi, L., et al. (2019). Transcription Factor EB activation rescues advanced αB−crystallin mutation−induced cardiomyopathy by normalizing desmin localization. *J. Am. Heart Assoc.* 8:e010866. doi: 10.1161/JAHA.118.010866

Malik, A., and Kanneganti, T.-D. (2017). Inflammasome activation and assembly at a glance. *J. Cell Sci.* 130, 3955–3963. doi: 10.1242/jcs.207365

Margineantu, D. H., Emerson, C. B., Diaz, D., and Hockenbery, D. M. (2007). Hsp90 inhibition decreases mitochondrial protein turnover. *PLoS One* 2:e1066. doi: 10.1371/journal.pone.0001066

Matheoud, D., Sugiura, A., Bellemare-Pelletier, A., Laplante, A., Rondeau, C., Chemali, M., et al. (2016). Parkinson's disease-related proteins PINK1 and Parkin repress mitochondrial antigen presentation. *Cell* 166, 314–327. doi: 10.1016/j.cell.2016.05.039

Matsuda, N., Sato, S., Shiba, K., Okatsu, K., Saisho, K., Gautier, C. A., et al. (2010). PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. *J. Cell Biol.* 189, 211–221. doi: 10.1083/jcb.200910140

McLelland, G.-L., Goiran, T., Yi, W., Dorval, G., Chen, C. X., Lauinger, N. D., et al. (2018). Mfn2 ubiquitination by PINK1/parkin gates the p97-dependent release of ER from mitochondria to drive mitophagy. *eLife* 7:e32866. doi: 10.7554/eLife.32866

McLelland, G.-L., Lee, S. A., Mcbride, H. M., and Fon, E. A. (2016). Syntaxin-17 delivers PINK1/parkin-dependent mitochondrial vesicles to the endolysosomal system. *J. Cell Biol.* 214, 275–291. doi: 10.1083/jcb.201603105

McLelland, G. L., Soubannier, V., Chen, C. X., Mcbride, H. M., and Fon, E. A. (2014). Parkin and PINK1 function in a vesicular trafficking pathway regulating mitochondrial quality control. *EMBO J.* 33, 282–295. doi: 10.1002/embj.201385902

Meng, F., Yao, D., Shi, Y., Kabakoff, J., Wu, W., Reicher, J., et al. (2011). Oxidation of the cysteine-rich regions of parkin perturbs its E3 ligase activity and contributes to protein aggregation. *Mol. Neurodegener.* 6:34. doi: 10.1186/1750-1326-6-34

Merry, T. L., and Ristow, M. (2016). Nuclear factor erythroid−derived 2−like 2 (NFE2L2, Nrf2) mediates exercise−induced mitochondrial biogenesis and the anti−oxidant response in mice. *J. Physiol.* 594, 5195–5207. doi: 10.1113/JP271957

Münch, C., and Harper, J. W. (2016). Mitochondrial unfolded protein response controls matrix pre-RNA processing and translation. *Nature* 534, 710–713. doi: 10.1038/nature18302

Narendra, D. P., Jin, S. M., Tanaka, A., Suen, D. F., Gautier, C. A., Shen, J., et al. (2010). PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol.* 8:e1000298. doi: 10.1371/journal.pbio.1000298

Nargund, A. M., Fiorese, C. J., Pellegrino, M. W., Deng, P., and Haynes, C. M. (2015). Mitochondrial and nuclear accumulation of the transcription factor ATFS-1 promotes OXPHOS recovery during the UPRmt. *Mol. Cell* 58, 123–133. doi: 10.1016/j.molcel.2015.02.008

Nargund, A. M., Pellegrino, M. W., Fiorese, C. J., Baker, B. M., and Haynes, C. M. (2012). Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation. *Science* 337, 587–590. doi: 10.1126/science.1223560

Nishida, Y., Arakawa, S., Fujitani, K., Yamaguchi, H., Mizuta, T., Kanaseki, T., et al. (2009). Discovery of Atg5/Atg7-independent alternative macroautophagy. *Nature* 461, 654–658. doi: 10.1038/nature08455

Palikaras, K., Lionaki, E., and Tavernarakis, N. (2015). Coordination of mitophagy and mitochondrial biogenesis during ageing in C. elegans. *Nature* 521, 525–528. doi: 10.1038/nature14300

Pan, B., Zhang, H., Cui, T., and Wang, X. (2017). TFEB activation protects against cardiac proteotoxicity via increasing autophagic flux. *J. Mol. Cell. Cardiol.* 113, 51–62. doi: 10.1016/j.yjmcc.2017.10.003

Petersen, C. A. H., Alikhani, N., Behbahani, H., Wiehager, B., Pavlov, P. F., Alafuzoff, I., et al. (2008). The amyloid β-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13145–13150. doi: 10.1073/pnas.0806192105

Pickles, S., Vigié, P., and Youle, R. J. (2018). Mitophagy and quality control mechanisms in mitochondrial maintenance. *Curr. Biol.* 28, R170–R185. doi: 10.1016/j.cub.2018.01.004

Pifferi, F., Terrien, J., Marchal, J., Dal-Pan, A., Djelti, F., Hardy, I., et al. (2018). Caloric restriction increases lifespan but affects brain integrity in grey mouse lemur primates. *Commun. Biol.* 1, 1–8. doi: 10.1038/s42003-018-0024-8

Piquereau, J., Godin, R., Deschênes, S., Bessi, V. L., Mofarrahi, M., Hussain, S. N., et al. (2013). Protective role of PARK2/Parkin in sepsis-induced cardiac contractile and mitochondrial dysfunction. *Autophagy* 9, 1837–1851. doi: 10.4161/auto.26502

Predmore, J. M., Wang, P., Davis, F., Bartolone, S., Westfall, M. V., Dyke, D. B., et al. (2010). Ubiquitin proteasome dysfunction in human hypertrophic and dilated cardiomyopathies. *Circulation* 121, 997–1004. doi: 10.1161/CIRCULATIONAHA.109.904557

Quirós, P. M., Prado, M. A., Zamboni, N., D'amico, D., Williams, R. W., Finley, D., et al. (2017). Multi-omics analysis identifies ATF4 as a key regulator of the mitochondrial stress response in mammals. *J. Cell Biol.* 216, 2027–2045. doi: 10.1083/jcb.201702058

Radke, S., Chander, H., Schäfer, P., Meiss, G., Krüger, R., Schulz, J. B., et al. (2008). Mitochondrial protein quality control by the proteasome involves ubiquitination and the protease Omi. *J. Biol. Chem.* 283, 12681–12685. doi: 10.1074/jbc.C800036200

Rajasekaran, N. S., Varadharaj, S., Khanderao, G. D., Davidson, C. J., Kannan, S., Firpo, M. A., et al. (2011). Sustained activation of nuclear erythroid 2-related factor 2/antioxidant response element signaling promotes reductive stress in the human mutant protein aggregation cardiomyopathy in mice. *Antioxid. Redox Signal.* 14, 957–971. doi: 10.1089/ars.2010.3587

Ranek, M. J., Zheng, H., Huang, W., Kumarapeli, A. R., Li, J., Liu, J., et al. (2015). Genetically induced moderate inhibition of 20S proteasomes in cardiomyocytes facilitates heart failure in mice during systolic overload. *J. Mol. Cell. Cardiol.* 85, 273–281. doi: 10.1016/j.yjmcc.2015.06.014

Rawat, S., Anusha, V., Jha, M., Sreedurgalakshmi, K., and Raychaudhuri, S. (2019). Aggregation of respiratory complex subunits marks the onset of proteotoxicity in proteasome inhibited cells. *J. Mol. Biol.* 431, 996–1015. doi: 10.1016/j.jmb.2019.01.022

Regula, K. M., Ens, K., and Kirshenbaum, L. A. (2002). Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. *Circ. Res.* 91, 226–231. doi: 10.1161/01.res.0000029232.42227.16

Ruan, L., Zhou, C., Jin, E., Kucharavy, A., Zhang, Y., Wen, Z., et al. (2017). Cytosolic proteostasis through importing of misfolded proteins into mitochondria. *Nature* 543, 443–446. doi: 10.1038/nature21695

Ryu, D., Mouchiroud, L., Andreux, P. A., Katsyuba, E., Moullan, N., Nicolet-Dit-Félix, A. A., et al. (2016). Urolithin A induces mitophagy and prolongs lifespan in C. elegans and increases muscle function in rodents. *Nat. Med.* 22, 879–888. doi: 10.1038/nm.4132

Saito, T., Nah, J., Oka, S. I., Mukai, R., Monden, Y., Maejima, Y., et al. (2019). An alternative mitophagy pathway mediated by Rab9 protects the heart against ischemia. *J. Clin. Invest.* 129, 802–819. doi: 10.1172/JCI122035

Sandoval, H., Thiagarajan, P., Dasgupta, S. K., Schumacher, A., Prchal, J. T., Chen, M., et al. (2008). Essential role for Nix in autophagic maturation of erythroid cells. *Nature* 454, 232–235. doi: 10.1038/nature07006

Sarraf, S. A., Raman, M., Guarani-Pereira, V., Sowa, M. E., Huttlin, E. L., Gygi, S. P., et al. (2013). Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. *Nature* 496, 372–376. doi: 10.1038/nature12043

Sepuri, N. B., Angireddy, R., Srinivasan, S., Guha, M., Spear, J., Lu, B., et al. (2017). Mitochondrial LON protease-dependent degradation of cytochrome c oxidase subunits under hypoxia and myocardial ischemia. *Biochim. Biophys. Acta Bioenergetics* 1858, 519–528. doi: 10.1016/j.bbabio.2017.04.003

Shanmugam, G., Wang, D., Gounder, S. S., Fernandes, J., Litovsky, S. H., Whitehead, K., et al. (2020). Reductive stress causes pathological cardiac remodeling and diastolic dysfunction. *Antioxid. Redox Signal.* [Epub ahead of print]. doi: 10.1089/ars.2019.7808

Shao, L.-W., Niu, R., and Liu, Y. (2016). Neuropeptide signals cell non-autonomous mitochondrial unfolded protein response. *Cell Res.* 26, 1182–1196. doi: 10.1038/cr.2016.118

Shcherbakov, D., Teo, Y., Boukari, H., Cortes-Sanchon, A., Mantovani, M., Osinnii, I., et al. (2019). Ribosomal mistranslation leads to silencing of the unfolded protein response and increased mitochondrial biogenesis. *Commun. Biol.* 2, 1–16. doi: 10.1038/s42003-019-0626-9

Shin, J.-H., Ko, H. S., Kang, H., Lee, Y., Lee, Y.-I., Pletinkova, O., et al. (2011). PARIS (ZNF746) repression of PGC-1α contributes to neurodegeneration in Parkinson's disease. *Cell* 144, 689–702. doi: 10.1016/j.cell.2011.02.010

Shirakabe, A., Zhai, P., Ikeda, Y., Saito, T., Maejima, Y., Hsu, C.-P., et al. (2016). Drp1-dependent mitochondrial autophagy plays a protective role against pressure overload–induced mitochondrial dysfunction and heart failure. *Circulation* 133, 1249–1263. doi: 10.1161/CIRCULATIONAHA.116.023667

Shpilka, T., and Haynes, C. M. (2018). The mitochondrial UPR: mechanisms, physiological functions and implications in ageing. *Nat. Rev. Mol. Cell Biol.* 19, 109–120. doi: 10.1038/nrm.2017.110

Siddall, H. K., Yellon, D. M., Ong, S. B., Mukherjee, U. A., Burke, N., Hall, A. R., et al. (2013). Loss of PINK1 increases the heart's vulnerability to ischemia-reperfusion injury. *PLoS One* 8:e62400. doi: 10.1371/journal.pone.0062400

Smyrnias, I., Gray, S. P., Okonko, D. O., Sawyer, G., Zoccarato, A., Catibog, N., et al. (2019). Cardioprotective effect of the mitochondrial unfolded protein response during chronic pressure overload. *J. Am. Coll. Cardiol.* 73, 1795–1806. doi: 10.1016/j.jacc.2018.12.087

Sorrentino, V., Romani, M., Mouchiroud, L., Beck, J. S., Zhang, H., D'amico, D., et al. (2017). Enhancing mitochondrial proteostasis reduces amyloid-β proteotoxicity. *Nature* 552, 187–193. doi: 10.1038/nature25143

Soubannier, V., Mclelland, G.-L., Zunino, R., Braschi, E., Rippstein, P., Fon, E. A., et al. (2012a). A vesicular transport pathway shuttles cargo from mitochondria to lysosomes. *Curr. Biol.* 22, 135–141. doi: 10.1016/j.cub.2011.11.057

Soubannier, V., Rippstein, P., Kaufman, B. A., Shoubridge, E. A., and Mcbride, H. M. (2012b). Reconstitution of mitochondria derived vesicle formation demonstrates selective enrichment of oxidized cargo. *PLoS One* 7:e52830. doi: 10.1371/journal.pone.0052830

Stekovic, S., Hofer, S. J., Tripolt, N., Aon, M. A., Royer, P., Pein, L., et al. (2019). Alternate day fasting improves physiological and molecular markers of aging in healthy, non-obese humans. *Cell Metab.* 30, 462–476.e6. doi: 10.1016/j.cmet.2019.07.016

Stevens, D. A., Lee, Y., Kang, H. C., Lee, B. D., Lee, Y.-I., Bower, A., et al. (2015). Parkin loss leads to PARIS-dependent declines in mitochondrial mass and respiration. *Proc. Natl. Acad. Sci. U.S.A.* 112, 11696–11701. doi: 10.1073/pnas.1500624112

Sun, Y., Vashisht, A. A., Tchieu, J., Wohlschlegel, J. A., and Dreier, L. (2012). Voltage-dependent anion channels (VDACs) recruit Parkin to defective mitochondria to promote mitochondrial autophagy. *J. Biol. Chem.* 287, 40652–40660. doi: 10.1074/jbc.M112.419721

Sun, Y., Yao, X., Zhang, Q.-J., Zhu, M., Liu, Z.-P., Ci, B., et al. (2018). Beclin-1-dependent autophagy protects the heart during sepsis. *Circulation* 138, 2247–2262. doi: 10.1161/CIRCULATIONAHA.117.032821

Szczepanowska, K., Maiti, P., Kukat, A., Hofsetz, E., Nolte, H., Senft, K., et al. (2016). CLPP coordinates mitoribosomal assembly through the regulation of ERAL1 levels. *EMBO J.* 35, 2566–2583. doi: 10.15252/embj.201694253

Tanase, M., Urbanska, A. M., Zolla, V., Clement, C. C., Huang, L., Morozova, K., et al. (2016). Role of carbonyl modifications on aging-associated protein aggregation. *Sci. Rep.* 6:19311. doi: 10.1038/srep19311

Tang, L., Mo, Y., Li, Y., Zhong, Y., He, S., Zhang, Y., et al. (2017). Urolithin A alleviates myocardial ischemia/reperfusion injury via PI3K/Akt pathway. *Biochem. Biophys. Res. Commun.* 486, 774–780. doi: 10.1016/j.bbrc.2017.03.119

Thai, P. N., Daugherty, D. J., Frederich, B. J., Lu, X., Deng, W., Bers, D. M., et al. (2018). Cardiac-specific conditional knockout of the 18-kDa mitochondrial translocator protein protects from pressure overload induced heart failure. *Sci. Rep.* 8:16213. doi: 10.1038/s41598-018-34451-2

Thai, P. N., Seidlmayer, L. K., Miller, C., Ferrero, M., Dorn, G. W. II, Schaefer, S., et al. (2019). Mitochondrial quality control in aging and heart failure: influence of ketone bodies and mitofusin-stabilizing peptides. *Front. Physiol.* 10:382. doi: 10.3389/fphys.2019.00382

Tong, M., Saito, T., Zhai, P., Oka, S.-I., Mizushima, W., Nakamura, M., et al. (2019). Mitophagy is essential for maintaining cardiac function during high fat diet-induced diabetic cardiomyopathy. *Circ. Res.* 124, 1360–1371. doi: 10.1161/CIRCRESAHA.118.314607

Vainshtein, A., Desjardins, E. M., Armani, A., Sandri, M., and Hood, D. A. (2015). PGC-1α modulates denervation-induced mitophagy in skeletal muscle. *Skelet. Muscle* 5:9. doi: 10.1186/s13395-015-0033-y

Venkatesh, S., Li, M., Saito, T., Tong, M., Rashed, E., Mareedu, S., et al. (2019). Mitochondrial LonP1 protects cardiomyocytes from ischemia/reperfusion injury in vivo. *J. Mol. Cell. Cardiol.* 128, 38–50. doi: 10.1016/j.yjmcc.2018.12.017

Vives-Bauza, C., Zhou, C., Huang, Y., Cui, M., De Vries, R. L., Kim, J., et al. (2010). PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. *Proc. Natl. Acad. Sci. U.S.A.* 107, 378–383. doi: 10.1073/pnas.0911187107

Walker, M. A., and Tian, R. (2018). Raising NAD in heart failure: time to translate? *Circulation* 137, 2274–2277. doi: 10.1161/CIRCULATIONAHA.117.032626

Wang, X., and Chen, X. J. (2015). A cytosolic network suppressing mitochondria-mediated proteostatic stress and cell death. *Nature* 524, 481–484. doi: 10.1038/nature14859

Wang, Y. T., Lim, Y., Mccall, M. N., Huang, K.-T., Haynes, C. M., Nehrke, K., et al. (2019). Cardioprotection by the mitochondrial unfolded protein response requires ATF5. *Am. J. Physiol. Heart Circ. Physiol.* 317, H472–H478. doi: 10.1152/ajpheart.00244.2019

Weinhäupl, K., Lindau, C., Hessel, A., Wang, Y., Schütze, C., Jores, T., et al. (2018). Structural basis of membrane protein chaperoning through the mitochondrial intermembrane space. *Cell* 175, 1365–1379.e25. doi: 10.1016/j.cell.2018.10.039

Woodall, B. P., Orogo, A. M., Najor, R. H., Cortez, M. Q., Moreno, E. R., Wang, H., et al. (2019). Parkin does not prevent accelerated cardiac aging in mitochondrial DNA mutator mice. *JCI Insight* 5:e127713. doi: 10.1172/jci.insight.127713

Wrobel, L., Topf, U., Bragoszewski, P., Wiese, S., Sztolsztener, M. E., Oeljeklaus, S., et al. (2015). Mistargeted mitochondrial proteins activate a proteostatic response in the cytosol. *Nature* 524, 485–488. doi: 10.1038/nature14951

Wu, K. L., Wu, C.-W., Chao, Y.-M., Hung, C.-Y., and Chan, J. Y. (2016). Impaired Nrf2 regulation of mitochondrial biogenesis in rostral ventrolateral medulla on hypertension induced by systemic inflammation. *Free Radic. Biol. Med.* 97, 58–74. doi: 10.1016/j.freeradbiomed.2016.05.012

Xiong, W., Ma, Z., An, D., Liu, Z., Cai, W., Bai, Y., et al. (2019). Mitofusin 2 participates in mitophagy and mitochondrial fusion against angiotensin II-induced cardiomyocyte injury. *Front. Physiol.* 10:411. doi: 10.3389/fphys.2019.00411

Xu, N., Gulick, J., Osinska, H., Yu, Y., Mclendon, P. M., Shay-Winkler, K., et al. (2020). Ube2v1 positively regulates protein aggregation by modulating ubiquitin proteasome system performance partially through K63 Ubiquitination. *Circ. Res.* 126, 907–922. doi: 10.1161/CIRCRESAHA.119.316444

Xu, S., Peng, G., Wang, Y., Fang, S., and Karbowski, M. (2011). The AAA-ATPase p97 is essential for outer mitochondrial membrane protein turnover. *Mol. Biol. Cell* 22, 291–300. doi: 10.1091/mbc.E10-09-0748

Yamamoto, K., Hamada, H., Shinkai, H., Kohno, Y., Koseki, H., and Aoe, T. (2003). The KDEL receptor modulates the endoplasmic reticulum stress response through mitogen-activated protein kinase signaling cascades. *J. Biol. Chem.* 278, 34525–34532. doi: 10.1074/jbc.M304188200

Yamano, K., and Youle, R. J. (2013). PINK1 is degraded through the N-end rule pathway. *Autophagy* 9, 1758–1769. doi: 10.4161/auto.24633

Yogalingam, G., Hwang, S., Ferreira, J. C., and Mochly-Rosen, D. (2013). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) phosphorylation by protein kinase Cδ (PKCδ) inhibits mitochondria elimination by lysosomal-like structures following ischemia and reoxygenation-induced injury. *J. Biol. Chem.* 288, 18947–18960. doi: 10.1074/jbc.M113.466870

Yu, W., Xu, M., Zhang, T., Zhang, Q., and Zou, C. (2019). Mst1 promotes cardiac ischemia–reperfusion injury by inhibiting the erk-creb pathway and repressing fundc1-mediated mitophagy. *J. Physiol. Sci.* 69, 113–127. doi: 10.1007/s12576-018-0627-3

Zhang, J., and Ney, P. A. (2009). Role of BNIP3 and NIX in cell death, autophagy, and mitophagy. *Cell Death Differ.* 16, 939–946. doi: 10.1038/cdd.2009.16

Zhang, Y., Sano, M., Shinmura, K., Tamaki, K., Katsumata, Y., Matsuhashi, T., et al. (2010). 4-Hydroxy-2-nonenal protects against cardiac ischemia–reperfusion injury via the Nrf2-dependent pathway. *J. Mol. Cell. Cardiol.* 49, 576–586. doi: 10.1016/j.yjmcc.2010.05.011

Zhao, Q., Wang, J., Levichkin, I. V., Stasinopoulos, S., Ryan, M. T., and Hoogenraad, N. J. (2002). A mitochondrial specific stress response in mammalian cells. *EMBO J.* 21, 4411–4419. doi: 10.1093/emboj/cdf445

Zhong, Q., Gao, W., Du, F., and Wang, X. (2005). Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. *Cell* 121, 1085–1095. doi: 10.1016/j.cell.2005.06.009

Zhong, Z., Umemura, A., Sanchez-Lopez, E., Liang, S., Shalapour, S., Wong, J., et al. (2016). NF-kappaB restricts inflammasome activation via elimination of damaged mitochondria. *Cell* 164, 896–910. doi: 10.1016/j.cell.2015.12.057

Zhou, B., and Tian, R. (2018). Mitochondrial dysfunction in pathophysiology of heart failure. *J. Clin. Invest.* 128, 3716–3726. doi: 10.1172/JCI120849

Zhou, H., Zhu, P., Guo, J., Hu, N., Wang, S., Li, D., et al. (2017). Ripk3 induces mitochondrial apoptosis via inhibition of FUNDC1 mitophagy in cardiac IR injury. *Redox. Biol.* 13, 498–507. doi: 10.1016/j.redox.2017.07.007

Zhou, H., Zhu, P., Wang, J., Zhu, H., Ren, J., and Chen, Y. (2018). Pathogenesis of cardiac ischemia reperfusion injury is associated with CK2α-disturbed mitochondrial homeostasis via suppression of FUNDC1-related mitophagy. *Cell Death Differ.* 25, 1080–1093. doi: 10.1038/s41418-018-0086-7

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 12 November 2020
doi: 10.3389/fphys.2020.593585

Phosphorylation Modifications Regulating Cardiac Protein Quality Control Mechanisms

Sumita Mishra[1†], Brittany L. Dunkerly-Eyring[2†], Gizem Keceli[1] and Mark J. Ranek[1]*

[1] Division of Cardiology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD, United States, [2] Department of Pharmacology and Molecular Sciences, Johns Hopkins University, Baltimore, MD, United States

Many forms of cardiac disease, including heart failure, present with inadequate protein quality control (PQC). Pathological conditions often involve impaired removal of terminally misfolded proteins. This results in the formation of large protein aggregates, which further reduce cellular viability and cardiac function. Cardiomyocytes have an intricately collaborative PQC system to minimize cellular proteotoxicity. Increased expression of chaperones or enhanced clearance of misfolded proteins either by the proteasome or lysosome has been demonstrated to attenuate disease pathogenesis, whereas reduced PQC exacerbates pathogenesis. Recent studies have revealed that phosphorylation of key proteins has a potent regulatory role, both promoting and hindering the PQC machinery. This review highlights the recent advances in phosphorylations regulating PQC, the impact in cardiac pathology, and the therapeutic opportunities presented by harnessing these modifications.

Keywords: phosphorylation, chaperones, proteasome, autophagy, ubiquitin enzymes, cardiac disease

OPEN ACCESS

Edited by:
Julian Stelzer, Case Western Reserve University, United States

Reviewed by:
Nazha Hamdani, Ruhr University Bochum, Germany
John Jeshurun Michael, Cornell University, United States

****Correspondence:***
Mark J. Ranek
mranek1@jhmi.edu;
mranek1@jh.edu

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *11 August 2020*
Accepted: *28 September 2020*
Published: *12 November 2020*

Citation:
Mishra S, Dunkerly-Eyring BL, Keceli G and Ranek MJ (2020) Phosphorylation Modifications Regulating Cardiac Protein Quality Control Mechanisms. Front. Physiol. 11:593585. doi: 10.3389/fphys.2020.593585

Abbreviations: Akt, protein kinase B; Ambra1, activating molecule in beclin1-regulated autophagy 1; AMPK, AMP-activated protein kinase; AR, androgen receptor; ARG, Abl-related gene product; ASK1, apoptosis signal-regulating kinase 1; Atg, autophagy-related gene; CamKII, calcium/calmodulin dependent protein kinase; CASA, chaperone-assisted autophagy; CDK, cyclin dependent kinase; CHIP, carboxyl terminus of HSC70 interacting protein; c-IAP1, cellular inhibitor of apoptosis protein 1; CK2, casein kinase 2; CMA, chaperone-mediated autophagy; CryAB, αB-crystallin; DRC, desmin-related cardiomyopathy; DYRK2, dual-specificity tyrosine-regulated kinase 2; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; ERK, extracellular signal regulated protein kinase; FGF, fibroblast growth factor; FGFR1, fibroblast growth factor receptor 1; FLNC, filamin-c; Foxo, forkhead transcription factor; HECT, homologous to E6-AP carboxyl terminus; HFpEF, heart failure with a preserved ejection fraction; HOP, HSP70-HSP90 organizing protein; HSP, heat shock protein; I/R, ischemia/reperfusion; IRS, insulin receptor substrate 1; JNK, c-Jun N-terminal Kinase; MDM2, murine double minute 2; MI, myocardial infarction; MK2, mitogen kinase 2; MKK, mitogen-activated protein kinase kinase; mTORC1, mechanistic target of rapamycin complex 1; NEDD4, neural precursor cell expressed developmentally down-regulated protein 4; OA, okadaic acid; PAS, pre-autophagosomal structure; PCNA, proliferating cell nuclear antigen; PFI, proteasome functional insufficiency; PI3K, phosphoinositide 3-kinase; PINK1, PTEN-induced kinase 1; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PO, pressure overload; PP1γ, protein phosphatase 1γ; PP2A, protein phosphatase 2A; PQ, expanded polyglutamine; PQC, protein quality control; PTM, post-translational modification; RBR, ring between ring; RING, really interesting new gene; SCF, skp, cullin, F-box containing complex; SOD, super oxide dismutase; TAC, transverse aortic constriction; TFEB, transcription factor EB; TSC2, tuberous sclerosis complex 2; UBE2A, ubiquitin-conjugating enzyme E2A; UBE2J1, ubiquitin-conjugating enzyme E2J1; Ulk1, Unc-51 like autophagy activating protein kinase; UPS, ubiquitin proteasome system.

INTRODUCTION

Hearts are tasked with an immense challenge of maintaining protein homeostasis (proteostasis) in the face of disease conditions stemming from genetic mutations or environmental stressors (Willis and Patterson, 2013; Henning and Brundel, 2017). Pathological stressors can produce non-functional, misfolding-prone proteins. The failure to remove these proteins in a timely manner can compromise the integrity of intracellular proteins and impair the contractile apparatus and organelles; this results in decreased cell survival and function, culminating in the development of heart failure (Wang and Robbins, 2006; Wang et al., 2008). Indeed, many forms of heart disease present with the accumulation of ubiquitinated proteins and/or protein aggregates: hallmarks of inadequate or impaired PQC (Weekes et al., 2003; Day, 2013; Day et al., 2013). Therapeutic strategies to enhance PQC and induce cardioprotection are of great interest, however a better understanding of the molecular mechanisms regulating cardiomyocyte PQC is needed to facilitate the development of such a novel strategy.

Cardiomyocytes utilize elaborate intracellular PQC mechanisms to maintain proteostasis and counter disease progression (**Figure 1**). Briefly stated the first line of defense against impaired proteostasis are the molecular chaperones which bind to misfolded proteins, whereas degradation of proteins is carried out by the proteasome and lysosome (Wang et al., 2008; Ranek et al., 2018). Protein misfolding can expose a stretch of hydrophobic amino acids to the cytosol. These amino acids are recognized and bound by molecular chaperones to either promote protein re-folding, prevent misfolded proteins from aggregating, and/or facilitate the interaction between the misfolded protein and a ubiquitin ligase (Ranek et al., 2018). The UPS degrades proteins that have been targeted for degradation via a ubiquitin chain, canonically a lysine-48 linked chain (Wang et al., 2008). The UPS consists of the ubiquitination enzymes: an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin ligase; along with the barrel shaped structure known as the proteasome which contains the proteolytic activities (Wang et al., 2008, 2011). The lysosome degrades proteins, macromolecules, and whole organelles by processes encompassing autophagy, which involves the delivery of substrates to the lysosome for internalization and degradation (Zheng et al., 2011; Sciarretta et al., 2018b). Autophagy comes in many forms: microautophagy, macroautophagy, chaperone-mediated autophagy (CMA), CASA, and organelle-specific autophagies (e.g., mitophagy) (Henning and Brundel, 2017; Ghosh and Pattison, 2018; Sciarretta et al., 2018b).

A growing area of interest surrounds the role of PTMs in the regulation of PQC pathways. Various PTMs have been reported to promote or hinder cardiomyocyte PQC, including nitrosylation, oxidation, and ubiquitination among others (Wang et al., 2013). By doing so, PTMs have greatly expanded the specificity and capacity of protein degradation during cardiac disease. This review highlights the recent advances of arguably the most established PQC-regulating PTM, phosphorylation. Excellent reviews of the other PTMs have been described elsewhere (Christians and Benjamin, 2012; Scruggs et al., 2012; Wang et al., 2013; Penna et al., 2018; VerPlank and Goldberg, 2018). This review details the recent PQC phosphorylations that have been identified (**Supplementary Table 1**), especially those with influential roles in cardiac pathophysiology and some ubiquitous signaling pathways, and that can be clinically interrogated.

Impaired Protein Quality Control (PQC) in Cardiac Disease

Many forms of cardiac disease are characterized by an accumulation of ubiquitinated proteins and the presence of aberrant protein aggregation in the form of pre-amyloid oligomers (Ranek and Wang, 2009; Parry et al., 2015; Gilda and Gomes, 2017). Indeed, the majority of failing human hearts are characterized by impaired proteostasis, termed cardiac proteinopathies (Su and Wang, 2010; Zheng et al., 2011; Willis and Patterson, 2013). Studies conducted in mice revealed that inadequate cardiomyocyte PQC precedes and exacerbates cardiac pathogenesis (**Figure 2**) (Wang et al., 2011; Gilda and Gomes, 2017; Henning and Brundel, 2017). The Robbins lab overexpressed a misfolded protein surrogate with a polyglutamine expansion pre-amyloid oligomer (PQ83) in the heart, which impaired PQC leading to reduced cardiac function and ultimately failure (Pattison et al., 2008). Similar results were obtained in another mouse model expressing a mutated αB-crystallin ($CryAB^{R120G}$), a misfolded and aggregation prone protein, in cardiomyocytes alone (Wang et al., 2001, 2003). PQ83 and $CryAB^{R120G}$ mice exemplify a pathological process termed proteotoxicity, which refers to the adverse effects induced by the presence of damaged or misfolded proteins (Sandri and Robbins, 2014). If not corrected, cardiac proteotoxicity will result in apoptosis/necrosis at the cellular level, decreased function at the organ level, and even premature death for the organism (Sandri and Robbins, 2014). Indeed, $CryAB^{R120G}$ mice showed impaired PQC within 3 months of age and heart failure within 6 months of age (Wang et al., 2001, 2003; Kumarapeli and Wang, 2004). Stimulation of protein degradation via genetic upregulation of autophagy rescued the $CryAB^{R120G}$ phenotype (Pattison et al., 2011). Interestingly exercise was also shown to attenuate pre-amyloid deposition, proteotoxicity, and heart failure progression (Maloyan et al., 2007). A recent study by the Wang lab utilized the $CryAB^{R120G}$ as a model of proteinopathy that occurs with HFpEF, a devastating disease currently without a treatment (Sharma and Kass, 2014). $CryAB^{R120G}$ mice were treated with a phosphodiesterase 1 inhibitor, which activated protein kinases A (PKA) and G (PKG), stimulated proteasome activities, and attenuated proteotoxic stress (Zhang et al., 2019). This study demonstrates the potential of targeting protein degradation as a heart failure treatment. Chronic inhibition of any one of the PQC machineries exacerbates cardiac pathogenesis following MI via closure of the left ventricular anterior descending artery or left ventricular PO via transaortic constriction (TAC), whereas the enhancement of cardiomyocyte PQC protects the myocardium facilitating increased proteostasis, cardiac function, and lifespan (Wang and Robbins, 2006; Wang et al., 2008, 2011; Su and Wang, 2010; Ranek et al., 2015).

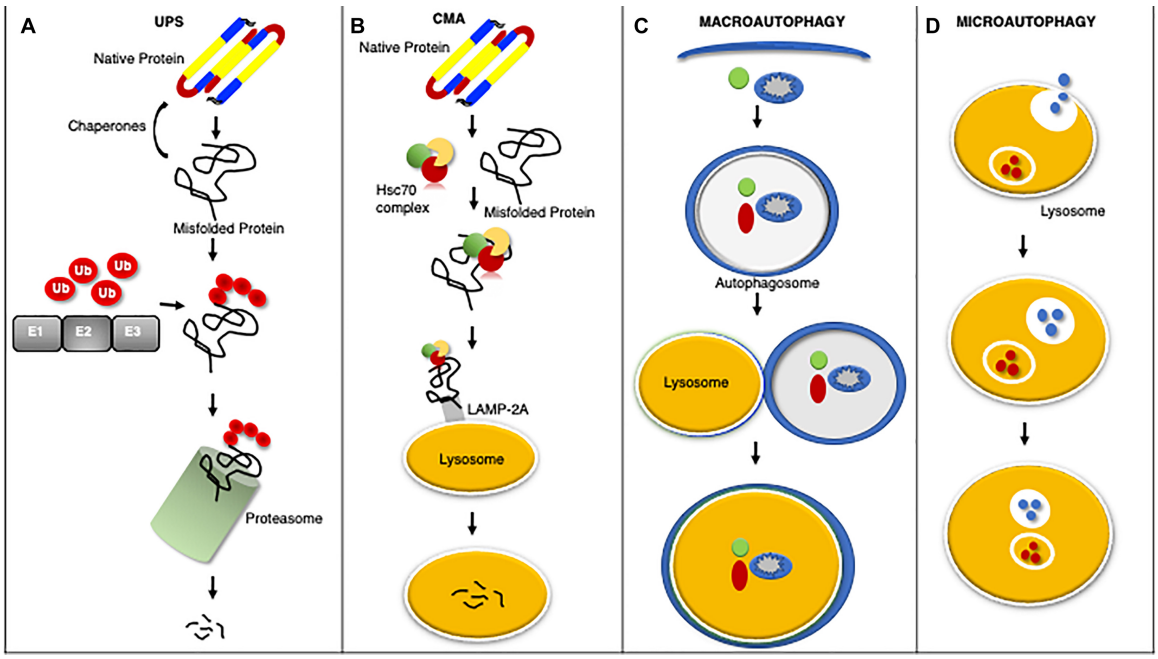


FIGURE 1 | An overview of protein quality control systems. Misfolded proteins may regain native structure with the assistance of chaperones, however if proper re-folding cannot occur the misfolded protein will be catalyzed by the UPS through ubiquitination via a series of enzymatic reactions involving an ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3) for degradation by the proteasome **(A)**. Chaperone mediated autophagy (CMA) is a process by which the heat shock cognate 70 (HSC70) complex recognizes and binds select protein targets for internalization and degradation to the lysosome through the lysosome associated membrane protein 2A (LAMP2A) receptor **(B)**. Macroautophagy is the bulk removal of proteins, protein aggregates, and organelles by first forming an autophagosome to surround the cargo followed by merger with the lysosome for degradation **(C)**. Microautophagy is a process by which the lysosome invaginates to bring protein substrates into the lysosome for degradation **(D)**.

Chaperones

The first line of defense against proteotoxicity are chaperones, which are a broad class of proteins that assist with refolding misfolded proteins, facilitating an interaction between misfolded proteins and a ubiquitination enzyme, and preventing proteins from aggregating together (**Figure 1**) (Willis and Patterson, 2010; Ranek et al., 2018). There are chaperones that are constitutively expressed (e.g., heat shock cognate 70, HSC70) and others, whose expression are induced by a cardiac stress [e.g., heat shock protein 70 (HSP70)] (Wu et al., 1985; Dworniczak and Mirault, 1987). Chaperones can protect cardiomyocytes against proteotoxicity and subsequent cell death during a stressful/pathological condition (Willis and Patterson, 2010; Tarone and Brancaccio, 2014; Penna et al., 2018). Indeed, overexpression of certain chaperones has protected hearts from cardiac disease (Willis and Patterson, 2010; Tarone and Brancaccio, 2014). Multiple studies have reported overexpression of HSP70 to be protective in mouse models of ischemia and ischemia-reperfusion injury (Marber et al., 1995; Plumier et al., 1995; Radford et al., 1996). Another study determined that HSP70 overexpression is protective in periods of brief ischemia where there is myocardial dysfunction but no infarct (Trost et al., 1998). In a pig model of ischemia-reperfusion, HSP90 transfection was protective and reduced the ischemic region (Kupatt et al., 2004). It was suggested that this protective effect was due to HSP90 mediated enhancement of NO formation (Depre et al., 2006). Injection of HSP22 into the swine heart was protective against MI in a NO-dependent manner (Chen et al., 2011), however, it seems that this is only effective acutely, as more recent work shows that chronic overexpression is deleterious (Morin et al., 2019). This discrepancy was due to increased ROS production and oxidative stress that occurs with chronic HSP22 expression that results in cardiac hypertrophy and shortened lifespan (Morin et al., 2019). Another study using a HSP22 overexpression transgenic mouse model reported cardioprotection with increased preconditioning to attenuate MI (Depre et al., 2006). Here, the authors noted a metabolic switch to favor glucose utilization and expression of anti-apoptotic factors over pro-apoptotic factors. These findings were supported by a recent study describing protection against doxorubicin induced cardiotoxicity in HSP22 transgenic mice (Lan et al., 2020).

One of the most recognized protective roles of cardiomyocyte chaperones is to maintain cardiac systole and diastole, through modulation of contractile proteins (Willis et al., 2009, 2010). Impaired cardiac function with increased ventricular stiffness are hallmarks of heart failure. Altered sarcomeric and calcium handling proteins contribute to diastolic left ventricular stiffness. Phospholamban regulates calcium uptake to the sarcoplasmic reticulum via SERCA for cardiomyocyte relaxation to occur. Qian et al. (2011) identified that HSP20 negatively regulates protein phosphatase 1 (PP1) activity via a direct interaction, favoring phosphorylation of phospholamban and increased cardiac function. The small heat shock protein HSPB7 modulates

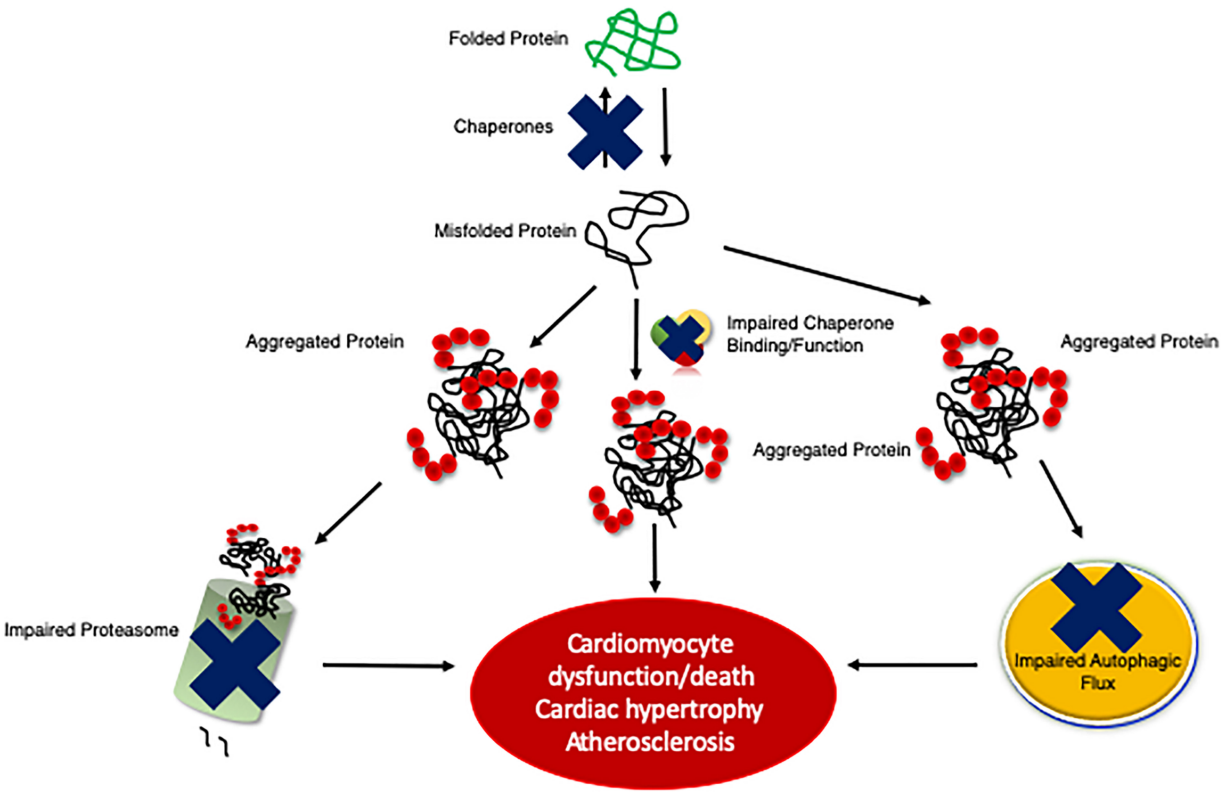


FIGURE 2 | An illustration of the development of cardiac proteinopathy. Cardiomyocytes accumulate ubiquitinated and aggregated proteins as a result of impaired PQC mechanisms. The failure of cardiomyocyte chaperones activity to efficiently re-fold misfolded proteins results in misfolded proteins aggregating, which impairs the proteasome and overwhelms autophagy. Insufficient PQC exacerbates disease pathogeneses (e.g., cardiac hypertrophy and atherosclerosis).

actin thin filament length by binding to monomeric actin and limiting its availability for polymerization (Wu et al., 2017). Zhu et al. (2009) applied single molecule force spectroscopy to determine the contour length and bending rigidity of the N2B-Us of titin and the effect of wild type and mutant R157H (harboring the dilated cardiomyopathy missense mutation), or R120G (desmin-related myopathy mutation) αB-crystallin (CryAB) on the molecular mechanics of the N2B-Us and its flanking Ig domains. CryAB functions as a chaperone that lowers the probability of Ig domain unfolding and the persistence length of the titin N2B-Us spring region. HSP27 or CryAB suppresses cardiomyocyte stiffness caused by stretch and low pH (Kotter et al., 2014). Collectively these studies demonstrate the strong cardioprotective potential of chaperones, however before this potential can be therapeutically leveraged, a greater understanding of the regulatory mechanisms involved in chaperone expression and function is needed (Willis and Patterson, 2010; Tarone and Brancaccio, 2014; Ranek et al., 2018).

Chaperones Regulated by Phosphorylation

Recent studies have identified protein kinases and phosphatases responsible for phosphorylation and dephosphorylation, respectively, of chaperones and the consequent effects on cardiac function (**Figure 3**). The role of phosphorylation of chaperones and co-chaperones varies depending on the site and even on the temporal regulation of the sites. Some phosphorylation events stabilize the chaperones and their interactions with their client proteins, while others are specific for their client proteins and will only function properly when phosphorylated at the appropriate sites.

The HSP70 family of chaperones support proper protein structure by unfolding and refolding misfolded proteins in an ATP-dependent manner (Sharma et al., 2010; Finka et al., 2015). Phosphorylation has a key role in regulating the function of HSP70s. HSC70 phosphorylation at threonine 38 controls cell cycle progression in a CDK-dependent manner by promoting G1 cyclin binding and subsequent degradation to promote G1/S transition in the cell cycle (Truman et al., 2012). While this study was done in yeast, this phosphorylation site is highly conserved and may play an important role in cardiovascular diseases which typically exhibit high expression of HSC70 (Boehm and Nabel, 2003). The vital role of HSP70s in redox homeostasis is precisely regulated by its phosphorylation at serine 631, which is required for superoxide dismutase 2 (SOD2) import and activation in mitochondria (Zemanovic et al., 2018). Once SOD2 restores the redox balance in mitochondria, HSP70 is dephosphorylated, and both HSP70 and SOD2 are ubiquitinated by CHIP and degraded (Zemanovic et al., 2018). Increased oxidative stress has been associated with the development and pathogenesis of cardiovascular disease. This precise regulation

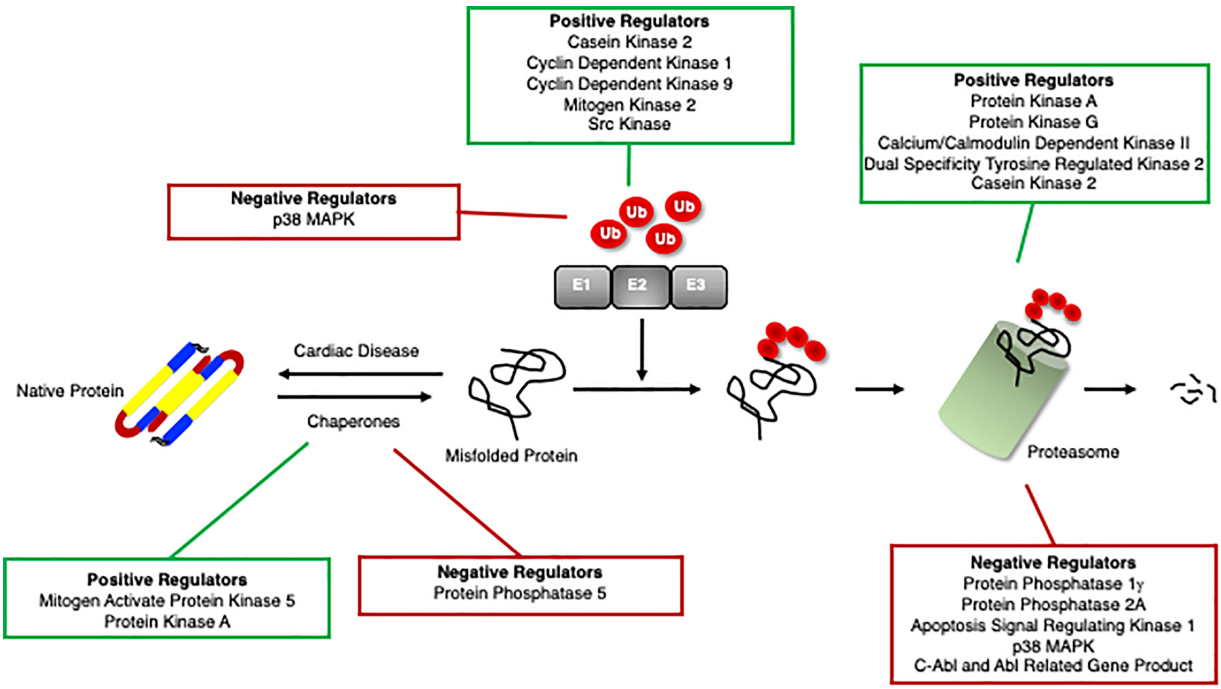


FIGURE 3 | Positive and negative regulators of the ubiquitin proteasome system. Proteins that positively (green box) and negatively (red box) regulate cardiomyocyte chaperones, ubiquitination enzymes, and the proteasome.

via phosphorylation is pivotal to both maintaining a homeostatic redox state and then binding to proteins damaged by oxidation. Regulatory phosphorylation can occur at the level of the chaperone as well as at the level of the co-chaperone. HSP40, a co-chaperone with HSP70, is phosphorylated by mitogen activated protein kinase 5 (MK5), resulting in enhanced ATP hydrolase function to increase the refolding capacity, thus PQC-maintaining ability, of HSP40/70 (Kostenko et al., 2014).

HSP90 proteins are chaperones with some similar, but different, functions to HSP70s in facilitating protein folding, and also directing proteins for degradation in order to avoid protein aggregation (Hohfeld et al., 2001). The decision-making process for whether to facilitate folding or to direct for degradation is mediated by binding to co-chaperones CHIP and HSP70-HSP90 organizing protein (HOP) (Muller et al., 2013). Phosphorylation is responsible for determining co-chaperone binding and macro-complex formation, such that phosphorylation in the C-termini of HSP70 and HSP90 prevents CHIP binding, but enhances HOP binding, which can assist with protein folding (Muller et al., 2013). Cells in a proliferative state have higher levels of phosphorylated HSP70 and HSP90 and those chaperones are preferentially bound to HOP (Muller et al., 2013). This has major implications in cancer due to the proliferative nature of those cells, but could also impact cardiovascular diseases like atherosclerosis, where vascular smooth muscle cell proliferation is problematic (Bennett et al., 2016). Bachman et al. (2018) demonstrated that the co-chaperone Cdc37 complexes with HSP90 and recruits client kinases, which phosphorylates HSP90 to provide a mechanism for highly specific tuning of the chaperone cycle depending on the client kinase that is recruited. Similarly, dephosphorylation of HSP90 by protein phosphatase 5 was shown to regulate the activity and interacting proteins of HSP90 (Haslbeck et al., 2015; Oberoi et al., 2016). These findings are of particular relevance to the heart as HSP90 is known to be involved in functioning of multiple steroid receptors (Pratt and Toft, 2003; Zhao et al., 2005) ARs play a role in multiple cardiovascular diseases including hypertension, stroke, and atherosclerosis, thus highlighting the therapeutic potential of modulating chaperones (Huang et al., 2016). HSP90 is bound to unliganded AR in the cytosol, however following phosphorylation of HSP90 at threonine 89 by PKA, the AR is released into the cytosol where it binds to HSP27 and migrates into the nucleus to modulates transcription via binding to androgen response elements (AREs) (Dagar et al., 2019).

The cardioprotection elicited by increased molecular chaperones in the heart is well-established (Willis and Patterson, 2010; Tarone and Brancaccio, 2014; Ranek et al., 2018), however, a better understanding of the regulation of chaperones via phosphorylation is desirable for the ability to better fine-tune chaperones' functions. A mouse model that overexpressed a constitutively phosphorylated HSP20 at serine 16 (previously found to be PKA mediated), promoted fibrotic remodeling and heart failure (Gardner et al., 2019). This came as a surprise, as prior studies suggested that HSP20 phosphorylation at serine 16 was protective against beta-agonist-induced apoptosis in cardiomyocytes (Fan et al., 2004; Qian et al., 2009). A possible explanation for this disparity is the former study examining constitutive HSP20 phosphorylation at serine 16 was done in

isolated cardiomyocytes, suggesting that acute phosphorylation of this site is protective, while the effects of chronic HSP20 activation are harmful. Gardner et al. (2019) utilized a ubiquitous knock-in mouse model and attributed this maladaptive response to a pro-fibrotic role of phosphorylated HSP20 that mediates IL-6 activation of cardiac fibroblasts. Another study identified HSP27 phosphorylation at serine 82 as being protective in biomechanically stressed mouse hearts (Collier et al., 2019). Here, they found that actin-binding protein filamin C (FLNC) and HSP27 are upregulated in mouse hearts subjected to left ventricular PO induced by TAC surgery (Collier et al., 2019). It was determined that these proteins interact, with phosphorylation of HSP27 facilitating their binding in a region of FLNC that is mechanosensing, while FLNC localizes to load-bearing sites (Collier et al., 2019). This suggests the mechanism chaperones are recruited in response to force destabilization is to maintain the integrity of the sarcomere and protect the myocardium. HSP27 phosphorylation has also been found to be upregulated in human platelets during ST-elevation post-MI, possibly offering a new measurable stress response for patients with MI (Kraemer et al., 2019).

Another chaperone with significant implications in cardiovascular disease is p97/valosin-containing protein (VCP)/Cdc48, an ATPase involved in a variety of functions to include ERAD, cell cycle progression, autophagy, and DNA repair (Parisi et al., 2018). This ATPase has been demonstrated to play a role in myofibrillar formation, both in limiting normal muscle growth but also contributing to muscle wasting diseases (Piccirillo and Goldberg, 2012). The function of p97 is regulated by phosphorylation at multiple sites (Baek et al., 2013). Phosphorylation of tyrosine 805 disrupts the interaction between p97 and PNGase, attenuating the ERAD pathway, thus PQC (Li et al., 2008). Additional studies found that tyrosine phosphorylation inhibits p97-ER membrane association and transitional ER assembly (Lavoie et al., 2000). Phosphorylation has also been shown to impact the ATPase activity of p97 as well as its association with ubiquitinated proteins (Klein et al., 2005; Mori-Konya et al., 2009). p97 has a role in the DNA damage response, as it is phosphorylated at serine 784 and accumulates at sites of DNA damage, possibly for DNA repair (Livingstone et al., 2005). Taken together, these studies demonstrate the regulatory role of phosphorylation and dephosphorylation on the function of chaperones, presenting many possibilities for targeting these modifications as a therapy.

Ubiquitination Enzymes

Proteins are targeted for degradation by the proteasome via ubiquitination, a sequential system involving the following enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3), and possibly a ubiquitin elongation factor (E4) (**Figure 1**) (Hershko and Ciechanover, 1998; Goldberg, 2003; Zuo et al., 2020). In humans, there are only 2 E1s that have been identified, and as their name suggests, they are responsible for activating ubiquitin so that it can be conjugated to a protein. This process occurs in an ATP-dependent manner in which the C-terminus of ubiquitin is bound to an E1 cysteine via a thioester bond (Hershko and Ciechanover, 1998; Zuo et al., 2020). The E1 then transfers ubiquitin to one of approximately 40 E2s in humans through another thioester bond (Hershko and Ciechanover, 1998; Zuo et al., 2020). Finally, with an E3 the ubiquitin will be attached to a substrate protein. There are three families of E3s: HECT, RING and RBR, and these families differ slightly in the way that the ubiquitin is transferred to its substrate (Zheng and Shabek, 2017; George et al., 2018). HECT E3s will accept the ubiquitin first from the E2 to form a thioester bond, and will then transfer the ubiquitin to the substrate directly from the E3 (Zuo et al., 2020). RING E3s will bring together the ubiquitin-E2 complex and the substrate and simply mediate the transfer of ubiquitin from the E2 to the substrate (Zheng and Shabek, 2017). RBR ubiquitin ligases act as a hybrid protein of two domains, a canonical RING domain as well as a catalytic cysteine residue similar to the HECT domain (George et al., 2018). The specificity of the UPS is thought to lie within the ubiquitination step (Wang et al., 2008; Ranek et al., 2018). Given the importance of ubiquitination in PQC and degradation, it comes as no surprise that this process and selectivity is further regulated through phosphorylation of these ubiquitination enzymes (**Figure 3**).

Phosphorylations That Regulate Ubiquitination Enzymes

There is limited data into the sites phosphorylated and potential functional change of the two E1s in humans. Stephen et al. (1997) described multiple phosphorylation sites at the N-terminus of E1a. It was previously suggested that E1 could be phosphorylated by PKC, and that E1a serine 4 could be phosphorylated by Cdc2, a kinase that is key in cell cycle regulation (Kong and Chock, 1992; Nagai et al., 1995). In these studies, the function of these phosphorylation sites was not fully described. It was determined that while phosphorylation increases in various states of the cell cycle, the enzymatic activity of E1 was not changed (Stephen et al., 1996). Also, while E1a was found to localize to the nucleus, it was determined that phosphorylation was not required for this localization (Stephen et al., 1997). It is possible that this phosphorylation may increase the rate of nuclear targeting or possibly change the stability of interactions with E2 enzymes, but how phosphorylation may alter these roles is yet to be determined.

Functional alterations of E2 enzymes have been attributed to phosphorylation, albeit the knowledge about them in the heart is limited. A common theme of E2 enzymes and their regulation via phosphorylation is their involvement in the cell cycle. There is evidence of the cell cycle playing a role in cardiovascular diseases such as hypertrophy and atherosclerosis, so these mechanisms of regulation could have interesting therapeutic potential (Bicknell et al., 2003; Ahuja et al., 2007). In one such study of the involvement of an E2 in cell cycle regulation, Cdc34, in yeast was shown to be phosphorylated at serines 203, 222, and 231 in the acidic C-terminal tail domain by casein kinase 2 (CK2) (Sadowski et al., 2007). In an effort to understand the biological importance of these sites, they mutated them to alanine (phospho-null), or a glutamate or aspartate (phospho-mimetics). With the phospho-mutants, they observed differential kinetics through the cell cycle, from G_1 through S into the G_2/M phase (Sadowski et al.,

2007). The authors were able to associate the cells with faster cell cycle kinetics with an increased rate of Sic1 degradation, and increased Skp, cullin, F-box containing complex (SCF)-mediated ubiquitination of Sic1 (Sadowski et al., 2007). Overall, this was a demonstration that phosphorylation can regulate the catalytic and cell cycle functions of Cdc34. In another study, Shchebet et al. (2012) demonstrated that the ubiquitin-conjugating enzyme E2 A (UBE2A) is directly phosphorylated by CDK9 at serine 120. Knocking down CDK9 reduced UBE2A phosphorylation, which then decreased monoubiquitination of histone H2B and PCNA, suggesting phosphorylation at serine 120 activates UBE2A (Shchebet et al., 2012). This finding is of particular interest to the heart, as a recent study identified monoubiquitination of H2B is a transcriptional regulator that controls expression of cilia genes, where mutations have been implicated in congenital heart disease (Robson et al., 2019). ER stress occurs in various cardiovascular diseases with impaired proteostasis (Ochoa et al., 2018). A study from 2017 identified ubiquitin-conjugating enzyme E2 J1 (UBE2J1) phosphorylation at serine 184 as being important for recovery of ER stress to maintain proteostasis (Elangovan et al., 2017). It was reported that UBE2J1 is phosphorylated at serine 184 by mitogen kinase 2 (MK2) as a mechanism to alleviate ER stress (Menon et al., 2013). Phosphorylated UBE2J1 has higher affinity for the E3 ligase, c-IAP1, and that cells expressing a phospho-null UBE2J1 cannot recover from ER stress (Elangovan et al., 2017). The authors also reported that phosphorylated UBE2J1 is degraded by the proteasome, potentially as a feedback loop (Elangovan et al., 2017).

An E3 ligase that is implicated in multiple diseases, including cardiovascular and neurodegenerative diseases, is Parkin, which is known to be regulated by phosphorylation (Wang et al., 2018). One study demonstrates phosphorylation negatively regulates Parkin's protective function (Chen et al., 2018). Here, they use a mutant α-synuclein model that, when overexpressed, activates p38 MAPK, which then directly phosphorylates Parkin at serine 131. This disrupts PTEN-induced kinase 1 (PINK1)-Parkin mediated mitophagy and exacerbates the mitochondrial impairment induced by α-synuclein accumulation (Chen et al., 2018). A few studies have highlighted the importance of Parkin in the heart by performing knockout experiments. One such experiment knocked out Parkin in flies and observed accumulation of dysfunctional mitochondria and dilated cardiomyopathy (Bhandari et al., 2014). This phenotype could be rescued with cardiomyocyte-specific Parkin expression (Bhandari et al., 2014). Another study found that in response to MI the parkin knockout mice fared worse than WT mice in terms of survival and infarct size (Kubli et al., 2013). Collectively these studies suggest determining the kinases capable of phosphorylating Parkin in the heart and the role of this PTM during cardiac pathogenesis could provide a novel therapeutic avenue.

The neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) is a HECT domain ubiquitin ligase that is required for heart development, turnover of potassium and sodium channels, and maintenance of proteostasis (Fouladkou et al., 2010; Gilda and Gomes, 2017). FGFR1 activation results in tyrosine phosphorylation of four key sites (43, 365, 366, and 585) on NEDD4 (Persaud et al., 2014). Site-directed mutagenesis of these four tyrosines revealed that expression of a phospho-mimetic results in constitutive activation of NEDD4 ubiquitin ligase activity, while phospho-null NEDD4 decreased its ubiquitin ligase activity (Persaud et al., 2014). FGF signaling is known to play a pathophysiological role in the heart as evidenced by worsened cardiac hypertrophy in isoproterenol stimulated hearts of FGF2 transgenic mice (House et al., 2010; Itoh and Ohta, 2013). FGF2 KO mice were protected against cardiac hypertrophy and fibrosis following isoproterenol stimulation (Itoh and Ohta, 2013). FGF2 is mostly expressed in non-myocytes within the heart, suggesting cell-to-cell communication as the mode of cardiomyocyte regulation (Itoh and Ohta, 2013). Considering the requirement of NEDD4 in cardiac development, the presence of FGF in all cell types of the heart, and its role in heart disease, this signaling axis presents an interesting therapeutic target.

SUMOylation and neddylation are other PTMs that are analogous to ubiquitination, utilize a similar E1, E2, and E3 mechanism of conjugating either SUMO or NEDD8 to proteins, and are known to be involved in many different biological processes (Wilkinson and Henley, 2010; Zhou et al., 2019). There is evidence of the modifiers SUMO-1 and NEDD8 themselves being phosphorylated, but the functional implications are still unknown (Matic et al., 2008; Enchev et al., 2015). We are only beginning to understand the regulation of neddylation E1 (NEDD8-activating enzyme NAE) and E2s (UBE2M and UBE2F) by phosphorylation. Interestingly, the E3s that have been identified for neddylation can also function as ubiquitination E3s (Enchev et al., 2015). Furthermore, phosphorylation is responsible for converting an E3 from ubiquitin conjugating to NEDD8 conjugating (Batuello et al., 2015). Under growth factor stimulation, src kinase phosphorylates Mdm2 at tyrosines 281 and 302, recruits the NEDD8 E2 (Ubc12) and Mdm2, to neddylate p53 in human cancer cell lines (Batuello et al., 2015). This has interesting implications if this mechanism applies to other E3s. While phosphorylations have been identified on SUMOylation E1 and E2, the function of these modifications is not known (Tomanov et al., 2018). However, there are multiple studies that have found that phosphorylation modifies the function of SUMO E3s. One such study identified Ubc9 as being phosphorylated by CDK1 at serine 71 to enhance SUMOylation activity *in vitro* (Su et al., 2012). Another study in HEK 293T cells elucidates a feedback system whereby DNA-damage induced homeodomain interacting protein kinase 2 (HIPK2) phosphorylates proprotein convertase (Pc2) at threonine 495, which in turn controls Pc2 SUMOylation of HIPK2 in order to enhance its ability to mediate transcriptional repression (Roscic et al., 2006). Additional studies are needed to determine the potential role phosphorylation of the neddylation and SUMOylation proteins may have in cardiac disease pathogenesis.

The Proteasome

The UPS plays a key role in eukaryotic cells by degrading damaged, defective, and non-functional proteins (**Figure 1**) (Jentsch, 1992; Schwartz and Ciechanover, 1992; Ciechanover,

1994). This pathway represents non-lysosomal degradation of cellular proteins and plays a critical role in PQC (Goldberg, 2003; Divald and Powell, 2006). Proteins are destined to proteasome-mediated degradation by the formation of a ubiquitin chain on the target protein. Ubiquitinated proteins are fed through the proteasome core complex for degradation *via* an ATP driven process (Collins and Goldberg, 2017). The mammalian proteasome is a highly sophisticated multi subunit protease complex composed of the 20S catalytic core particle, within which proteins are degraded, and two 19S regulatory cap particles which locates and binds to ubiquitinated substrate (Budenholzer et al., 2017; Collins and Goldberg, 2017). The 20S particle is formed by two copies of 14 different subunits (α1–α7 and β1–β7) stacked in heptameric rings (Groll et al., 1997; Budenholzer et al., 2017). The active sites of the catalytic subunits (β1, β2, and β5) lines the central lumen of a chamber gated by an α subunit at its either end (Groll et al., 1997; Budenholzer et al., 2017). The proteasome activator 700 (PA700) or 19S cap has two distinct sub-components (Lasker et al., 2012). The base consists of six Rpt (1→6) subunits that have constitutive ATPase activity, plus two non-ATPase subunits, Rpn1 and Rpn2; and the lid structure contains 10 Rpn (3→12) subunits (Lasker et al., 2012; Budenholzer et al., 2017). The 19S subunit is responsible for binding and unfolding ubiquitinated proteins and feeding the protein substrate to the 20S proteasome for catalysis (Wang et al., 2008).

Several studies have shown a central role of the UPS in cardiovascular physiology and pathophysiology (Razeghi et al., 2006; Drews et al., 2010; Powell and Divald, 2010; Predmore et al., 2010; Zu et al., 2010; Li et al., 2011). Cardiac proteasomes exhibit distinct functional and subcellular distribution (Gomes et al., 2006, 2009; Drews et al., 2007; Powell et al., 2008; Dahlmann, 2016). A substantial number of studies have shown impaired proteasomal function contributes to heart disease (Su and Wang, 2010; Willis et al., 2010; Wang et al., 2011; Day, 2013), which is supported by findings of insufficient UPS activity in human end stage heart failure (Hein et al., 2003; Weekes et al., 2003; Predmore et al., 2010). Proteasome dysfunction has been reported in hypertrophic, ischemic, atrophic, desmin-related, and diabetic cardiomyopathies (Razeghi et al., 2006; Drews et al., 2010; Powell and Divald, 2010; Predmore et al., 2010; Zu et al., 2010; Li et al., 2011). Arguably the most well-studied cardiac proteinopathy mouse model is DRC caused by mutations in desmin, αB-crystallin (CryAB), and other related genes (Wang and Robbins, 2006). The DRC model features aberrant protein aggregates in myocytes, reduced cardiac function, and a shortened lifespan (Wang et al., 2001, 2003; Pattison et al., 2011). These hearts also have PFI, which was postulated to contribute further to the impaired proteostasis and the pathogenesis of DRC (Wang and Robbins, 2006; Su and Wang, 2010; Wang et al., 2011). Studies enhancing proteasome activity in DRC have supported this notion (Li et al., 2011; Ranek et al., 2013). PFI has also been observed in mouse hearts subjected to ischemia/reperfusion (I/R) injury and transaortic constriction (TAC)-induced cardiac PO (Wang et al., 2011). Expression of a catalytically inactive proteasome beta5 subunit negates the chymotrypsin-like activity, but not the caspase- or trypsin-like activities, and worsened cardiac PQC and function following I/R or TAC (Ranek et al., 2015).

Methods to enhance proteasome activities have been shown to be protective. Post-translational modifications are key regulatory mechanisms for proteasome function. More than 300 phosphorylation sites in proteasome subunits have been detected that can regulate protein stability, abundance, assembly, subcellular localization, substrate recognition and enzymatic activity of proteasome subunits (Benedict and Clawson, 1996; Satoh et al., 2001; Bose et al., 2004; Zong et al., 2006; Bingol et al., 2010; Guo et al., 2011, 2016; Djakovic et al., 2012; Sledz et al., 2013; Yuan et al., 2013; Li et al., 2015; Lokireddy et al., 2015; Wani et al., 2016). In humans, 20S cap and the 19S base subunits are more frequently phosphorylated than the 19S lid. Many kinases have been demonstrated to regulate proteasome activity in general and in heart tissues (**Figure 3**) (Powell and Divald, 2010; Sledz et al., 2013; Wang et al., 2013; Collins and Goldberg, 2017; VerPlank and Goldberg, 2018).

Phosphorylations Regulating the Proteasome

Protein Kinase A (PKA)

Protein kinase A was the first kinase identified to phosphorylate proteasome subunits and stimulate proteasome activities *in vitro* as well as *in vivo* (Zong et al., 2006, 2008; Asai et al., 2009; Gomes et al., 2009; Drews et al., 2010). A series of studies using bovine pituitaries, and later from murine hearts, identified PKA in a complex with endogenous 20S proteasomes (Pereira and Wilk, 1990; Zong et al., 2006; Drews et al., 2010). PKA phosphorylation of the proteasome has been detected on serine residues of the α 1-, α 2-, α 3-, β 2-, β 3-, and β7-subunits, and the threonine residues of the α 3-, β 3-, and β7-subunits of the 20S proteasome (Zong et al., 2006). Additionally studies demonstrated enhancement of proteasome activity via PKA stimulation. The addition of cAMP stimulates PKA activation and its interaction with the proteasome, which resulted in increased phosphorylation of 19S cap subunit Rpn6 at Ser14 (Lokireddy et al., 2015). Pathare and others have shown that raising cAMP levels increases the amount of double-capped 26S proteasomes, indicating increased stabilization of the proteasome complexes (Pathare et al., 2012; VerPlank et al., 2019). Rpn6 serine 14 phosphorylation has been detected *in vivo* in response to fasting, intense exercise, and hormonal cues which increases intracellular cAMP and PKA activity (VerPlank et al., 2019). PKA has also been reported to activate the proteasome via phosphorylation of the ATPase subunit Rpt6 (Zhang et al., 2007). Recombinant PKA directly phosphorylates Rpt6 at serine 120 *in vitro*, and expression of a Rpt6 phospho-silenced Ser120Ala mutation reduced the proteasome activity (Zhang et al., 2007). Phosphorylation of the 19S cap Rpt6 ATPase subunit initiates assembly of 26S proteasome, by stimulating the association of the 19S particle with the 20S proteasome (Satoh et al., 2001). Subsequent studies have reproduced the changes in proteasome activity by modulating PKA activity, however, they have failed to confirm phosphorylation of several proteasome subunit sites (VerPlank and Goldberg, 2018). There are various explanations

for this discrepancy: a kinase may modify additional targets in an artificial setting, *in vitro* but not *in vivo*, only in specific cell types, and that kinases and phosphatases may co-purify with the proteasome which can add or remove phosphate groups during isolation (Guo et al., 2017; VerPlank and Goldberg, 2018). Nonetheless, these studies demonstrate PKA is a critical regulator of proteasome assembly and activity during physiological and pathological conditions.

Protein Phosphatases 1γ (PP1γ) and 2A (PP2A)

Dephosphorylation via protein phosphatases of the proteasome negatively regulates the activity of the proteasome. Protein phosphatase 1γ (PP1γ) was postulated to reverse the effect of PKA phosphorylation on 20S proteasome activity (Zhang et al., 2007). Protein phosphatase 2A (PP2A) has also been reported to interact with and dephosphorylate the native 20S subunits in cardiac proteasome (Zong et al., 2006). PP2A reduced serine phosphorylation on α1 and β7, and threonine phosphorylation on α1, were linked to suppressed proteasome activity. Echoing these results, pharmacological inhibition of PP2A by OA conferred increased proteasome activity (Gomes et al., 2006; Zong et al., 2006). These data demonstrate the regulatory role of these phosphatases, however whether they can be targeted during disease pathogenesis to enhance proteostasis remains to be determined.

Calcium/Calmodulin-Dependent Protein Kinase II (CaMKII)

A rise of intracellular calcium stimulates proteasome activity by way of calcium/calmodulin dependent protein kinase (CaMKII) phosphorylation of the proteasome (Djakovic et al., 2009). Purified CaMKIIα phosphorylated Rpt6 at serine 120 resulting in increased 26S proteasome activity in both neurons and HEK 293T cells (Djakovic et al., 2009). Phosphorylation of Rpt6 at serine 120 was found to be important for neuronal plasticity (Jarome et al., 2013). Activated CaMKIIα interacts with proteasome complex which is essential for proteasome redistribution to dendritic spines for degradation of polyubiquitinated proteins (Bingol et al., 2010; Djakovic et al., 2012; Hamilton et al., 2012). Interestingly in the presence of calcium, calmodulin binds to several proteasome subunits and proteasome-interacting proteins, which may alter 26S function allosterically without involvement of a kinase (Shen et al., 2005; Djakovic et al., 2009, 2012; Bingol et al., 2010). These findings are of particular relevance to the cardiac field as both intracellular calcium and activation of CaMKII are known to worsen cardiac disease pathogenesis (Anderson et al., 2011), although CaMKII regulation of the cardiac proteasomes and proteostasis in a diseased state is yet to be explored.

Dual-Specificity Tyrosine-Regulated Kinase 2 (DYRK2)

The proteasome regulates many cellular processes, including cell cycle progression where accumulating evidence suggests a pivotal role for proteasome phosphorylation. Guo and colleagues reported proteasomes purified from S phase of the cell cycle contained increased phosphorylated Rpt3 (of the 19S cap) at threonine 25 and remained high through G2 and M phases (Guo et al., 2016). Utilizing an unbiased screen of human kinases, they found that dual-specificity tyrosine-regulated kinase 2 (DYRK2) could catalyze Rpt3 phosphorylation at threonine 25 to promote cell proliferation (Guo et al., 2016). Prevention of this phosphorylation through expression of a phospho-null threonine to valine (Thr25Val) mutation in human breast cancer cells blocked the stimulation of the proteasomes by DYRK2, thus slowing the degradation of essential cell cycle regulatory factors like cyclin-dependent kinase inhibitor 1B ($p27^{Kip1}$) and cyclin-dependent kinase inhibitor 1 ($p21^{Cip1}$) (Guo et al., 2016). DYRK2 phosphorylation of Rpt3 threonine 25 increased substrate-stimulated ATP-hydrolysis, without changing basal ATPase activity, indicating that the modification promotes substrate translocation and degradation (Guo et al., 2016). The role of DYRK2 on the proteasome remains to be explored in striated muscle and as a potential therapeutic target for cardiac proteotoxicities.

Apoptosis Signal-Regulating Kinase 1 (ASK1)

Proteasome phosphorylation does not always result in increased activity. The ASK1 is a member of the mitogen-activated protein kinase kinase kinase (MKK) family and is activated by several cytotoxic stressors and apoptotic stimuli (Nishida et al., 2017). ASK1 negatively regulates the 26S proteasome under oxidative stress or apoptosis through phosphorylation of the 19S cap subunit Rpt5, however it remains unclear which sites of Rpt5 are modified by ASK1 (Um et al., 2010). Proteasome regulation by ASK1 could be an underlying mechanism of its role in cardiac pathogenesis. Deletion of ASK1 protected hearts from MI and PO as evidenced by decreased fibrotic remodeling and apoptosis with improved cardiac function (Yamaguchi et al., 2003). Similar results were obtained with pharmacological inhibition of ASK1 in a model of pulmonary arterial hypertension (Budas et al., 2018). Together these studies suggest the cardioprotective effects of ASK1 inhibition could be mediated by enhanced proteasome activities.

P38 Mitogen Activated Protein Kinase (MAPK)

The Rpn2 subunit of the 19S cap acts as a scaffold for other proteasome subunits and with other 19S subunits, regulates its gate opening of the 26S proteasome into the 20S core (Chen et al., 2016; Finley et al., 2016; Schweitzer et al., 2016). Phosphorylation of Rpn2 at threonine 273 by p38 MAPK suppresses proteasome function (Lee et al., 2010). It has been speculated that this phosphorylation of Rpn2 causes a conformational change, affecting the accessibility of substrates to the 20S core (Kors et al., 2019). Lee et al. (2010) reported purified 26S proteasomes from HeLa cells expressing activated p38 MAPK, had reduced proteolytic activities. The authors also showed that overexpression of an active mutant of p38 MAPK caused the accumulation of proteins that normally undergo rapid proteasomal degradation by ubiquitin-dependent and -independent pathways. p38 MAPK mediated phosphorylation of Rpn2 at threonine 273 is stimulated by sorbitol and NaCl-induced osmotic stress (Lee et al., 2010). Small molecule inhibitors of p38 MAPK and siRNA-mediated knockdown of

its α-isoform stimulated proteasome peptidase activity and intracellular degradation of α-synuclein in neurons (Leestemaker et al., 2017). Of note, Tsai et al. (2015) has shown that another MAPK, ERK 2, could also phosphorylate the 19S cap subunit Rpn2 at threonine 273 *in vitro*. While p38 is known to be involved in that pathogenesis of cardiac disease (Yokota and Wang, 2016) its effect on the proteasome in the heart remains to be elucidated.

Casein Kinase II (CK2)

Casein kinase II (CK2) is a pivotal regulator of the proteasome and is dysregulated in heart failure. CK2 is co-purified with the 20S proteasome and has been demonstrated to phosphorylate the α7 subunit at serines 243 and 250, which is associated with stabilization of the 26S proteasome (Ludemann et al., 1993; Castano et al., 1996; Bose et al., 2004). The phosphorylation of serines is important for the interaction between the 19S cap proteins with the 20S regulatory proteins of proteasome complex (Bose et al., 2004). Interestingly, phosphorylation of the 20S proteasome α7 subunit at serine 250 is found to be significantly less in the tissues from end stage heart failure patients, which is consistent with the diminished activity of the UPS in these patients (Hein et al., 2003; Weekes et al., 2003; Predmore et al., 2010). However, CK2 expression was found to be higher by almost 70% ($P = 0.019$) in failing hearts (Day et al., 2013). The possible explanation could be due to by increased expression of PP1 and PP2A in myocardium of such patients which can dephosphorylate this site (Neumann et al., 1997; Zong et al., 2006; Hamdani et al., 2010), or increased oxidation and carbonylation of CK2 resulting in diminished activity of the kinase (Murtaza et al., 2008).

C-Abl and Abl-Related Gene Product (ARG)

As key regulators of cardiac growth and development, C-Abl and ARG (Abl-related gene product) are multi-functional tyrosine kinases, which also directly phosphorylate and regulate the proteasome (Liu et al., 2006; Qiu et al., 2010; Li et al., 2016). Phosphorylation of the α4-subunit at tyrosine 153 by c-Abl led to the inhibition of the 20S and 26S proteasome and decreased the degradation of ubiquitinated short-lived proteins in mouse and human cells (Liu et al., 2006; Li et al., 2015). Activation of c-Abl by H_2O_2 or γ-irradiation increased its interaction with the α4-subunit and inhibited proteasome function (Liu et al., 2006). Expression of a phospho-null α4 subunit tyrosine 153 mutant in HEK 293T cells resulted in downregulation of several cell cycle regulatory proteins and G1/S cell cycle arrest, indicating the prominent role of c-Abl/ARG in cell cycle control (Liu et al., 2006). In a separate study, it was shown that phosphorylation of α4 at a different site, tyrosine 106, by c-Abl/ARG regulated the turnover of the proteasome subunit itself (Li et al., 2015). These interesting studies could provide insight into the role of the proteasome in cardiac growth and development as well as its regulation.

Protein Kinase G (PKG)

Activation of PKG protects the myocardium against pressure-overload and ischemia-reperfusion (Takimoto et al., 2005; Nagayama et al., 2009; Zhang and Kass, 2011; Sasaki et al., 2014; Kokkonen and Kass, 2017; Ranek et al., 2019). There are also a plethora of therapeutic strategies capable of activating PKG in the heart (Dunkerly-Eyring and Kass, 2019). PKG activators have shown such immense promise in pre-clinical studies that many clinical trials were initiated with various PKG activation strategies to treat human heart failure (Dunkerly-Eyring and Kass, 2019; Pinilla-Vera et al., 2019; Oeing et al., 2020a). Stimulation of PKG via pharmacological activation of the muscarinic 2 receptor or inhibition of phosphodiesterase 5A, positively regulates cardiomyocyte proteasomal proteolytic activity (Ranek et al., 2013, 2014). Stimulation of PKG enhanced proteasome-mediated degradation of a misfolded protein substrate (a modified GFP harboring a degron sequence) and a bona fide misfolded protein $CryAB^{R120G}$ (Ranek et al., 2013). The mechanism of action involved increased phosphorylation of the 20S subunit β5 and the 19S ATPase subunit Rpt6 upon PKG activation in cardiomyocytes (Ranek et al., 2013). The residues phosphorylated by PKG were not identified in this study. It is noteworthy that proteasome-mediated degradation of misfolded proteins was enhanced but not that of normal (properly folded) proteins with PKG stimulation, increasing the safety profile as a potential therapy (Ranek et al., 2013). Accordingly, PKG activators are safe and well-tolerated in human patients (Dunkerly-Eyring and Kass, 2019; Pinilla-Vera et al., 2019). A recent study that utilized human HFpEF myocardium biopsies found that *in vitro* treatment with an guanylyl cyclase 1 (GC-1) activator (to activate PKG) resulted in lower levels of inflammatory cytokines and oxidative stress (Kolijn et al., 2020). With oxidative stress being a known cause of protein damage and activation of the protein damage response, this reduction in oxidative stress can alleviate the burden on the PQC systems such as the proteasomal degradation pathway (Aiken et al., 2011). Taken together, the combination of promising pre-clinical studies, human clinical trials, and availability of pharmacological agents make activators of PKG an attractive therapeutic strategy for cardiac proteinopathies.

AUTOPHAGY

Autophagy is an intracellular self-degradative process responsible for the removal of misfolded proteins damaged organelles and by the lysosome. Cardiomyocytes utilize many forms of autophagy which differ by the method of degradation and the substrates targeted for degradation (**Figure 1**) (Sciarretta et al., 2018b). Macroautophagy begins with the formation of a double-membrane vesicle called phagophore, through a series of highly coordinated steps termed induction, nucleation and elongation, which engulf malformed proteins or whole organelles in the cytoplasm to form autophagosomes (Klionsky, 2005; Suzuki and Ohsumi, 2007; Levine and Kroemer, 2008). A fully formed autophagosome will then fuse with the acidic compartment of lysosome for the degradation of its cargo molecules (Sciarretta et al., 2018b). The non-selective lysosomal degradative process called microautophagy involves direct engulfment of cytoplasmic cargo at a boundary membrane by autophagic tubes, which mediate both invagination and vesicle scission into the lumen (Su et al., 2011). There are two types of autophagy in mammalian

cells which involves degradation of cellular components via a chaperone. Chaperone-mediated autophagy (CMA) is a selective autophagic process mediated by a HSC70 chaperone complex, wherein the proteins for degradation are selected based on a consensus sequence of amino acid sequence present on the surface of protein (Kaushik and Cuervo, 2018; Juste and Cuervo, 2019). On the other hand, CASA is a highly selective autophagy of misfolded proteins following a chaperone-mediated formation of protein aggregates that are targeted to form autophagosomes (Ulbricht et al., 2015). The vast majority of studies into the role of autophagy in the heart and disease pathogenesis have investigated macroautophagy. This section describes our current knowledge into the roles and regulations of autophagy by phosphorylation in cardiac disease, primarily discussing macroautophagy due to the limited understanding of the regulations of other forms of autophagy.

Evidence of autophagic impairment in human heart disease was first reported in tissue samples from patients with dilated cardiomyopathy (Shimomura et al., 2001). Autophagy is repressed in failing hearts as evidenced by the accumulation of numerous autophagic vacuoles containing cytoplasmic material and organelles to be degraded within the degenerated cardiomyocytes, which is thought to contribute to pathological remodeling and heart failure (Shimomura et al., 2001). Reduced autophagy is also implicated in cardiovascular decline and aging (Eisenberg et al., 2016; Shirakabe et al., 2016). Autophagy protects the heart during ischemia and starvation by supplying substrates for maintaining cellular bioenergetics (Matsui et al., 2007; Hariharan et al., 2010; Zhai et al., 2011; Sciarretta et al., 2012a; Kubli et al., 2013). Activation of autophagy has protected the myocardium against pathological cardiac hypertrophy and proteotoxicity (Gao et al., 2006; Kuzman et al., 2007; Flynn et al., 2013; Dai et al., 2014). These seminal studies utilized a genetic overexpression of an autophagy protein to stimulate autophagy, while recent studies have identified critical PTMs that regulate autophagy. More than 300 PTMs have been characterized for various autophagic proteins that influence their structure and function (Xie et al., 2015; Botti-Millet et al., 2016). Here, we discuss the role of phosphorylation as a critical PTM and its functional relevance in fine-tuning the autophagic process (**Figure 4**).

Phosphorylations Regulating Autophagic Flux

Mechanistic Target of Rapamycin Complex 1 (mTORC1)

Arguably the most well-defined regulator of macroautophagy is the mechanistic (mammalian) target of rapamycin complex 1 (mTORC1), an atypical serine/threonine kinase that also regulates cellular metabolism and bioenergetics. Pro-growth signals and a nutrient-rich environment are associated with mTORC1 activation which negatively regulate macroautophagy. Cardiac PO, obesity, and metabolic syndrome are associated with hyperactivation of mTORC1 (Choi et al., 2012; Li et al., 2012; Ramos et al., 2012; Guo et al., 2013; Pires et al., 2017). Indeed, the hearts from pressure overloaded mice, *ob/ob* obese mice, mice with hypertrophic cardiomyopathies, and hearts from a swine model of metabolic syndrome exhibit mTORC1 activation, depressed autophagic flux, and cardiac functional impairment, all of which were rescued by inhibiting mTORC1 via rapamycin administration (Choi et al., 2012; Li et al., 2012; Ramos et al., 2012; Guo et al., 2013; Pires et al., 2017).

Macroautophagy is influenced by mTORC1 by various methods and pathways. Autophagosome formation is reduced through mTORC1 phosphorylation of autophagy related (Atg) 13, which decreases its affinity for Atg1 (also known as Unc-51 like autophagy activating protein kinase, Ulk1), resulting in the dissociation of the Atg13-Atg1-FIP200 complex (Papinski and Kraft, 2016). Direct phosphorylation of Atg1 (Ulk1) by mTORC1 is another strategy by which mTORC1 suppresses autophagosome formation (Sciarretta et al., 2012b, 2018a). Recently protein phosphatase 2A (PP2A) was identified as a phosphatase for Ulk1 at serine 637, which counteracts the autophagy inhibiting action of mTORC1 (Wong et al., 2015). The Ambra1 is a positive regulator of autophagy that is inhibited by mTORC1 phosphorylation at serine 52 (Nazio et al., 2013). The role of Ambra1 is not well-defined in cardiac disease, however it has been associated with the pathogenesis of Alzheimer's Disease, aging, and tumor growth (Cianfanelli et al., 2015). Cardiomyocyte macroautophagy is tightly linked to the protein expression levels of Atg7 (Bhuiyan et al., 2013), which is negatively regulated by mTORC1 by reducing its expression level (Ramos et al., 2012). Overexpression of Atg7 enhances macroautophagy to protect the myocardium from cardiac proteinopathy, as evidenced by improved cardiac function and extended lifespan (Pattison et al., 2011). The TFEB is a master regulator, driving genes encoding autophagic proteins like ultraviolet radiation resistance associated gene (*UVRAG)*, WD repeat domain phosphoinositide-interacting protein (*WIPI)*, microtubule-associated protein 1 light chain 3 beta (*MAPLC3B), sequestosome 1 (SQSTM1), vacuolar protein sorting-associated protein 11 (VPS11), VPS18,* and *Atg9B* (Settembre et al., 2011). mTORC1 negatively regulates TFEB through phosphorylation at serines 142 and 211, which prevents the nuclear localization and transcriptional activity of TFEB and thereby decreasing macroautophagy (Napolitano et al., 2018). Buss et al. (2009) and others have shown that hyperactivated mTORC1 in the heart blunts autophagy and profoundly increases pathological cardiac remodeling in response to chronic ischemic injury, and can be reversed by direct mTORC1 inhibition with everolimus. Taneike et al. (2016) have shown that knocking out TSC2 (tuberin), an upstream negative regulator of mTORC1, results in constitutive stimulation of mTORC1 in the heart and subsequent cardiac chamber dilatation and dysfunction associated with an impairment of autophagic flux, which were reversed by pharmacological inhibition of mTORC1. GSK-3α knockout mice display premature death and age-related cardiac abnormalities, such as hypertrophy and sarcomere disruption, as a result of mTORC1 activation and autophagy inhibition (Zhou et al., 2013). Inactivation of Ras homolog enriched in brain (Rheb) (a positive mTORC1 regulator) protects cardiomyocytes during energy deprivation via depressing mTORC1 hyperactivation resulting in activation of autophagy, reduction of energy expenditure

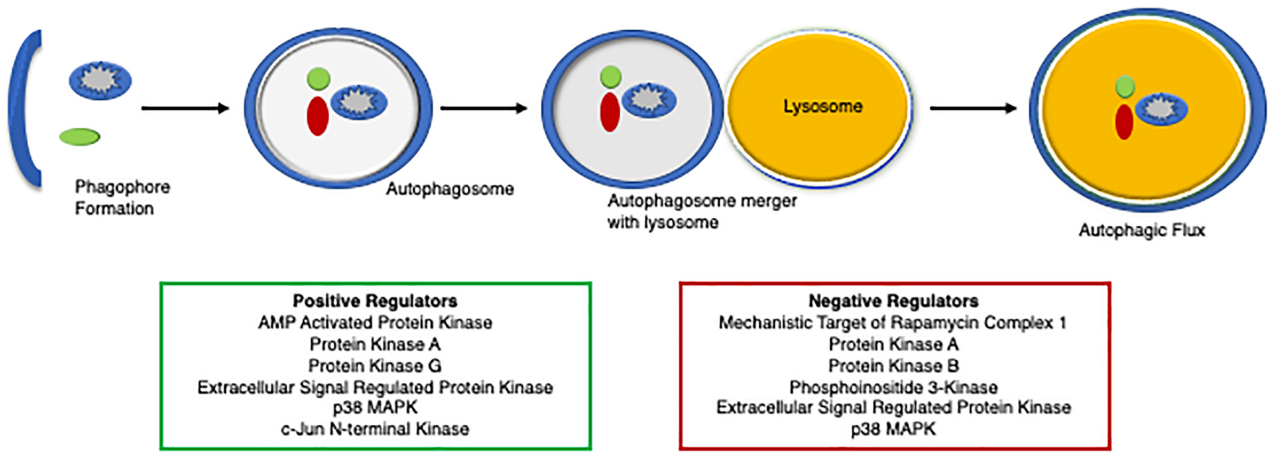


FIGURE 4 | A diagram of the regulators of autophagy. An overview of protein degradation via autophagy and its regulators, both positively (green box) and negatively (red box).

and attenuation of ER stress in high fat diet-induced metabolic syndrome (Sciarretta et al., 2012a). Similarly, long-term caloric restriction attenuates the LV diastolic dysfunction of the aged rat heart by reducing mTORC1 activity and enhancing autophagic flux (Shinmura et al., 2011). These studies demonstrate the regulatory capacity of mTORC1 on macroautophagy and cardioprotective effects that mTORC1 inhibition may have, however targeting mTORC1 as a treatment regimen is complex.

Considering mTORC1 is a critical regulator of many physiological processess, the role of mTORC1 and autophagy during cardiac development and pathogenesis is nuanced. Riehle et al. (2013) has shown that insulin/IGF-1 signaling through the insulin receptor substrate 1 proteins are vital to perinatal development of the heart. Reduced IRS signaling prevents the physiological activation of mTORC1 to suppress autophagy resulting in unrestrained autophagy in cardiomyocytes, which contributes to myocyte loss, heart failure, and premature death (Riehle et al., 2013). Knocking out raptor, the scaffolding protein of mTORC1, inhibits mTORC1 and leads to dilated cardiomyopathy (Shende et al., 2011). Furthermore, chronic mTORC1 inhibition with rapamycin is associated with immunosuppression (Thomson et al., 2009). Collectively these studies highlight the risk and reward that comes with broad mTORC1 inhibition. Inhibitors of mTORC1 that inhibit the pathological but not physiological roles of mTORC1 are of great interest.

AMP-Activated Protein Kinase (AMPK)

AMP-activated protein kinase is an intracellular metabolic energy sensor that functions to maintain homeostatic levels of ATP. Stimulation of AMPK results in inhibition of anabolic pathways consuming ATP and activation of catabolic pathways generating ATP. Cardiac insults such as myocardial ischemia, hypertrophy, and heart failure are associated with reduced intracellular ATP and subsequent activation of AMPK as a compensation to increase autophagy (Kubli and Gustafsson, 2014). Many studies have demonstrated that AMPK inhibits pathological cardiac hypertrophy by activating autophagy (Li et al., 2014). A study by Xie et al. (2011) suggested activation of AMPK and subsequent increase in cardiac autophagy reduced myocyte apoptosis in diabetic mouse hearts. AMPK activity was reduced in a mouse model of severe early-onset type-1 diabetes, which subsequently suppressed autophagy and increased cardiomyocyte apoptosis. Restoration of AMPK activity with metformin prevented the development of cardiomyopathy (Xie et al., 2011).

AMPK can stimulate autophagy through multiple mechanisms. AMPK activates TSC2 (an upstream negative of mTORC1) though phosphorylation at serine 1387 to inhibit mTORC1 (Inoki et al., 2003). AMPK also directly phosphorylates raptor (mTORC1 scaffold protein) at serines 722 and 792 to reduce mTORC1 activity by inducing 14-3-3 association (Gwinn et al., 2008). Atg1 (Ulk1) is positively and negatively regulated by phosphorylation. mTORC1-mediated phosphorylation of Ulk1 at serine 757 disrupts the Ulk1-AMPK interaction by inducing an unstructured and intrinsically disordered region in the protein (Kim J. et al., 2011; Shang et al., 2011; Khan and Kumar, 2012). Kim J. et al. (2011) have shown that under glucose starvation, AMPK promotes autophagy by phosphorylating Ulk1 at serines 317 and 777, thus activating Ulk1. Under conditions of mitochondrial stress, AMPK phosphorylates Ulk1 at serine 555 to enhance mitophagy by promoting translocation of Ulk1 to mitochondria. Tian et al. (2015) created a phosho-silenced Ulk1 by mutating serine 555, which blocked AMPK phosphorylation and subsequent mitophagy, demonstrating the necessity for this site to be targeted. AMPK phosphorylates beclin1 at serines 91 and 94 in response to glucose starvation, and serines 93 and 14 following exposure to ethanol (Kim et al., 2013; Hong-Brown et al., 2017). AMPK mediated phosphorylation of beclin1 at threonine 388 enhances the association of beclin1 with the VPS34-ATG14-VPS15 complex to stimulate autophagy and also reduces beclin1-Bcl2 complex formation (Zhang et al., 2016), which is known to inhibit autophagy and induce apoptosis. A beclin1^{T388A} phospho-silenced mutant suppresses autophagy by inhibiting its interaction with ATG14 (Zhang et al., 2016). The

role that phosphorylation of these sites on beclin1 has during cardiac disease remains to be explored but may hold potential as multiple studies demonstrated the important role of becin in the heart (Zhu and He, 2015; Maejima et al., 2016; Sun et al., 2018). AMPK is considered to be cardioprotective and is associated with enhancing cardiomyocyte PQC, thus represents a potential therapeutic target if the exact mechanisms of action can be well-defined during cardiac disease.

Protein Kinase A (PKA)

Autophagic flux has been reported to be negatively and positively regulated by PKA phosphorylation of various targets (Torres-Quiroz et al., 2015). In a nutrient-rich environment Atg1 is phosphorylated by PKA which keeps Atg1 largely cytosolic and dissociated from the pre-autophagosomal structure (PAS), which inhibits autophagosome formation to suppress autophagy. During starvation Atg1 is dephosphorylated and localized to the PAS to facilitate autophagy (He and Klionsky, 2009). Atg13 is an essential subunit of the Atg1 autophagy initiation complex, which following phosphorylation by PKA at serine 437 will translocate away from the PAS. Conversely, inactivation of PKA induces autophagy by reducing the inhibitory phosphorylations of Atg13 (serines 344, 437, and 581) and Atg1 (serines 508 and 515) allowing Atg13 localization at the PAS (Budovskaya et al., 2005; Stephan et al., 2009). PKA phosphorylation of microtubule-associated protein 1 light chain 3 (LC3) at serine 12 inhibits its lipidation, a critical step involved in the incorporation of LC3 into autophagosomes, resulting in inhibition of autophagy (Cherra et al., 2010). In a model of diabetic cardiomyopathy, Lin28a overexpression improved cardiac function and prevented apoptosis by activating PKA/RhoA/ROCK2-dependent signaling and by upregulating autophagy (Sun et al., 2016). Taken together, these studies suggest that, much like the positive and negative reports of stimulating autophagy in cardiac disease, PKA can also elicit protective or detrimental responses. These disparities are likely due to compartmentalization of activation within specific micro-domains. Further studies are needed to tease out the differential PKA regulation of autophagy.

Protein Kinase G (PKG)

The cardioprotective abilities of PKG have been demonstrated in the setting of various cardiac diseases including PO, MI, ischemia/reperfusion injury and is the subject of many clinical trials as a heart failure treatment (Kokkonen-Simon et al., 2018; Dunkerly-Eyring and Kass, 2019; Pinilla-Vera et al., 2019; Oeing et al., 2020b). ER stress during cardiac aging and heart failure was abrogated by activating PKG by way of sildenafil treatment (PDE5 inhibition) in isoproterenol-induced or TAC-induced hypertrophy, or swimming exercise (Gong et al., 2013; Chang et al., 2020). A new signaling paradigm was revealed in early 2019 whereby PKG phosphorylates TSC2 (aka tuberin) at serine 1365, which results in mTORC1 inhibition but only in the presence of a pathological stimulus (Ranek et al., 2019). Intriguingly, oxidation of PKG impairs the ability of PKG to target TSC2 S1365 in response to a pathological stimulus adding a layer of complexity to this pathway (Oeing et al., 2020b). Phosphorylation of TSC2 serine 1365 or expression of a knock in phosho-mimetic (S1365E) mouse had no effect on basal mTORC1 activity or macroautophagy, however during cardiac PO mTORC1 hyperactivation was blocked resulting in stimulated autophagic flux, reduced cardiomyocyte hypertrophy, and increased cardiac function and lifespan (Ranek et al., 2019). These findings are unique as inhibition of mTORC1, including phosphorylation regulations are associated with a total inhibition of mTORC1 activity, whereas modulation of TSC2 serine 1365 does not influence mTORC1 activity at baseline but has a potent suppression (phosphorylated or phospho-mimetic) or exacerbation (unphosphorylated or phospho-silenced) in the presence of a pathological hypertrophy stimulus (Ranek et al., 2019; Oeing et al., 2020b). In a recent study, activating PKG with an GC-1 activator has also been shown to reduce oxidative stress and enhance autophagy in H9c2 cardiomyocytes (Zhao et al., 2020). In this study, H9c2 cardiomyocytes were pretreated with an GC-1 activator, followed by doxorubicin, a known inducer of oxidative stress. These cells had an increase in autophagosome formation, indicating an increase in autophagic flux due to the activation of PKG. Collectively these findings make PKG an attractive therapeutic target.

Phosphoinositide 3-Kinase (PI3K) and Protein Kinase B (PKB)

Activation of the class I phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt) pathway negatively regulates autophagy during cardiomyocyte stress (Mellor et al., 2013). Macroautophagy has been associated with attenuation and exacerbation of cardiac disease following myocardial ischemia-reperfusion (I/R) injury, which appears to be due to the timing and the method of autophagy activation (Sciarretta et al., 2012b, 2018b). Excessive autophagy is deleterious to cardiac function, and as Li X. et al. (2018) demonstrated, stimulation of the PI3K/Akt/mTOR pathway reduced myocardial I/R injury by suppressing excessive autophagy. Similarly, in a 12-weeks of high fructose diet model Mellor et al. (2011) found that PI3K-Akt pathway suppression resulted in a dramatic upregulation of autophagy. A recent study noted that in mice subjected to aortic banding to induce left ventricular PO, there was enhanced Akt activity with reductions in autophagic flux and cardiac function, whereas inhibiting Akt activation increased autophagic flux an cardiac function (Xu et al., 2019). A study out of the Ghigo lab in 2018 investigated the role of PI3Kγ on autophagy in anthracycline induced cardiotoxicity (Li M. et al., 2018). Wild type mice treated with doxycycline exhibited increased PI3Kγ activity, Akt activation, and Ulk1 phosphorylation which were associated with reduced autophagic flux, cell survival, and cardiac function (Li M. et al., 2018). PI3Kγ-inhibited mice had suppressed Akt activation and Ulk1 phosphorylation which coupled to increased autophagic flux, cell survival, and cardiac function (Li M. et al., 2018). In addition to increasing mTORC1 activity, activation of the PI3K/Akt pathway also negatively regulates autophagy by phosphorylating and inhibiting the Ulk1/2 complex in diabetic hearts (Mellor et al., 2013). Additionally, Akt inhibits autophagy

through phosphorylation of forkhead transcription factor (FoxO) protein to impede transcription of autophagy related genes *LC3*, gamma-aminobutyric acid receptor-associated protein-like 1 (*Gabarapl1*), and *Atg12* (Sengupta et al., 2009).

Extracellular Signal Regulated Protein Kinase (ERK)

Activation of the ERK regulates autophagy through maturation of the autophagic vacuoles (Corcelle et al., 2007). Treatment with trastuzumab (Herceptin), a monoclonal antibody commonly used as a therapy in HER2+ breast cancer, activated the ERK/mTORC1 pathway and inhibited autophagy in human primary cardiomyocytes resulting in increased production of ROS. Trastuzumab treatment interferes with cardiac HER2 signaling that leads to the phosphorylation of HER1-Y845/HER2-Y1248 and the activation of Erk. This in turn results in upregulation of the mTOR–Ulk1 pathway to mediate inhibition of autophagy in cardiomyocytes (Mohan et al., 2016). ERK is a negative regulator of TSC2, thereby activating mTORC1 which is known to suppress autophagy and accelerate cellular growth (Huang and Manning, 2008; Sciarretta et al., 2014, 2018a; Wang et al., 2019). Mitogen-activated protein kinase kinase (MEK) inhibition counteracted the protective effects of rapamycin on the induction of autophagy and attenuation of phenylephrine (PE)-induced cardiac hypertrophy, suggesting that ERK can also inhibit autophagy independent of mTORC1 (Gu et al., 2016). A highly selective inhibitor of MEK/ERK (U0126), reduced excessive autophagy, apoptosis, and infarct size in a model of cardiac I/R injury via the MEK/ERK/EGR-1 pathway (Wang et al., 2016). Studies in renal tubular epithelial cells and malignant human breast cells demonstrated that ERK positively regulates autophagy by facilitating the transition from LC3-I to LC3-II and increased production of beclin1 (Choi et al., 2010; Zeng et al., 2012). Further studies are needed to further determine the role of ERK regulation of autophagy in cardiac disease and identify the targets by which this occurs.

p38 MAPK

Similar to ERK, both activation and inhibition of autophagy have been reported with p38 MAPK stimulation (Webber and Tooze, 2010b). Autophagy-related (Atg) 5 is a crucial protein for autophagy to proceed. Atg5 is phosphorylated at threonine 75 by p38 MAPK leading to the inhibition of starvation-induced autophagy (Keil et al., 2013). The authors went on to create Atg5 phospho-silenced (threonine 75 to alanine, T75A) and phospho-mimetic (threonine 75 to glutamic acid, T75E) constructs, and these were used to transfect Atg5 deficient mouse embryonic fibroblasts (MEFs), thus the only Atg5 being expressed was the mutant constructs. Atg5 T75A MEFs contained autophagosomes in both the starved and serum fed conditions, suggesting enhanced basal autophagy, while the Atg5 T75E MEFs showed no autophagosome formation in either condition and had decreased autophagic flux (Keil et al., 2013). Another mechanism of inhibition is following a proinflammatory signal, whereupon p38α MAPK phosphorylates Atg1 (Ulk1) at serines 504 and 757 to disrupt its functional complex with Atg13 and reduce autophagy (He et al., 2018). Similarly, inhibition of p38 MAPK in myelogenous leukemic K562 cells increased beclin 1 expression and induction of autophagy (Colosetti et al., 2009). Alternatively, p38 MAPK was shown to phosphorylate the tumor suppressor protein p53 at serine 392, which enhanced its transcriptional activity causing increased expression of beclin1 resulting in stimulation of autophagy (Liu et al., 2009; Younce and Kolattukudy, 2010; Duan et al., 2011). Accordingly, in cultured neonatal rat cardiomyocytes exposed to 48 h of mechanical stretch and in mice following TAC, p38 MAPK inhibition caused a decrease in the autophagy marker LC3-II, suggesting a positive p38-autophagy relationship (Lin et al., 2014). Furthermore, Webber and Tooze provided evidence that the mAtg9–p38 MAPK interaction is important for autophagy and is regulated by the p38α activity (Webber and Tooze, 2010a). They identified p38 as a mAtg9 interacting protein and showed the interaction of mAtg9–p38 is required for starvation-induced mAtg9 trafficking and autophagosome formation, allowing the process of autophagy to proceed (Webber and Tooze, 2010a). The regulation of autophagy by p38 MAPK appears to be driven by the nature of the stimulus with potential sub-domain specificity.

c-Jun N-Terminal Kinase (JNK)

Increased autophagy has been reported with activation of c-Jun N-terminal Kinase (JNK), which has been attributed to a compensatory mechanism (Borsello et al., 2003; Jia et al., 2006; Li et al., 2006). JNK-dependent pathways are involved in the pathological mechanisms of myocardial hypertrophy and ischemia/reperfusion injury (He et al., 1999; Sun et al., 2012; Javadov et al., 2014). Other studies have shown that JNK activation is transient and varies depending on the severity and timing of oxidative stress during ischemia and reperfusion (Knight and Buxton, 1996; Laderoute and Webster, 1997; He et al., 1999; Fryer et al., 2001; Armstrong, 2004). JNK stimulates autophagy by phosphorylating anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) (Chen et al., 2008; Wei et al., 2008a; Geeraert et al., 2010; Nopparat et al., 2010), which is typically bound to beclin1 thereby inhibiting autophagy (Pattingre et al., 2005), however once phosphorylated will dissociate from beclin1, resulting in the induction of autophagy (Wei et al., 2008b; Mrakovcic and Frohlich, 2018). JNK phosphorylation of Bcl-2 is associated with cell survival (Pattingre et al., 2009). JNK has also been shown to induce expression of pro-autophagic proteins SQSTM1 (p62), Atg5 and Atg7 in response to oxidative stress and oncogenic transformations as a method to enhance autophagy (Byun et al., 2009; Wong et al., 2010; Kim M. J. et al., 2011). JNK can also induce the expression of beclin1 via phosphorylation of c-Jun (Li et al., 2009; Ren et al., 2010). In contrast to the above mentioned pro-autophagic role of JNK, Xu et al. (2011) have demonstrated that targeted deletion of *JNK1*, *JNK2* and *JNK3* in neurons increases autophagy. Furthermore, in a diabetic cardiomyopathy model and in Angiotensin II-treated hearts, induction of miR-221 stimulated the transcription of JNK/c-Jun which resulted in depressed autophagy and exacerbated cardiac hypertrophy (Qian et al., 2017). Collectively, these studies demonstrate that much like the other MAP kinases, the regulatory role of JNK is context and stimuli dependent.

FUTURE DIRECTIONS

Therapeutic strategies to enhance cardiac PQC are attractive, presenting a new direction for treatment of cardiac disease. Post-translational modifications, specifically phosphorylation and dephosphorylation, of key PQC proteins add selectivity and specificity to the protein degradation processes and provide a unique opportunity for pharmacological intervention. Indeed, some studies described here have demonstrated the cardioprotective potential of PQC enhancers. Here we have summarized the studies that investigated cardiac PQC (**Supplementary Table 2**). We also detailed other kinases that currently have not been linked to a protective pharmacological strategy, but may have immense potential. Phosphorylation of the proteins responsible for maintaining cardiac PQC during a pathological stimulus has added a new mechanism of regulation to this powerful system. With new drugs to target kinases and phosphatases, and advances in gene editing to create phospho-mimetic and -silenced proteins, the opportunities to manipulate these PQC systems are endless. Thus, elucidating the phosphorylations that regulate cardiac PQC is essential to advance our understanding and provide new therapeutic opportunities to interrogate cardiomyocyte PQC to treat cardiac disease.

AUTHOR CONTRIBUTIONS

SM and BD-E wrote the manuscript in consultation with GK. MR conceived, designed, and directed the writing. All authors provided critical feedback, interpretation, and contributed to the final manuscript.

FUNDING

Supported by NIH – National Heart Lung and Blood Institute grant F31-HL143905 (BD-E), National Institute on Aging T32 training grant T32AG058527 (GK), American Heart Association 16SFRN28620000 (SM), NIH-NHLBI HL-135827 to David Kass, an American Heart Association Career Development Award 18CDA34110140 and Amyloidosis Foundation Donald C. Brockman Memorial Research Grant (MR).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.593585/full#supplementary-material

REFERENCES

Ahuja, P., Sdek, P., and MacLellan, W. R. (2007). Cardiac myocyte cell cycle control in development, disease, and regeneration. *Physiol. Rev.* 87, 521–544. doi: 10.1152/physrev.00032.2006

Aiken, C. T., Kaake, R. M., Wang, X., and Huang, L. (2011). Oxidative stress-mediated regulation of proteasome complexes. *Mol. Cell. Proteomics* 10:R110006924. doi: 10.1074/mcp.R110.006924

Anderson, M. E., Brown, J. H., and Bers, D. M. (2011). CaMKII in myocardial hypertrophy and heart failure. *J. Mol. Cell Cardiol.* 51, 468–473. doi: 10.1016/j.yjmcc.2011.01.012

Armstrong, S. C. (2004). Protein kinase activation and myocardial ischemia/reperfusion injury. *Cardiovasc. Res.* 61, 427–436. doi: 10.1016/j.cardiores.2003.09.031

Asai, M., Tsukamoto, O., Minamino, T., Asanuma, H., Fujita, M., Asano, Y., et al. (2009). PKA rapidly enhances proteasome assembly and activity in in vivo canine hearts. *J. Mol. Cell Cardiol.* 46, 452–462. doi: 10.1016/j.yjmcc.2008.11.001

Bachman, A. B., Keramisanou, D., Xu, W., Beebe, K., Moses, M. A., and Gelis, I. (2018). Phosphorylation induced cochaperone unfolding promotes kinase recruitment and client class-specific Hsp90 phosphorylation. *Nat. Commun.* 9:265. doi: 10.1038/s41467-017-02711-w

Baek, G. H., Cheng, H., Choe, V., Bao, X., Shao, J., Luo, S., et al. (2013). Cdc48: a swiss army knife of cell biology. *J. Amino Acids* 2013:183421. doi: 10.1155/2013/183421

Batuello, C. N., Hauck, P. M., Gendron, J. M., Lehman, J. A., and Mayo, L. D. (2015). Src phosphorylation converts Mdm2 from a ubiquitinating to a neddylating E3 ligase. *Proc. Natl. Acad. Sci. U.S.A.* 112, 1749–1754. doi: 10.1073/pnas.1416656112

Benedict, C. M., and Clawson, G. A. (1996). Nuclear multicatalytic proteinase subunit RRC3 is important for growth regulation in hepatocytes. *Biochemistry* 35, 11612–11621. doi: 10.1021/bi960889p

Bennett, M. R., Sinha, S., and Owens, G. K. (2016). Vascular smooth muscle cells in atherosclerosis. *Circ. Res.* 118, 692–702. doi: 10.1161/CIRCRESAHA.115.306361

Bhandari, P., Song, M., Chen, Y., Burelle, Y., and Dorn, G. W. (2014). Mitochondrial contagion induced by Parkin deficiency in Drosophila hearts and its containment by suppressing mitofusin. *Circ. Res.* 114, 257–265. doi: 10.1161/CIRCRESAHA.114.302734

Bhuiyan, M. S., Pattison, J. S., Osinska, H., James, J., Gulick, J., McLendon, P. M., et al. (2013). Enhanced autophagy ameliorates cardiac proteinopathy. *J. Clin. Invest.* 123, 5284–5297. doi: 10.1172/JCI70877

Bicknell, K. A., Surry, E. L., and Brooks, G. (2003). Targeting the cell cycle machinery for the treatment of cardiovascular disease. *J. Pharm. Pharmacol.* 55, 571–591. doi: 10.1211/002235703765344487

Bingol, B., Wang, C. F., Arnott, D., Cheng, D., Peng, J., and Sheng, M. (2010). Autophosphorylated CaMKIIalpha acts as a scaffold to recruit proteasomes to dendritic spines. *Cell* 140, 567–578. doi: 10.1016/j.cell.2010.01.024

Boehm, M., and Nabel, E. G. (2003). The cell cycle and cardiovascular diseases. *Prog. Cell Cycle Res.* 5, 19–30.

Borsello, T., Croquelois, K., Hornung, J. P., and Clarke, P. G. (2003). N-methyl-d-aspartate-triggered neuronal death in organotypic hippocampal cultures is endocytic, autophagic and mediated by the c-Jun N-terminal kinase pathway. *Eur. J. Neurosci.* 18, 473–485. doi: 10.1046/j.1460-9568.2003.02757.x

Bose, S., Stratford, F. L., Broadfoot, K. I., Mason, G. G., and Rivett, A. J. (2004). Phosphorylation of 20S proteasome alpha subunit C8 (alpha7) stabilizes the 26S proteasome and plays a role in the regulation of proteasome complexes by gamma-interferon. *Biochem. J.* 378, 177–184. doi: 10.1042/bj20031122

Botti-Millet, J., Nascimbeni, A. C., Dupont, N., Morel, E., and Codogno, P. (2016). Fine-tuning autophagy: from transcriptional to posttranslational regulation. *Am. J. Physiol. Cell Physiol.* 311, C351–C362. doi: 10.1152/ajpcell.00129.2016

Budas, G. R., Boehm, M., Kojonazarov, B., Viswanathan, G., Tian, X., Veeroju, S., et al. (2018). ASK1 inhibition halts disease progression in preclinical models of pulmonary arterial hypertension. *Am. J. Respir. Crit. Care Med.* 197, 373–385. doi: 10.1164/rccm.201703-0502OC

Budenholzer, L., Cheng, C. L., Li, Y., and Hochstrasser, M. (2017). Proteasome structure and assembly. *J. Mol. Biol.* 429, 3500–3524. doi: 10.1016/j.jmb.2017.05.027

Budovskaya, Y. V., Stephan, J. S., Deminoff, S. J., and Herman, P. K. (2005). An evolutionary proteomics approach identifies substrates of the cAMP-dependent

protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* 102, 13933–13938. doi: 10.1073/pnas.0501046102

Buss, S. J., Muenz, S., Riffel, J. H., Malekar, P., Hagenmueller, M., and Weiss, C. S. (2009). Beneficial effects of Mammalian target of rapamycin inhibition on left ventricular remodeling after myocardial infarction. *J. Am. Coll. Cardiol.* 54, 2435–2446. doi: 10.1016/j.jacc.2009.08.031

Byun, J. Y., Yoon, C. H., An, S., Park, I. C., Kang, C. M., Kim, M. J., et al. (2009). The Rac1/MKK7/JNK pathway signals upregulation of Atg5 and subsequent autophagic cell death in response to oncogenic Ras. *Carcinogenesis* 30, 1880–1888. doi: 10.1093/carcin/bgp235

Castano, J. G., Mahillo, E., Arizti, P., and Arribas, J. (1996). Phosphorylation of C8 and C9 subunits of the multicatalytic proteinase by casein kinase II and identification of the C8 phosphorylation sites by direct mutagenesis. *Biochemistry* 35, 3782–3789. doi: 10.1021/bi952540s

Chang, P., Zhang, X., Zhang, M., Li, G., Hu, L., Zhao, H., et al. (2020). Swimming exercise inhibits myocardial ER stress in the hearts of aged mice by enhancing cGMPPKG signaling. *Mol. Med. Rep.* 21, 549–556. doi: 10.3892/mmr.2019.10864

Chen, J., Ren, Y., Gui, C., Zhao, M., Wu, X., Mao, K., et al. (2018). Phosphorylation of Parkin at serine 131 by p38 MAPK promotes mitochondrial dysfunction and neuronal death in mutant A53T alpha-synuclein model of Parkinson's disease. *Cell Death Dis.* 9:700. doi: 10.1038/s41419-018-0722-7

Chen, J. L., Lin, H. H., Kim, K. J., Lin, A., Forman, H. J., and Ann, D. K. (2008). Novel roles for protein kinase Cdelta-dependent signaling pathways in acute hypoxic stress-induced autophagy. *J. Biol. Chem.* 283, 34432–34444. doi: 10.1074/jbc.M804239200

Chen, L., Lizano, P., Zhao, X., Sui, X., Dhar, S. K., Shen, Y. T., et al. (2011). Preemptive conditioning of the swine heart by H11 kinase/Hsp22 provides cardiac protection through inducible nitric oxide synthase. *Am. J. Physiol. Heart Circ. Physiol.* 300, H1303–H1310. doi: 10.1152/ajpheart.00979.2010

Chen, S., Wu, J., Lu, Y., Ma, Y. B., Lee, B. H., Yu, Z., et al. (2016). Structural basis for dynamic regulation of the human 26S proteasome. *Proc. Natl. Acad. Sci. U.S.A.* 113, 12991–12996. doi: 10.1073/pnas.1614614113

Cherra, S. J. III, Kulich, S. M., Uechi, G., Balasubramani, M., Mountzouris, J., Day, B. W., et al. (2010). Regulation of the autophagy protein LC3 by phosphorylation. *J. Cell Biol.* 190, 533–539. doi: 10.1083/jcb.201002108

Choi, C. H., Jung, Y. K., and Oh, S. H. (2010). Autophagy induction by capsaicin in malignant human breast cells is modulated by p38 and extracellular signal-regulated mitogen-activated protein kinases and retards cell death by suppressing endoplasmic reticulum stress-mediated apoptosis. *Mol. Pharmacol.* 78, 114–125. doi: 10.1124/mol.110.063495

Choi, J. C., Wu, W., Muchir, A., Iwata, S., Homma, S., and Worman, H. J. (2012). Dual specificity phosphatase 4 mediates cardiomyopathy caused by lamin A/C (LMNA) gene mutation. *J. Biol. Chem.* 287, 40513–40524. doi: 10.1074/jbc.M112.404541

Christians, E. S., and Benjamin, I. J. (2012). Proteostasis and REDOX state in the heart. *Am. J. Physiol. Heart Circ. Physiol.* 302, H24–H37. doi: 10.1152/ajpheart.00903.2011

Cianfanelli, V., Nazio, F., and Cecconi, F. (2015). Connecting autophagy: AMBRA1 and its network of regulation. *Mol. Cell Oncol.* 2:e970059. doi: 10.4161/23723548.2014.970059

Ciechanover, A. (1994). The ubiquitin-proteasome proteolytic pathway. *Cell* 79, 13–21. doi: 10.1016/0092-8674(94)90396-4

Collier, M. P., Alderson, T. R., Nicholls, D., Gastall, H. Y., Allison, T. M., and Degiacomi, M. T. (2019). HspB1 phosphorylation regulates its intramolecular dynamics and mechanosensitive molecular chaperone interaction with filamin C. *Sci. Adv.* 5:eaav8421. doi: 10.1126/sciadv.aav8421

Collins, G. A., and Goldberg, A. L. (2017). The logic of the 26S proteasome. *Cell* 169, 792–806. doi: 10.1016/j.cell.2017.04.023

Colosetti, P., Puissant, A., Robert, G., Luciano, F., Jacquel, A., Gounon, P., et al. (2009). Autophagy is an important event for megakaryocytic differentiation of the chronic myelogenous leukemia K562 cell line. *Autophagy* 5, 1092–1098. doi: 10.4161/auto.5.8.9889

Corcelle, E., Djerbi, N., Mari, M., Nebout, M., Fiorini, C., Fenichel, P., et al. (2007). Control of the autophagy maturation step by the MAPK ERK and p38: lessons from environmental carcinogens. *Autophagy* 3, 57–59. doi: 10.4161/auto.3424

Dagar, M., Singh, J. P., Dagar, G., Tyagi, R. K., and Bagchi, G. (2019). Phosphorylation of HSP90 by protein kinase A is essential for the nuclear translocation of androgen receptor. *J. Biol. Chem.* 294, 8699–8710. doi: 10.1074/jbc.RA119.007420

Dahlmann, B. (2016). Mammalian proteasome subtypes: their diversity in structure and function. *Arch. Biochem. Biophys.* 591, 132–140. doi: 10.1016/j.abb.2015.12.012

Dai, D. F., Karunadharma, P. P., Chiao, Y. A., Basisty, N., Crispin, D., Hsieh, E. J., et al. (2014). Altered proteome turnover and remodeling by short-term caloric restriction or rapamycin rejuvenate the aging heart. *Aging Cell* 13, 529–539. doi: 10.1111/acel.12203

Day, S. M. (2013). The ubiquitin proteasome system in human cardiomyopathies and heart failure. *Am. J. Physiol. Heart Circ. Physiol.* 304, H1283–H1293. doi: 10.1152/ajpheart.00249.2012

Day, S. M., Divald, A., Wang, P., Davis, F., Bartolone, S., Jones, R., et al. (2013). Impaired assembly and post-translational regulation of 26S proteasome in human end-stage heart failure. *Circ, Heart Fail.* 6, 544–549. doi: 10.1161/CIRCHEARTFAILURE.112.000119

Depre, C., Wang, L., Sui, X., Qiu, H., Hong, C., Hedhli, N., et al. (2006). H11 kinase prevents myocardial infarction by preemptive preconditioning of the heart. *Circ. Res.* 98, 280–288. doi: 10.1161/01.RES.0000201284.45482.e8

Divald, A., and Powell, S. R. (2006). Proteasome mediates removal of proteins oxidized during myocardial ischemia. *Free Radic. Biol. Med.* 40, 156–164. doi: 10.1016/j.freeradbiomed.2005.09.022

Djakovic, S. N., Marquez-Lona, E. M., Jakawich, S. K., Wright, R., Chu, C., Sutton, M. A., et al. (2012). Phosphorylation of Rpt6 regulates synaptic strength in hippocampal neurons. *J. Neurosci.* 32, 5126–5131. doi: 10.1523/JNEUROSCI.4427-11.2012

Djakovic, S. N., Schwarz, L. A., Barylko, B., DeMartino, G. N., and Patrick, G. N. (2009). Regulation of the proteasome by neuronal activity and calcium/calmodulin-dependent protein kinase II. *J. Biol. Chem.* 284, 26655–26665. doi: 10.1074/jbc.M109.021956

Drews, O., Tsukamoto, O., Liem, D., Streicher, J., Wang, Y., and Ping, P. (2010). Differential regulation of proteasome function in isoproterenol-induced cardiac hypertrophy. *Circ. Res.* 107, 1094–1101. doi: 10.1161/CIRCRESAHA.110.222364

Drews, O., Wildgruber, R., Zong, C., Sukop, U., Nissum, M., Weber, G., et al. (2007). Mammalian proteasome subpopulations with distinct molecular compositions and proteolytic activities. *Mol. Cell. Proteomics* 6, 2021–2031. doi: 10.1074/mcp.M700187-MCP200

Duan, W. J., Li, Q. S., Xia, M. Y., Tashiro, S., Onodera, S., and Ikejima, T. (2011). Silibinin activated p53 and induced autophagic death in human fibrosarcoma HT1080 cells via reactive oxygen species-p38 and c-Jun N-terminal kinase pathways. *Biol. Pharm. Bull.* 34, 47–53. doi: 10.1248/bpb.34.47

Dunkerly-Eyring, B., and Kass, D. A. (2019). Myocardial phosphodiesterases and their role in cGMP regulation. *J. Cardiovasc. Pharmacol.* 75, 483–493. doi: 10.1097/FJC.0000000000000773

Dworniczak, B., and Mirault, M. E. (1987). Structure and expression of a human gene coding for a 71 kd heat shock 'cognate' protein. *Nucleic Acids Res.* 15, 5181–5197. doi: 10.1093/nar/15.13.5181

Eisenberg, T., Abdellatif, M., Schroeder, S., Primessnig, U., Stekovic, S., Pendl, T., et al. (2016). Cardioprotection and lifespan extension by the natural polyamine spermidine. *Nat. Med.* 22, 1428–1438. doi: 10.1038/nm.4222

Elangovan, M., Chong, H. K., Park, J. H., Yeo, E. J., and Yoo, Y. J. (2017). The role of ubiquitin-conjugating enzyme Ube2j1 phosphorylation and its degradation by proteasome during endoplasmic stress recovery. *J. Cell Commun. Signal.* 11, 265–273. doi: 10.1007/s12079-017-0386-6

Enchev, R. I., Schulman, B. A., and Peter, M. (2015). Protein neddylation: beyond cullin-RING ligases. *Nat. Rev. Mol. Cell Biol.* 16, 30–44. doi: 10.1038/nrm3919

Fan, G. C., Chu, G., Mitton, B., Song, Q., Yuan, Q., and Kranias, E. G. (2004). Small heat-shock protein Hsp20 phosphorylation inhibits beta-agonist-induced cardiac apoptosis. *Circ. Res.* 94, 1474–1482. doi: 10.1161/01.RES.0000129179.66631.00

Finka, A., Sharma, S. K., and Goloubinoff, P. (2015). Multi-layered molecular mechanisms of polypeptide holding, unfolding and disaggregation by HSP70/HSP110 chaperones. *Front. Mol. Biosci.* 2:29. doi: 10.3389/fmolb.2015.00029

Finley, D., Chen, X., and Walters, K. J. (2016). Gates, channels, and switches: elements of the proteasome machine. *Trends Biochem. Sci.* 41, 77–93. doi: 10.1016/j.tibs.2015.10.009

Flynn, J. M., O'Leary, M. N., Zambataro, C. A., Academia, E. C., Presley, M. P., and Garrett, B. J. (2013). Late-life rapamycin treatment reverses age-related heart dysfunction. *Aging Cell* 12, 851–862. doi: 10.1111/acel.12109

Fouladkou, F., Lu, C., Jiang, C., Zhou, L., She, Y., Walls, J. R., et al. (2010). The ubiquitin ligase Nedd4-1 is required for heart development and is a suppressor of thrombospondin-1. *J. Biol. Chem.* 285, 6770–6780. doi: 10.1074/jbc.M109.082347

Fryer, R. M., Patel, H. H., Hsu, A. K., and Gross, G. J. (2001). Stress-activated protein kinase phosphorylation during cardioprotection in the ischemic myocardium. *Am. J. Physiol. Heart Circ. Physiol.* 281, H1184–H1192. doi: 10.1152/ajpheart.2001.281.3.H1184

Gao, X. M., Wong, G., Wang, B., Kiriazis, H., Moore, X. L., Su, Y. D., et al. (2006). Inhibition of mTOR reduces chronic pressure-overload cardiac hypertrophy and fibrosis. *J. Hypertens.* 24, 1663–1670. doi: 10.1097/01.hjh.0000239304.01496.83

Gardner, G. T., Travers, J. G., Qian, J., Liu, G. S., Haghighi, K., Robbins, N., et al. (2019). Phosphorylation of Hsp20 promotes fibrotic remodeling and heart failure. *JACC Basic Transl. Sci.* 4, 188–199. doi: 10.1016/j.jacbts.2018.11.007

Geeraert, C., Ratier, A., Pfisterer, S. G., Perdiz, D., Cantaloube, I., Rouault, A., et al. (2010). Starvation-induced hyperacetylation of tubulin is required for the stimulation of autophagy by nutrient deprivation. *J. Biol. Chem.* 285, 24184–24194. doi: 10.1074/jbc.M109.091553

George, A. J., Hoffiz, Y. C., Charles, A. J., Zhu, Y., and Mabb, A. M. (2018). A comprehensive atlas of E3 ubiquitin ligase mutations in neurological disorders. *Front. Genet.* 9:29. doi: 10.3389/fgene.2018.00029

Ghosh, R., and Pattison, J. S. (2018). Macroautophagy and chaperone-mediated autophagy in heart failure: the known and the unknown. *Oxid. Med. Cell. Longev.* 2018:8602041. doi: 10.1155/2018/8602041

Gilda, J. E., and Gomes, A. V. (2017). Proteasome dysfunction in cardiomyopathies. *J. Physiol.* 595, 4051–4071. doi: 10.1113/JP273607

Goldberg, A. L. (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature* 426, 895–899. doi: 10.1038/nature02263

Gomes, A. V., Young, G. W., Wang, Y., Zong, C., Eghbali, M., Drews, O., et al. (2009). Contrasting proteome biology and functional heterogeneity of the 20 S proteasome complexes in mammalian tissues. *Mol. Cell. Proteom.* 8, 302–315. doi: 10.1074/mcp.M800058-MCP200

Gomes, A. V., Zong, C., Edmondson, R. D., Li, X., Stefani, E., Zhang, J., et al. (2006). Mapping the murine cardiac 26S proteasome complexes. *Circ. Res.* 99, 362–371. doi: 10.1161/01.RES.0000237386.98506.f7

Gong, W., Duan, Q., Cai, Z., Chen, C., Ni, L., Yan, M., et al. (2013). Chronic inhibition of cGMP-specific phosphodiesterase 5 suppresses endoplasmic reticulum stress in heart failure. *Br. J. Pharmacol.* 170, 1396–1409. doi: 10.1111/bph.12346

Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D., et al. (1997). Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature* 386, 463–471. doi: 10.1038/386463a0

Gu, J., Hu, W., Song, Z. P., Chen, Y. G., Zhang, D. D., and Wang, C. Q. (2016). Rapamycin inhibits cardiac hypertrophy by promoting autophagy via the MEK/ERK/Beclin-1 pathway. *Front. Physiol.* 7:104. doi: 10.3389/fphys.2020.00104

Guo, R., Zhang, Y., Turdi, S., and Ren, J. (2013). Adiponectin knockout accentuates high fat diet-induced obesity and cardiac dysfunction: role of autophagy. *Biochim. Biophys. Acta* 1832, 1136–1148. doi: 10.1016/j.bbadis.2013.03.013

Guo, X., Engel, J. L., Xiao, J., Tagliabracci, V. S., Wang, X., Huang, L., et al. (2011). UBLCP1 is a 26S proteasome phosphatase that regulates nuclear proteasome activity. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18649–18654. doi: 10.1073/pnas.1113170108

Guo, X., Huang, X., and Chen, M. J. (2017). Reversible phosphorylation of the 26S proteasome. *Protein Cell* 8, 255–272. doi: 10.1007/s13238-017-0382-x

Guo, X., Wang, X., Wang, Z., Banerjee, S., Yang, J., Huang, L., et al. (2016). Site-specific proteasome phosphorylation controls cell proliferation and tumorigenesis. *Nat. Cell Biol.* 18, 202–212. doi: 10.1038/ncb3289

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., et al. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell.* 30, 214–226. doi: 10.1016/j.molcel.2008.03.003

Hamdani, N., Borbely, A., Veenstra, S. P., Kooij, V., Vrydag, W., Zaremba, R., et al. (2010). More severe cellular phenotype in human idiopathic dilated cardiomyopathy compared to ischemic heart disease. *J. Muscle Res. Cell Motil.* 31, 289–301. doi: 10.1007/s10974-010-9231-8

Hamilton, A. M., Oh, W. C., Vega-Ramirez, H., Stein, I. S., Hell, J. W., Patrick, G. N., et al. (2012). Activity-dependent growth of new dendritic spines is regulated by the proteasome. *Neuron* 74, 1023–1030. doi: 10.1016/j.neuron.2012.04.031

Hariharan, N., Maejima, Y., Nakae, J., Paik, J., Depinho, R. A., and Sadoshima, J. (2010). Deacetylation of FoxO by sirt1 plays an essential role in mediating starvation-induced autophagy in cardiac myocytes. *Circ. Res.* 107, 1470–1482. doi: 10.1161/CIRCRESAHA.110.227371

Haslbeck, V., Eckl, J. M., Drazic, A., Rutz, D. A., Lorenz, O. R., Zimmermann, K., et al. (2015). The activity of protein phosphatase 5 towards native clients is modulated by the middle- and C-terminal domains of Hsp90. *Sci. Rep.* 5:17058. doi: 10.1038/srep17058

He, C., and Klionsky, D. J. (2009). Regulation mechanisms and signaling pathways of autophagy. *Annu. Rev. Genet.* 43, 67–93. doi: 10.1146/annurev-genet-102808-114910

He, H., Li, H. L., Lin, A., and Gottlieb, R. A. (1999). Activation of the JNK pathway is important for cardiomyocyte death in response to simulated ischemia. *Cell Death. Differ.* 6, 987–991. doi: 10.1038/sj.cdd.4400572

He, Y., She, H., Zhang, T., Xu, H., Cheng, L., Yepes, M., et al. (2018). p38 MAPK inhibits autophagy and promotes microglial inflammatory responses by phosphorylating ULK1. *J. Cell Biol.* 217, 315–328. doi: 10.1083/jcb.201701049

Hein, S., Arnon, E., Kostin, S., Schonburg, M., Elsasser, A., Polyakova, V., et al. (2003). Progression from compensated hypertrophy to failure in the pressure-overloaded human heart: structural deterioration and compensatory mechanisms. *Circulation* 107, 984–991. doi: 10.1161/01.CIR.0000051865.66123.B7

Henning, R. H., and Brundel, B. (2017). Proteostasis in cardiac health and disease. *Nat. Rev. Cardiol.* 14, 637–653. doi: 10.1038/nrcardio.2017.89

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–479. doi: 10.1146/annurev.biochem.67.1.425

Hohfeld, J., Cyr, D. M., and Patterson, C. (2001). From the cradle to the grave: molecular chaperones that may choose between folding and degradation. *EMBO Rep.* 2, 885–890. doi: 10.1093/embo-reports/kve206

Hong-Brown, L. Q., Brown, C. R., Navaratnarajah, M., and Lang, C. H. (2017). FoxO1-AMPK-ULK1 regulates ethanol-induced autophagy in muscle by enhanced ATG14 association with the BECN1-PIK3C3 complex. *Alcohol. Clin. Exp. Res.* 41, 895–910. doi: 10.1111/acer.13377

House, S. L., House, B. E., Glascock, B., Kimball, T., Nusayr, E., Schultz, J. E., et al. (2010). Fibroblast growth factor 2 mediates isoproterenol-induced cardiac hypertrophy through activation of the extracellular regulated kinase. *Mol. Cell. Pharmacol.* 2, 143–154.

Huang, C. K., Lee, S. O., Chang, E., Pang, H., and Chang, C. (2016). Androgen receptor (AR) in cardiovascular diseases. *J. Endocrinol.* 229, R1–R16. doi: 10.1530/JOE-15-0518

Huang, J., and Manning, B. D. (2008). The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. *Biochem. J.* 412, 179–190. doi: 10.1042/BJ20080281

Inoki, K., Zhu, T., and Guan, K. L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. *Cell* 115, 577–590. doi: 10.1016/S0092-8674(03)00929-2

Itoh, N., and Ohta, H. (2013). Pathophysiological roles of FGF signaling in the heart. *Front. Physiol.* 4:247. doi: 10.3389/fphys.2013.00247

Jarome, T., Kwapis, J., Ruenzel, W., and Helmstetter, F. (2013). CaMKII, but not protein kinase A, regulates Rpt6 phosphorylation and proteasome activity during the formation of long-term memories. *Front. Behav. Neurosci.* 7:115. doi: 10.3389/fnbeh.2013.00115

Javadov, S., Jang, S., and Agostini, B. (2014). Crosstalk between mitogen-activated protein kinases and mitochondria in cardiac diseases: therapeutic perspectives. *Pharmacol. Ther.* 144, 202–225. doi: 10.1016/j.pharmthera.2014.05.013

Jentsch, S. (1992). Ubiquitin-dependent protein degradation: a cellular perspective. *Trends Cell. Biol.* 2, 98–103. doi: 10.1016/0962-8924(92)90013-D

Jia, G., Cheng, G., Gangahar, D. M., and Agrawal, D. K. (2006). Insulin-like growth factor-1 and TNF-alpha regulate autophagy through c-jun N-terminal kinase and Akt pathways in human atherosclerotic vascular smooth cells. *Immunol. Cell Biol.* 84, 448–454. doi: 10.1111/j.1440-1711.2006.01454.x

Juste, Y. R., and Cuervo, A. M. (2019). Analysis of chaperone-mediated autophagy. *Methods Mol. Biol.* 1880, 703–727. doi: 10.1007/978-1-4939-8873-0_47

Kaushik, S., and Cuervo, A. M. (2018). The coming of age of chaperone-mediated autophagy. *Nat. Rev. Mol. Cell Biol.* 19, 365–381. doi: 10.1038/s41580-018-0001-6

Keil, E., Hocker, R., Schuster, M., Essmann, F., Ueffing, N., Hoffman, B., et al. (2013). Phosphorylation of Atg5 by the Gadd45beta-MEKK4-p38 pathway inhibits autophagy. *Cell Death. Differ.* 20, 321–332. doi: 10.1038/cdd.2012.129

Khan, S. H., and Kumar, R. (2012). Role of an intrinsically disordered conformation in AMPK-mediated phosphorylation of ULK1 and regulation of autophagy. *Mol. Biosyst.* 8, 91–96. doi: 10.1039/C1MB05265A

Kim, J., Kim, Y. C., Fang, C., Russell, R. C., Kim, J. H., Fan, W., et al. (2013). Differential regulation of distinct Vps34 complexes by AMPK in nutrient stress and autophagy. *Cell* 152, 290–303. doi: 10.1016/j.cell.2012.12.016

Kim, J., Kundu, M., Viollet, B., and Guan, L. K. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat. Cell Biol.* 13, 132–141. doi: 10.1038/ncb2152

Kim, M. J., Woo, S. J., Yoon, C. H., Lee, J. S., An, S., Choi, Y. H., et al. (2011). Involvement of autophagy in oncogenic K-Ras-induced malignant cell transformation. *J. Biol. Chem.* 286, 12924–12932. doi: 10.1074/jbc.M110.138958

Klein, J. B., Barati, M. T., Wu, R., Gozal, D., and Sachleben, L. R. Jr. (2005). Akt-mediated valosin-containing protein 97 phosphorylation regulates its association with ubiquitinated proteins. *J. Biol. Chem.* 280, 31870–31881. doi: 10.1074/jbc.M501802200

Klionsky, D. J. (2005). The molecular machinery of autophagy: unanswered questions. *J. Cell Sci.* 118, 7–18. doi: 10.1242/jcs.01620

Knight, R. J., and Buxton, D. B. (1996). Stimulation of c-Jun kinase and mitogen-activated protein kinase by ischemia and reperfusion in the perfused rat heart. *Biochem. Biophys. Res. Commun.* 218, 83–88. doi: 10.1006/bbrc.1996.0016

Kokkonen, K., and Kass, D. A. (2017). Nanodomain regulation of cardiac cyclic nucleotide signaling by phosphodiesterases. *Annu. Rev. Pharmacol. Toxicol.* 57, 455–479. doi: 10.1146/annurev-pharmtox-010716-104756

Kokkonen-Simon, K. M., Saberi, A., Nakamura, T., Ranek, M. J., Zhu, G., Bedja, D., et al. (2018). Marked disparity of microRNA modulation by cGMP-selective PDE5 versus PDE9 inhibitors in heart disease. *JCI Insight.* 3:e121739. doi: 10.1172/jci.insight.121739

Kolijn, D., Kovacs, A., Herwig, M., Lodi, M., Sieme, M., Alhaj, A., et al. (2020). Enhanced cardiomyocyte function in hypertensive rats with diastolic dysfunction and human heart failure patients after acute treatment with soluble guanylyl cyclase (sGC) Activator. *Front. Physiol.* 11:345. doi: 10.3389/fphys.2020.00345

Kong, S. K., and Chock, P. B. (1992). Protein ubiquitination is regulated by phosphorylation. An in vitro study. *J. Biol. Chem.* 267, 14189–14192.

Kors, S., Geijtenbeek, K., Reits, E., and Schipper-Krom, S. (2019). Regulation of proteasome activity by (Post-)transcriptional mechanisms. *Front. Mol. Biosci.* 6:48. doi: 10.3389/fmolb.2019.00048

Kostenko, S., Jensen, K. L., and Moens, U. (2014). Phosphorylation of heat shock protein 40 (Hsp40/DnaJB1) by mitogen-activated protein kinase-activated protein kinase 5 (MK5/PRAK). *Int. J. Biochem. Cell Biol.* 47, 29–37. doi: 10.1016/j.biocel.2013.11.004

Kotter, S., Unger, A., Hamdani, N., Lang, P., Vorgerd, M., Nagel-Steger, L., et al. (2014). Human myocytes are protected from titin aggregation-induced stiffening by small heat shock proteins. *J. Cell Biol.* 204, 187–202. doi: 10.1083/jcb.201306077

Kraemer, B. F., Mannell, H., Lamkemeyer, T., Franz-Wachtel, M., and Lindemann, S. (2019). Heat-shock protein 27 (HSPB1) is upregulated and phosphorylated in human platelets during ST-elevation myocardial infarction. *Int. J. Mol. Sci.* 20:5968. doi: 10.3390/ijms20235968

Kubli, D. A., and Gustafsson, ÅB. (2014). Cardiomyocyte health: adapting to metabolic changes through autophagy. *Trends Endocrinol. Metab.* 25, 156–164. doi: 10.1016/j.tem.2013.11.004

Kubli, D. A., Zhang, X., Lee, Y., Hanna, R. A., Quinsay, M. N., Nguyen, C. K., et al. (2013). Parkin protein deficiency exacerbates cardiac injury and reduces survival following myocardial infarction. *J. Biol. Chem.* 288, 915–926. doi: 10.1074/jbc.M112.411363

Kumarapeli, A. R., and Wang, X. (2004). Genetic modification of the heart: chaperones and the cytoskeleton. *J. Mol. Cell Cardiol.* 37, 1097–1109. doi: 10.1016/j.yjmcc.2004.07.004

Kupatt, C., Dessy, C., Hinkel, R., Raake, P., Daneau, G., Bouzin, C., et al. (2004). Heat shock protein 90 transfection reduces ischemia-reperfusion-induced myocardial dysfunction via reciprocal endothelial NO synthase serine 1177 phosphorylation and threonine 495 dephosphorylation. *Arterioscler. Thromb. Vasc. Biol.* 24, 1435–1441. doi: 10.1161/01.ATV.0000134300.87476.d1

Kuzman, J. A., O'Connell, T. D., and Gerdes, A. M. (2007). Rapamycin prevents thyroid hormone-induced cardiac hypertrophy. *Endocrinology* 148, 3477–3484. doi: 10.1210/en.2007-0099

Laderoute, K. R., and Webster, K. A. (1997). Hypoxia/reoxygenation stimulates Jun kinase activity through redox signaling in cardiac myocytes. *Circ. Res.* 80, 336–344. doi: 10.1161/01.RES.80.3.336

Lan, Y., Wang, Y., Huang, K., and Zeng, Q. (2020). Heat shock protein 22 attenuates doxorubicin-induced cardiotoxicity via regulating inflammation and apoptosis. *Front. Pharmacol.* 11:257. doi: 10.3389/fphys.2018.0257

Lasker, K., Forster, F., Bohn, S., Walzthoeni, T., Villa, E., Unverdorben, P., et al. (2012). Molecular architecture of the 26S proteasome holocomplex determined by an integrative approach. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1380–1387. doi: 10.1073/pnas.1120559109

Lavoie, C., Chevet, E., Roy, L., Tonks, N. K., Fazel, A., Posner, B. I., et al. (2000). Tyrosine phosphorylation of p97 regulates transitional endoplasmic reticulum assembly in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 97, 13637–13642. doi: 10.1073/pnas.240278097

Lee, S. H., Park, Y., Yoon, S. K., and Yoon, J. B. (2010). Osmotic stress inhibits proteasome by p38 MAPK-dependent phosphorylation. *J. Biol. Chem.* 285, 41280–41289. doi: 10.1074/jbc.M110.182188

Leestemaker, Y., de Jong, A., Witting, K. F., Penning, R., Schuurman, K., Rodenko, B., et al. (2017). Proteasome activation by small molecules. *Cell Chem. Biol.* 24, 725–736.e7. doi: 10.1016/j.chembiol.2017.05.010

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell* 132, 27–42. doi: 10.1016/j.cell.2007.12.018

Li, C., Capan, E., Zhao, Y., Zhao, J., Stolz, D., Watkins, S. C., et al. (2006). Autophagy is induced in CD4+ T cells and important for the growth factor-withdrawal cell death. *J. Immunol.* 177, 5163–5168. doi: 10.4049/jimmunol.177.8.5163

Li, D., Dong, Q., Tao, Q., Gu, J., Cui, Y., Jiang, X., et al. (2015). c-Abl regulates proteasome abundance by controlling the ubiquitin-proteasomal degradation of PSMA7 subunit. *Cell Rep.* 10, 484–496. doi: 10.1016/j.celrep.2014.12.044

Li, D. D., Wang, L. L., Deng, R., Tang, J., Shen, Y., Guo, J. F., et al. (2009). The pivotal role of c-Jun NH2-terminal kinase-mediated Beclin 1 expression during anticancer agents-induced autophagy in cancer cells. *Oncogene* 28, 886–898. doi: 10.1038/onc.2008.441

Li, G., Zhao, G., Schindelin, H., and Lennarz, W. J. (2008). Tyrosine phosphorylation of ATPase p97 regulates its activity during ERAD. *Biochem. Biophys. Res. Commun.* 375, 247–251. doi: 10.1016/j.bbrc.2008.08.018

Li, J., Horak, K. M., Su, H., Sanbe, A., Robbins, J., and Wang, X. (2011). Enhancement of proteasomal function protects against cardiac proteinopathy and ischemia/reperfusion injury in mice. *J. Clin. Invest.* 121, 3689–3700. doi: 10.1172/JCI45709

Li, J., Wilkinson, B., Clementel, V. A., Hou, J., O'Dell, T. J., and Coba, M. P. (2016). Long-term potentiation modulates synaptic phosphorylation networks and reshapes the structure of the postsynaptic interactome. *Sci. Signal.* 9:rs8. doi: 10.1126/scisignal.aaf6716

Li, M., Sala, V., Cimino, J., Cappello, P., Pianca, N., Margaria, J. P., et al. (2018). Phosphoinositide 3-kinase gamma inhibition protects from Anthracycline cardiotoxicity and reduces tumor growth. *Circulation* 138, 696–711. doi: 10.1161/CIRCULATIONAHA.117.030352

Li, X., Hu, X., Wang, J., Xu, W., Yi, C., Ma, R., et al. (2018). Inhibition of autophagy via activation of PI3K/Akt/mTOR pathway contributes to the protection of hesperidin against myocardial ischemia/reperfusion injury. *Int. J. Mol. Med.* 42, 1917–1924. doi: 10.3892/ijmm.2018.3794

Li, Y., Chen, C., Yao, F., Su, Q., Liu, D., Xue, R., et al. (2014). AMPK inhibits cardiac hypertrophy by promoting autophagy via mTORC1. *Arch. Biochem. Biophys.* 558, 79–86. doi: 10.1016/j.abb.2014.06.023

Li, Z. L., Woollard, J. R., Ebrahimi, B., Crane, J. A., Jordan, K. L., Lerman, A., et al. (2012). Transition from obesity to metabolic syndrome is associated with

altered myocardial autophagy and apoptosis. *Arterioscler. Thromb. Vasc. Biol.* 32, 1132–1141. doi: 10.1161/ATVBAHA.111.244061

Lin, L., Tang, C., Xu, J., Ye, Y., Weng, L., Wei, W., et al. (2014). Mechanical stress triggers cardiomyocyte autophagy through angiotensin II type 1 receptor-mediated p38MAP kinase independently of angiotensin II. *PLoS One* 9:e89629. doi: 10.1371/journal.pone.089629

Liu, B., Cheng, Y., Zhang, B., Bian, H. J., and Bao, J. K. (2009). *Polygonatum cyrtonema* lectin induces apoptosis and autophagy in human melanoma A375 cells through a mitochondria-mediated ROS-p38-p53 pathway. *Cancer Lett.* 275, 54–60. doi: 10.1016/j.canlet.2008.09.042

Liu, X., Huang, W., Li, C., Li, P., Yuan, J., Li, X., et al. (2006). Interaction between c-Abl and Arg tyrosine kinases and proteasome subunit PSMA7 regulates proteasome degradation. *Mol. Cell.* 22, 317–327. doi: 10.1016/j.molcel.2006.04.007

Livingstone, M., Ruan, H., Weiner, J., Clauser, K. R., Strack, P., Jin, S., et al. (2005). Valosin-containing protein phosphorylation at Ser784 in response to DNA damage. *Cancer Res.* 65, 7533–7540. doi: 10.1158/0008-5472.CAN-04-3729

Lokireddy, S., Kukushkin, N. V., and Goldberg, A. L. (2015). cAMP-induced phosphorylation of 26S proteasomes on Rpn6/PSMD11 enhances their activity and the degradation of misfolded proteins. *Proc. Natl. Acad. Sci. U.S.A.* 112, E7176–E7185. doi: 10.1073/pnas.1522332112

Ludemann, R., Lerea, K. M., and Etlinger, J. D. (1993). Copurification of casein kinase II with 20 S proteasomes and phosphorylation of a 30-kDa proteasome subunit. *J. Biol. Chem.* 268, 17413–17417.

Maejima, Y., Isobe, M., and Sadoshima, J. (2016). Regulation of autophagy by Beclin 1 in the heart. *J. Mol. Cell Cardiol.* 95, 19–25. doi: 10.1016/j.yjmcc.2015.10.032

Maloyan, A., Gulick, J., Glabe, C. G., Kayed, R., and Robbins, J. (2007). Exercise reverses preamyloid oligomer and prolongs survival in alphaB-crystallin-based desmin-related cardiomyopathy. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5995–6000. doi: 10.1073/pnas.0609202104

Marber, M. S., Mestril, R., Chi, S. H., Sayen, M. R., Yellon, D. M., and Dillmann, W. H. (1995). Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J. Clin. Invest.* 95, 1446–1456. doi: 10.1172/JCI117815

Matic, I., Macek, B., Hilger, M., Walther, T. C., and Mann, M. (2008). Phosphorylation of SUMO-1 occurs in vivo and is conserved through evolution. *J. Proteome Res.* 7, 4050–4057. doi: 10.1021/pr800368m

Matsui, Y., Takagi, H., Qu, X., Abdellatif, M., Sakoda, H., Asano, T., et al. (2007). Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circ. Res.* 100, 914–922. doi: 10.1161/01.RES.0000261924.76669.36

Mellor, K. M., Bell, J. R., Young, M. J., Ritchie, R. H., and Delbridge, L. M. (2011). Myocardial autophagy activation and suppressed survival signaling is associated with insulin resistance in fructose-fed mice. *J. Mol. Cell Cardiol.* 50, 1035–1043. doi: 10.1016/j.yjmcc.2011.03.002

Mellor, K. M., Reichelt, M. E., and Delbridge, L. M. (2013). Autophagic predisposition in the insulin resistant diabetic heart. *Life Sci.* 92, 616–620. doi: 10.1016/j.lfs.2012.03.042

Menon, M. B., Tiedje, C., Lafera, J., Ronkina, N., Konen, T., Kotlyarov, A., et al. (2013). Endoplasmic reticulum-associated ubiquitin-conjugating enzyme Ube2j1 is a novel substrate of MK2 (MAPKAP kinase-2) involved in MK2-mediated TNFalpha production. *Biochem. J.* 456, 163–172. doi: 10.1042/BJ20130755

Mohan, N., Shen, Y., Endo, Y., ElZarrad, M. K., and Wu, W. J. (2016). Trastuzumab, but not pertuzumab, dysregulates HER2 signaling to mediate inhibition of autophagy and increase in reactive oxygen species production in human cardiomyocytes. *Mol. Cancer Ther.* 15, 1321–1331. doi: 10.1158/1535-7163.MCT-15-0741

Mori-Konya, C., Kato, N., Maeda, R., Yasuda, K., Higashimae, N., Noguchi, M., et al. (2009). p97/valosin-containing protein (VCP) is highly modulated by phosphorylation and acetylation. *Genes Cells* 14, 483–497. doi: 10.1111/j.1365-2443.2009.01286.x

Morin, D., Long, R., Panel, M., Laure, L., Taranu, A., Gueguen, C., et al. (2019). Hsp22 overexpression induces myocardial hypertrophy, senescence and reduced life span through enhanced oxidative stress. *Free Radic. Biol. Med.* 137, 194–200. doi: 10.1016/j.freeradbiomed.2019.04.035

Mrakovcic, M., and Frohlich, L. F. (2018). p53-mediated molecular control of autophagy in tumor cells. *Biomolecules* 8:14. doi: 10.3390/biom8020014

Muller, P., Ruckova, E., Halada, P., Coates, P. J., Hrstka, R., Lane, D. P., et al. (2013). C-terminal phosphorylation of Hsp70 and Hsp90 regulates alternate binding to co-chaperones CHIP and HOP to determine cellular protein folding/degradation balances. *Oncogene* 32, 3101–3110. doi: 10.1038/onc.2012.314

Murtaza, I., Wang, H. X., Feng, X., Alenina, N., Bader, M., Prabhakar, B. S., et al. (2008). Down-regulation of catalase and oxidative modification of protein kinase CK2 lead to the failure of apoptosis repressor with caspase recruitment domain to inhibit cardiomyocyte hypertrophy. *J. Biol. Chem.* 283, 5996–6004. doi: 10.1074/jbc.M706466200

Nagai, Y., Kaneda, S., Nomura, K., Yasuda, H., Seno, T., and Yamao, F. (1995). Ubiquitin-activating enzyme, E1, is phosphorylated in mammalian cells by the protein kinase Cdc2. *J. Cell Sci.* 108(Pt 6), 2145–2152.

Nagayama, T., Hsu, S., Zhang, M., Koitabashi, N., Bedja, D., Gabrielson, K. L., et al. (2009). Sildenafil stops progressive chamber, cellular, and molecular remodeling and improves calcium handling and function in hearts with pre-existing advanced hypertrophy caused by pressure overload. *J. Am. Coll. Cardiol.* 53, 207–215. doi: 10.1016/j.jacc.2008.08.069

Napolitano, G., Esposito, A., Choi, H., Matarese, M., Benedetti, V., Monfregola, J., et al. (2018). mTOR-dependent phosphorylation controls TFEB nuclear export. *Nat. Commun.* 9:3312. doi: 10.1038/s41467-018-05862-6

Nazio, F., Strappazzon, F., Antonioli, M., Bielli, P., Cianfanelli, V., Bordi, M., et al. (2013). mTOR inhibits autophagy by controlling ULK1 ubiquitylation, self-association and function through AMBRA1 and TRAF6. *Nat. Cell Biol.* 15, 406–416. doi: 10.1038/ncb2708

Neumann, J., Eschenhagen, T., Jones, L. R., Linck, B., Schmitz, W., Scholz, H., et al. (1997). Increased expression of cardiac phosphatases in patients with end-stage heart failure. *J. Mol. Cell Cardiol.* 29, 265–272. doi: 10.1006/jmcc.1996.0271

Nishida, T., Hattori, K., and Watanabe, K. (2017). The regulatory and signaling mechanisms of the ASK family. *Adv. Biol. Regul.* 66, 2–22. doi: 10.1016/j.jbior.2017.05.004

Nopparat, C., Porter, J. E., Ebadi, M., and Govitrapong, P. (2010). The mechanism for the neuroprotective effect of melatonin against methamphetamine-induced autophagy. *J. Pineal Res.* 49, 382–389. doi: 10.1111/j.1600-079X.2010.00805.x

Oberoi, J., Dunn, D. M., Woodford, M. R., Mariotti, L., Schulman, J., Bourboulia, D., et al. (2016). Structural and functional basis of protein phosphatase 5 substrate specificity. *Proc. Natl. Acad. Sci. U.S.A.* 113, 9009–9014. doi: 10.1073/pnas.1603059113

Ochoa, C. D., Wu, R. F., and Terada, L. S. (2018). ROS signaling and ER stress in cardiovascular disease. *Mol. Aspects Med.* 63, 18–29. doi: 10.1016/j.mam.2018.03.002

Oeing, C. U., Mishra, S., Dunkerly-Eyring, B. L., and Ranek, M. J. (2020a). Targeting protein kinase G to treat cardiac proteotoxicity. *Front. Physiol.* 11:858. doi: 10.3389/fphys.2020.00858

Oeing, C. U., Nakamura, T., Pan, S., Mishra, S., Lin, B. L., Chen, A., et al. (2020b). PKG1alpha cysteine-42 redox state controls mTORC1 activation in pathological cardiac hypertrophy. *Circ. Res.* 127, 522–533. doi: 10.1161/CIRCRESAHA.119.315714

Papinski, D., and Kraft, C. (2016). Regulation of autophagy by signaling through the Atg1/ULK1 complex. *J. Mol. Biol.* 428, 1725–1741. doi: 10.1016/j.jmb.2016.03.030

Parisi, E., Yahya, G., Flores, A., and Aldea, M. (2018). Cdc48/p97 segregase is modulated by cyclin-dependent kinase to determine cyclin fate during G1 progression. *EMBO J.* 37:e98724. doi: 10.15252/embj.201798724

Parry, T. L., Melehani, J. H., Ranek, M. J., and Willis, M. S. (2015). Functional amyloid signaling via the inflammasome, necrosome, and signalosome: new therapeutic targets in heart failure. *Front. Cardiovasc. Med.* 2:25. doi: 10.3389/fphys.2018.0025

Pathare, G. R., Nagy, I., Bohn, S., Unverdorben, P., Hubert, A., Korner, R., et al. (2012). The proteasomal subunit Rpn6 is a molecular clamp holding the core and regulatory subcomplexes together. *Proc. Natl. Acad. Sci. U.S.A.* 109, 149–154. doi: 10.1073/pnas.1117648108

Pattingre, S., Bauvy, C., Carpentier, S., Levade, T., Levine, B., and Codogno, P. (2009). Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy. *J. Biol. Chem.* 284, 2719–2728. doi: 10.1074/jbc.M805920200

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., et al. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122, 927–939. doi: 10.1016/j.cell.2005.07.002

Pattison, J. S., Osinska, H., and Robbins, J. (2011). Atg7 induces basal autophagy and rescues autophagic deficiency in CryABR120G cardiomyocytes. *Circ. Res.* 109, 151–160. doi: 10.1161/CIRCRESAHA.110.237339

Pattison, J. S., Sanbe, A., Maloyan, A., Osinska, H., Klevitsky, R., and Robbins, J. (2008). Cardiomyocyte expression of a polyglutamine preamyloid oligomer causes heart failure. *Circulation* 117, 2743–2751. doi: 10.1161/CIRCULATIONAHA.107.750232

Penna, C., Sorge, M., Femmino, S., Pagliaro, P., and Brancaccio, M. (2018). Redox aspects of chaperones in cardiac function. *Front. Physiol.* 9:216. doi: 10.3389/fphys.2018.00216

Pereira, M. E., and Wilk, S. (1990). Phosphorylation of the multicatalytic proteinase complex from bovine pituitaries by a copurifying cAMP-dependent protein kinase. *Arch. Biochem. Biophys.* 283, 68–74. doi: 10.1016/0003-9861(90)90613-4

Persaud, A., Alberts, P., Mari, S., Tong, J., Murchie, R., Maspero, E., et al. (2014). Tyrosine phosphorylation of NEDD4 activates its ubiquitin ligase activity. *Sci. Signal.* 7:ra95. doi: 10.1126/scisignal.2005290

Piccirillo, R., and Goldberg, A. L. (2012). The p97/VCP ATPase is critical in muscle atrophy and the accelerated degradation of muscle proteins. *EMBO J.* 31, 3334–3350. doi: 10.1038/emboj.2012.178

Pinilla-Vera, M., Hahn, V. S., and Kass, D. A. (2019). Leveraging signaling pathways to treat heart failure with reduced ejection fraction. *Circ. Res.* 124, 1618–1632. doi: 10.1161/CIRCRESAHA.119.313682

Pires, K. M., Buffolo, M., Schaaf, C., David Symons, J., Cox, J., Abel, E. D., et al. (2017). Activation of IGF-1 receptors and Akt signaling by systemic hyperinsulinemia contributes to cardiac hypertrophy but does not regulate cardiac autophagy in obese diabetic mice. *J. Mol. Cell Cardiol.* 113, 39–50. doi: 10.1016/j.yjmcc.2017.10.001

Plumier, J. C., Ross, B. M., Currie, R. W., Angelidis, C. E., Kazlaris, H., Kollias, G., et al. (1995). Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery. *J. Clin. Invest.* 95, 1854–1860. doi: 10.1172/JCI117865

Powell, S. R., and Divald, A. (2010). The ubiquitin-proteasome system in myocardial ischaemia and preconditioning. *Cardiovasc. Res.* 85, 303–311. doi: 10.1093/cvr/cvp321

Powell, S. R., Samuel, S. M., Wang, P., Divald, A., Thirunavukkarasu, M., Koneru, S., et al. (2008). Upregulation of myocardial 11S-activated proteasome in experimental hyperglycemia. *J. Mol. Cell Cardiol.* 44, 618–621. doi: 10.1016/j.yjmcc.2007.12.009

Pratt, W. B., and Toft, D. O. (2003). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp. Biol. Med.* 228, 111–133. doi: 10.1177/153537020322800201

Predmore, J. M., Wang, P., Davis, F., Bartolone, S., Westfall, M. V., Dyke, D. B., et al. (2010). Ubiquitin proteasome dysfunction in human hypertrophic and dilated cardiomyopathies. *Circulation* 121, 997–1004. doi: 10.1161/CIRCULATIONAHA.109.904557

Qian, J., Ren, X., Wang, X., Zhang, P., Jones, W. K., Molkentin, J. D., et al. (2009). Blockade of Hsp20 phosphorylation exacerbates cardiac ischemia/reperfusion injury by suppressed autophagy and increased cell death. *Circ. Res.* 105, 1223–1231. doi: 10.1161/CIRCRESAHA.109.200378

Qian, J., Vafiadaki, E., Florea, S. M., Singh, V. P., Song, W., Lam, C. K., et al. (2011). Small heat shock protein 20 interacts with protein phosphatase-1 and enhances sarcoplasmic reticulum calcium cycling. *Circ. Res.* 108, 1429–1438. doi: 10.1161/CIRCRESAHA.110.237644

Qian, L. B., Jiang, S. Z., Tang, X. Q., Zhang, J., Liang, Y. Q., Yu, H. T., et al. (2017). Exacerbation of diabetic cardiac hypertrophy in OVE26 mice by angiotensin II is associated with JNK/c-Jun/miR-221-mediated autophagy inhibition. *Oncotarget* 8, 106661–106671. doi: 10.18632/oncotarget.21302

Qiu, Z., Cang, Y., and Goff, S. P. (2010). c-Abl tyrosine kinase regulates cardiac growth and development. *Proc. Natl. Acad. Sci. U.S.A.* 107, 1136–1141. doi: 10.1073/pnas.0913131107

Radford, N. B., Fina, M., Benjamin, I. J., Moreadith, R. W., Graves, K. H., Zhao, P., et al. (1996). Cardioprotective effects of 70-kDa heat shock protein in transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 93, 2339–2342. doi: 10.1073/pnas.93.6.2339

Ramos, F. J., Chen, S. C., Garelick, M. G., Dai, D. F., Liao, C. Y., Schreiber, K. H., et al. (2012). Rapamycin reverses elevated mTORC1 signaling in lamin A/C-deficient mice, rescues cardiac and skeletal muscle function, and extends survival. *Sci. Transl. Med.* 4:144ra103. doi: 10.1126/scitranslmed.3003802

Ranek, M. J., Kost, C. K. Jr., Hu, C., Martin, D. S., and Wang, X. (2014). Muscarinic 2 receptors modulate cardiac proteasome function in a protein kinase G-dependent manner. *J. Mol. Cell Cardiol.* 69, 43–51. doi: 10.1016/j.yjmcc.2014.01.017

Ranek, M. J., Stachowski, M. J., Kirk, J. A., and Willis, M. S. (2018). The role of heat shock proteins and co-chaperones in heart failure. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 373:20160530. doi: 10.1098/rstb.2016.0530

Ranek, M. J., Terpstra, E. J., Li, J., Kass, D. A., and Wang, X. (2013). Protein kinase g positively regulates proteasome-mediated degradation of misfolded proteins. *Circulation* 128, 365–376. doi: 10.1161/CIRCULATIONAHA.113.001971

Ranek, M. J., Vera, M. P., Oeing, C. U., Patel, C. H., Nakamura, T., Zhu, G., et al. (2019). PKG1-modified TSC2 regulates mTORC1 activity to counter adverse cardiac stress. *Nature* 566, 264–269. doi: 10.1038/s41586-019-0895-y

Ranek, M. J., and Wang, X. (2009). Activation of the ubiquitin-proteasome system in doxorubicin cardiomyopathy. *Curr. Hypertens. Rep.* 11, 389–395. doi: 10.1007/s11906-009-0068-8

Ranek, M. J., Zheng, H., Huang, W., Kumarapeli, A. R., Li, J., Liu, J., et al. (2015). Genetically induced moderate inhibition of 20S proteasomes in cardiomyocytes facilitates heart failure in mice during systolic overload. *J. Mol. Cell Cardiol.* 85, 273–281. doi: 10.1016/j.yjmcc.2015.06.014

Razeghi, P., Baskin, K. K., Sharma, S., Young, M. E., Stepkowski, S., Essop, M. F., et al. (2006). Atrophy, hypertrophy, and hypoxemia induce transcriptional regulators of the ubiquitin proteasome system in the rat heart. *Biochem. Biophys. Res. Commun.* 342, 361–364. doi: 10.1016/j.bbrc.2006.01.163

Ren, H., Fu, K., Mu, C., Li, B., Wang, D., and Wang, G. (2010). DJ-1, a cancer and Parkinson's disease associated protein, regulates autophagy through JNK pathway in cancer cells. *Cancer Lett.* 297, 101–108. doi: 10.1016/j.canlet.2010.05.001

Riehle, C., Wende, A. R., Sena, S., Pires, K. M., Pereira, R. O., Zhu, Y., et al. (2013). Insulin receptor substrate signaling suppresses neonatal autophagy in the heart. *J. Clin. Invest.* 123, 5319–5333. doi: 10.1172/JCI71171

Robson, A., Makova, S. Z., Barish, S., Zaidi, S., Mehta, S., Drozd, J., et al. (2019). Histone H2B monoubiquitination regulates heart development via epigenetic control of cilia motility. *Proc. Natl. Acad. Sci. U.S.A.* 116, 14049–14054. doi: 10.1073/pnas.1808341116

Roscic, A., Moller, A., Calzado, M. A., Renner, F., Wimmer, V. C., Gresko, E., et al. (2006). Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. *Mol. Cell.* 24, 77–89. doi: 10.1016/j.molcel.2006.08.004

Sadowski, M., Mawson, A., Baker, R., and Sarcevic, B. (2007). Cdc34 C-terminal tail phosphorylation regulates Skp1/cullin/F-box (SCF)-mediated ubiquitination and cell cycle progression. *Biochem. J.* 405, 569–581. doi: 10.1042/BJ20061812

Sandri, M., and Robbins, J. (2014). Proteotoxicity: an underappreciated pathology in cardiac disease. *J. Mol. Cell Cardiol.* 71, 3–10. doi: 10.1016/j.yjmcc.2013.12.015

Sasaki, H., Nagayama, T., Blanton, R. M., Seo, K., Zhang, M., Zhu, G., et al. (2014). PDE5 inhibitor efficacy is estrogen dependent in female heart disease. *J. Clin. Invest.* 124, 2464–2471. doi: 10.1172/JCI70731

Satoh, K., Sasajima, H., Nyoumura, K. I., Yokosawa, H., and Sawada, H. (2001). Assembly of the 26S proteasome is regulated by phosphorylation of the p45/Rpt6 ATPase subunit. *Biochemistry* 40, 314–319. doi: 10.1021/bi001815n

Schwartz, A. L., and Ciechanover, A. (1992). Ubiquitin-mediated protein modification and degradation. *Am. J. Respir. Cell Mol. Biol.* 7, 463–468. doi: 10.1165/ajrcmb/7.5.463

Schweitzer, A., Aufderheide, A., Rudack, T., Beck, F., Pfeifer, G., Plitzko, J. M., et al. (2016). Structure of the human 26S proteasome at a resolution of 3.9 A. *Proc. Natl. Acad. Sci. U.S.A.* 113, 7816–7821. doi: 10.1073/pnas.1608050113

Sciarretta, S., Forte, M., Frati, G., and Sadoshima, J. (2018a). New insights into the role of mTOR signaling in the cardiovascular system. *Circ. Res.* 122, 489–505. doi: 10.1161/CIRCRESAHA.117.311147

Sciarretta, S., Maejima, Y., Zablocki, D., and Sadoshima, J. (2018b). The Role of Autophagy in the Heart. *Annu. Rev. Physiol.* 80, 1–26. doi: 10.1146/annurev-physiol-021317-121427

Sciarretta, S., Volpe, M., and Sadoshima, J. (2014). Mammalian target of rapamycin signaling in cardiac physiology and disease. *Circ. Res.* 114, 549–564. doi: 10.1161/CIRCRESAHA.114.302022

Sciarretta, S., Zhai, P., Shao, D., Maejima, Y., Robbins, J., Volpe, M., et al. (2012a). Rheb is a critical regulator of autophagy during myocardial ischemia: pathophysiological implications in obesity and metabolic syndrome. *Circulation* 125, 1134–1146. doi: 10.1161/CIRCULATIONAHA.111.078212

Sciarretta, S., Zhai, P., Volpe, M., and Sadoshima, J. (2012b). Pharmacological modulation of autophagy during cardiac stress. *J. Cardiovasc. Pharmacol.* 60, 235–241. doi: 10.1097/FJC.0b013e3182575f61

Scruggs, S. B., Zong, N. C., Wang, D., Stefani, E., and Ping, P. (2012). Post-translational modification of cardiac proteasomes: functional delineation enabled by proteomics. *Am. J. Physiol. Heart Circ. Physiol.* 303, H9–H18. doi: 10.1152/ajpheart.00189.2012

Sengupta, A., Molkentin, J. D., and Yutzey, K. E. (2009). FoxO transcription factors promote autophagy in cardiomyocytes. *J. Biol. Chem.* 284, 28319–28331. doi: 10.1074/jbc.M109.024406

Settembre, C., Vetrini, F., Erdin, S., Erdin, S. U., Huynh, T., Medina, D., et al. (2011). TFEB links autophagy to lysosomal biogenesis. *Science* 332, 1429–1433. doi: 10.1126/science.1204592

Shang, L., Chen, S., Du, F., Li, S., Zhao, L., and Wang, X. (2011). Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK. *Proc. Natl. Acad. Sci. U.S.A.* 108, 4788–4793. doi: 10.1073/pnas.1100844108

Sharma, K., and Kass, D. A. (2014). Heart failure with preserved ejection fraction: mechanisms, clinical features, and therapies. *Circ. Res.* 115, 79–96. doi: 10.1161/CIRCRESAHA.115.302922

Sharma, S. K., De los Rios, P., Christen, P., Lustig, A., and Goloubinoff, P. (2010). The kinetic parameters and energy cost of the Hsp70 chaperone as a polypeptide unfoldase. *Nat. Chem. Biol.* 6, 914–920. doi: 10.1038/nchembio.455

Shchebet, A., Karpiuk, O., Kremmer, E., Eick, D., and Johnsen, S. A. (2012). Phosphorylation by cyclin-dependent kinase-9 controls ubiquitin-conjugating enzyme-2A function. *Cell Cycle* 11, 2122–2127. doi: 10.4161/cc.20548

Shen, X., Valencia, C. A., Szostak, J. W., Dong, B., and Liu, R. (2005). Scanning the human proteome for calmodulin-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5969–5974. doi: 10.1073/pnas.0407928102

Shende, P., Plaisance, I., Morandi, C., Pellieux, C., Berthonneche, C., Zorzato, F., et al. (2011). Cardiac raptor ablation impairs adaptive hypertrophy, alters metabolic gene expression, and causes heart failure in mice. *Circulation* 123, 1073–1082. doi: 10.1161/CIRCULATIONAHA.110.977066

Shimomura, H., Terasaki, F., Hayashi, T., Kitaura, Y., Isomura, T., and Suma, H. (2001). Autophagic degeneration as a possible mechanism of myocardial cell death in dilated cardiomyopathy. *Jpn. Circ. J.* 65, 965–968. doi: 10.1253/jcj.65.965

Shinmura, K., Tamaki, K., Sano, M., Murata, M., Yamakawa, H., Ishida, H., et al. (2011). Impact of long-term caloric restriction on cardiac senescence: caloric restriction ameliorates cardiac diastolic dysfunction associated with aging. *J. Mol. Cell Cardiol.* 50, 117–127. doi: 10.1016/j.yjmcc.2010.10.018

Shirakabe, A., Ikeda, Y., Sciarretta, S., Zablocki, D. K., and Sadoshima, J. (2016). Aging and autophagy in the heart. *Circ. Res.* 118, 1563–1576. doi: 10.1161/CIRCRESAHA.116.307474

Sledz, P., Forster, F., and Baumeister, W. (2013). Allosteric effects in the regulation of 26S proteasome activities. *J. Mol. Biol.* 425, 1415–1423. doi: 10.1016/j.jmb.2013.01.036

Stephan, J. S., Yeh, Y. Y., Ramachandran, V., Deminoff, S. J., and Herman, P. K. (2009). The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. *Proc. Natl. Acad. Sci. U.S.A.* 106, 17049–17054. doi: 10.1073/pnas.0903316106

Stephen, A. G., Trausch-Azar, J. S., Ciechanover, A., and Schwartz, A. L. (1996). The ubiquitin-activating enzyme E1 is phosphorylated and localized to the nucleus in a cell cycle-dependent manner. *J. Biol. Chem.* 271, 15608–15614. doi: 10.1074/jbc.271.26.15608

Stephen, A. G., Trausch-Azar, J. S., Handley-Gearhart, P. M., Ciechanover, A., and Schwartz, A. L. (1997). Identification of a region within the ubiquitin-activating enzyme required for nuclear targeting and phosphorylation. *J. Biol. Chem.* 272, 10895–10903. doi: 10.1074/jbc.272.16.10895

Su, H., Li, F., Ranek, M. J., Wei, N., and Wang, X. (2011). COP9 signalosome regulates autophagosome maturation. *Circulation* 124, 2117–2128. doi: 10.1161/CIRCULATIONAHA.111.048934

Su, H., and Wang, X. (2010). The ubiquitin-proteasome system in cardiac proteinopathy: a quality control perspective. *Cardiovasc. Res.* 85, 253–262. doi: 10.1093/cvr/cvp287

Su, Y. F., Yang, T., Huang, H., Liu, L. F., and Hwang, J. (2012). Phosphorylation of Ubc9 by Cdk1 enhances SUMOylation activity. *PLoS One* 7:e34250. doi: 10.1371/journal.pone.0034250 doi: 10.1371/journal.pone.0034250

Sun, L., Isaak, C. K., Zhou, Y., Petkau, J. C., Liu, Y., and Siow, Y. L. (2012). Salidroside and tyrosol from Rhodiola protect H9c2 cells from ischemia/reperfusion-induced apoptosis. *Life Sci.* 91, 151–158. doi: 10.1016/j.lfs.2012.06.026

Sun, S., Zhang, M., Lin, J., Hu, J., Zhang, R., Li, C., et al. (2016). Lin28a protects against diabetic cardiomyopathy via the PKA/ROCK2 pathway. *Biochem. Biophys. Res. Commun.* 469, 29–36. doi: 10.1016/j.bbrc.2015.11.065

Sun, Y., Yao, X., Zhang, Q. J., Zhu, M., Liu, Z. P., Ci, B., et al. (2018). Beclin-1-dependent autophagy protects the heart during sepsis. *Circulation* 138, 2247–2262. doi: 10.1161/CIRCULATIONAHA.117.032821

Suzuki, K., and Ohsumi, Y. (2007). Molecular machinery of autophagosome formation in yeast, *Saccharomyces cerevisiae*. *FEBS Lett.* 581, 2156–2161. doi: 10.1016/j.febslet.2007.01.096

Takimoto, E., Champion, H. C., Li, M., Belardi, D., Ren, S., Rodriguez, E. R., et al. (2005). Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy. *Nat. Med.* 11, 214–222. doi: 10.1038/nm1175

Taneike, M., Nishida, K., Omiya, S., Zarrinpashneh, E., Misaka, T., Austin, R., et al. (2016). mTOR hyperactivation by ablation of tuberous sclerosis complex 2 in the mouse heart induces cardiac dysfunction with the increased number of small mitochondria mediated through the down-regulation of autophagy. *PLoS One* 11:e0152628. doi: 10.1371/journal.pone.0152628

Tarone, G., and Brancaccio, M. (2014). Keep your heart in shape: molecular chaperone networks for treating heart disease. *Cardiovasc. Res.* 102, 346–361. doi: 10.1093/cvr/cvu049

Thomson, A. W., Turnquist, H. R., and Raimondi, G. (2009). Immunoregulatory functions of mTOR inhibition. *Nat. Rev. Immunol.* 9, 324–337. doi: 10.1038/nri2546

Tian, W., Li, W., Chen, Y., Yan, Z., Huang, X., Zhuang, H., et al. (2015). Phosphorylation of ULK1 by AMPK regulates translocation of ULK1 to mitochondria and mitophagy. *FEBS Lett.* 589, 1847–1854. doi: 10.1016/j.febslet.2015.05.020

Tomanov, K., Nukarinen, E., Vicente, J., Mendiondo, G. M., Winter, N., and Nehlin, L. (2018). Sumoylation and phosphorylation: hidden and overt links. *J. Exp. Bot.* 69, 4583–4590. doi: 10.1093/jxb/ery167

Torres-Quiroz, F., Filteau, M., and Landry, C. R. (2015). Feedback regulation between autophagy and PKA. *Autophagy* 11, 1181–1183. doi: 10.1080/15548627.2015.1055440

Trost, S. U., Omens, J. H., Karlon, W. J., Meyer, M., Mestril, R., Covell, J. W., et al. (1998). Protection against myocardial dysfunction after a brief ischemic period in transgenic mice expressing inducible heat shock protein 70. *J. Clin. Invest.* 101, 855–862. doi: 10.1172/JCI265

Truman, A. W., Kristjansdottir, K., Wolfgeher, D., Hasin, N., Polier, S., Zhang, H., et al. (2012). CDK-dependent Hsp70 Phosphorylation controls G1 cyclin abundance and cell-cycle progression. *Cell* 151, 1308–1318. doi: 10.1016/j.cell.2012.10.051

Tsai, C. F., Wang, Y. T., Yen, H. Y., Tsou, C. C., Ku, W. C., Lin, P. Y., et al. (2015). Large-scale determination of absolute phosphorylation stoichiometries in human cells by motif-targeting quantitative proteomics. *Nat. Commun.* 6:6622. doi: 10.1038/ncomms7622

Ulbricht, A., Gehlert, S., Leciejewski, B., Schiffer, T., Bloch, W., and Hohfeld, J. (2015). Induction and adaptation of chaperone-assisted selective autophagy CASA in response to resistance exercise in human skeletal muscle. *Autophagy* 11, 538–546. doi: 10.1080/15548627.2015.1017186

Um, J. W., Im, E., Park, J., Oh, Y., Min, B., Lee, H. J., et al. (2010). ASK1 negatively regulates the 26 S proteasome. *J. Biol. Chem.* 285, 36434–36446. doi: 10.1074/jbc.M110.133777

VerPlank, J. S., and Goldberg, A. L. (2018). Exploring the regulation of proteasome function by subunit phosphorylation. *Methods Mol. Biol.* 1844, 309–319. doi: 10.1007/978-1-4939-8706-1_20

VerPlank, J. S., Lokireddy, S., Zhao, J., and Goldberg, A. L. (2019). 26S Proteasomes are rapidly activated by diverse hormones and physiological states that raise cAMP and cause Rpn6 phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* 116, 4228–4237. doi: 10.1073/pnas.1809254116

Wang, A., Zhang, H., Liang, Z., Xu, K., Qiu, W., Tian, Y., et al. (2016). U0126 attenuates ischemia/reperfusion-induced apoptosis and autophagy in myocardium through MEK/ERK/EGR-1 pathway. *Eur. J. Pharmacol* 788, 280–285. doi: 10.1016/j.ejphar.2016.06.038

Wang, B., Nie, J., Wu, L., Hu, Y., Wen, Z., Dong, L., et al. (2018). AMPKalpha2 protects against the development of heart failure by enhancing mitophagy via PINK1 phosphorylation. *Circ. Res.* 122, 712–729. doi: 10.1161/CIRCRESAHA.117.312317

Wang, H., Liu, Y., Wang, D., Xu, Y., Dong, R., Yang, Y., et al. (2019). The upstream pathway of mTOR-mediated autophagy in liver diseases. *Cells* 8:1597. doi: 10.3390/cells8121597

Wang, X., Klevitsky, R., Huang, W., Glasford, J., Li, F., and Robbins, J. (2003). AlphaB-crystallin modulates protein aggregation of abnormal desmin. *Circ. Res.* 93, 998–1005. doi: 10.1161/01.RES.0000102401.77712.ED

Wang, X., Li, J., Zheng, H., Su, H., and Powell, S. R. (2011). Proteasome functional insufficiency in cardiac pathogenesis. *Am. J. Physiol. Heart Circ. Physiol.* 301, H2207–H2219. doi: 10.1152/ajpheart.00714.2011

Wang, X., Osinska, H., Klevitsky, R., Gerdes, A. M., Nieman, M., Lorenz, J., et al. (2001). Expression of R120G-alphaB-crystallin causes aberrant desmin and alphaB-crystallin aggregation and cardiomyopathy in mice. *Circ. Res.* 89, 84–91. doi: 10.1161/hh1301.092688

Wang, X., Pattison, J. S., and Su, H. (2013). Posttranslational modification and quality control. *Circ. Res.* 112, 367–381. doi: 10.1161/CIRCRESAHA.112.268706

Wang, X., and Robbins, J. (2006). Heart failure and protein quality control. *Circ. Res.* 99, 1315–1328. doi: 10.1161/01.RES.0000252342.61447.a2

Wang, X., Su, H., and Ranek, M. J. (2008). Protein quality control and degradation in cardiomyocytes. *J. Mol. Cell Cardiol.* 45, 11–27. doi: 10.1016/j.yjmcc.2008.03.025

Wani, P. S., Suppahia, A., Capalla, X., Ondracek, A., and Roelofs, J. (2016). Phosphorylation of the C-terminal tail of proteasome subunit alpha7 is required for binding of the proteasome quality control factor Ecm29. *Sci. Rep.* 6:27873. doi: 10.1038/srep27873

Webber, J. L., and Tooze, S. A. (2010a). Coordinated regulation of autophagy by p38alpha MAPK through mAtg9 and p38IP. *EMBO J.* 29, 27–40. doi: 10.1038/emboj.2009.321

Webber, J. L., and Tooze, S. A. (2010b). New insights into the function of Atg9. *FEBS Lett.* 584, 1319–1326. doi: 10.1016/j.febslet.2010.01.020

Weekes, J., Morrison, K., Mullen, A., Wait, R., Barton, P., and Dunn, M. J. (2003). Hyperubiquitination of proteins in dilated cardiomyopathy. *Proteomics* 3, 208–216. doi: 10.1002/pmic.200390029

Wei, Y., Pattingre, S., Sinha, S., Bassik, M., and Levine, B. (2008a). JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Mol. Cell.* 30, 678–688. doi: 10.1016/j.molcel.2008.06.001

Wei, Y., Sinha, S., and Levine, B. (2008b). Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation. *Autophagy* 4, 949–951. doi: 10.4161/auto.6788

Wilkinson, K. A., and Henley, J. M. (2010). Mechanisms, regulation and consequences of protein SUMOylation. *Biochem. J.* 428, 133–145. doi: 10.1042/BJ20100158

Willis, M. S., and Patterson, C. (2010). Hold me tight: role of the heat shock protein family of chaperones in cardiac disease. *Circulation* 122, 1740–1751. doi: 10.1161/CIRCULATIONAHA.110.942250

Willis, M. S., and Patterson, C. (2013). Proteotoxicity and cardiac dysfunction–Alzheimer's disease of the heart? *N. Engl. J. Med.* 368, 455–464. doi: 10.1056/NEJMra1106180

Willis, M. S., Schisler, J. C., Portbury, A. L., and Patterson, C. (2009). Build it up-Tear it down: protein quality control in the cardiac sarcomere. *Cardiovasc. Res.* 81, 439–448. doi: 10.1093/cvr/cvn289

Willis, M. S., Townley-Tilson, W. H., Kang, E. Y., Homeister, J. W., and Patterson, C. (2010). Sent to destroy: the ubiquitin proteasome system regulates cell signaling and protein quality control in cardiovascular development and disease. *Circ. Res.* 106, 463–478. doi: 10.1161/CIRCRESAHA.109.208801

Wong, C. H., Iskandar, K. B., Yadav, S. K., Hirpara, J. L., Loh, T., and Pervaiz, S. (2010). Simultaneous induction of non-canonical autophagy and apoptosis in cancer cells by ROS-dependent ERK and JNK activation. *PLoS One* 5:e9996. doi: 10.1371/journal.pone.09996

Wong, P. M., Feng, Y., Wang, J., Shi, R., and Jiang, X. (2015). Regulation of autophagy by coordinated action of mTORC1 and protein phosphatase 2A. *Nat. Commun.* 6:8048. doi: 10.1038/ncomms9048

Wu, B., Hunt, C., and Morimoto, R. (1985). Structure and expression of the human gene encoding major heat shock protein HSP70. *Mol. Cell. Biol.* 5, 330–341. doi: 10.1128/MCB.5.2.330

Wu, T., Mu, Y., Bogomolovas, J., Fang, X., Veevers, J., Nowak, R. B., et al. (2017). HSPB7 is indispensable for heart development by modulating actin filament assembly. *Proc. Natl. Acad. Sci. U.S.A.* 114, 11956–11961. doi: 10.1073/pnas.1713763114

Xie, Y., Kang, R., Sun, X., Zhong, M., Huang, J., Klionsky, D. J., et al. (2015). Posttranslational modification of autophagy-related proteins in macroautophagy. *Autophagy* 11, 28–45. doi: 10.4161/15548627.2014.984267

Xie, Z., Lau, K., Eby, B., Lozano, P., He, C., Pennington, B., et al. (2011). Improvement of cardiac functions by chronic metformin treatment is associated with enhanced cardiac autophagy in diabetic OVE26 mice. *Diabetes Metab. Res. Rev.* 60, 1770–1778. doi: 10.2337/db10-0351

Xu, M., Wan, C. X., Huang, S. H., Wang, H. B., Fan, D., Wu, H. M., et al. (2019). Oridonin protects against cardiac hypertrophy by promoting P21-related autophagy. *Cell Death Dis.* 10:403. doi: 10.1038/s41419-019-1617-y

Xu, P., Das, M., Reilly, J., and Davis, R. J. (2011). JNK regulates FoxO-dependent autophagy in neurons. *Genes Dev.* 25, 310–322. doi: 10.1101/gad.1984311

Yamaguchi, O., Higuchi, Y., Hirotani, S., Kashiwase, K., Nakayama, H., Hikoso, S., et al. (2003). Targeted deletion of apoptosis signal-regulating kinase 1 attenuates left ventricular remodeling. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15883–15888. doi: 10.1073/pnas.2136717100

Yokota, T., and Wang, Y. (2016). p38 MAP kinases in the heart. *Gene* 575, 369–376. doi: 10.1016/j.gene.2015.09.030

Younce, C. W., and Kolattukudy, P. E. (2010). MCP-1 causes cardiomyoblast death via autophagy resulting from ER stress caused by oxidative stress generated by inducing a novel zinc-finger protein. *MCPIP Biochem. J.* 426, 43–53. doi: 10.1042/BJ20090976

Yuan, F., Ma, Y., You, P., Lin, W., Lu, H., Yu, Y., et al. (2013). A novel role of proteasomal beta1 subunit in tumorigenesis. *Biosci. Rep.* 33:e00050. doi: 10.1042/BSR20130013

Zemanovic, S., Ivanov, M. V., Ivanova, L. V., Bhatnagar, A., Michalkiewicz, T., Teng, R. J., et al. (2018). Dynamic phosphorylation of the C terminus of Hsp70 regulates the mitochondrial import of SOD2 and redox balance. *Cell Rep* 25, 2605–2616.e7. doi: 10.1016/j.celrep.2018.11.015

Zeng, Y., Yang, X., Wang, J., Fan, J., Kong, Q., and Yu, X. (2012). Aristolochic acid I induced autophagy extenuates cell apoptosis via ERK 1/2 pathway in renal tubular epithelial cells. *PLoS One* 7:e30312. doi: 10.1371/journal.pone.030312

Zhai, P., Sciarretta, S., Galeotti, J., Volpe, M., and Sadoshima, J. (2011). Differential roles of GSK-3beta during myocardial ischemia and ischemia/reperfusion. *Circ. Res.* 109, 502–511. doi: 10.1161/CIRCRESAHA.111.249532

Zhang, D., Wang, W., Sun, X., Xu, D., Wang, C., Zhang, Q., et al. (2016). AMPK regulates autophagy by phosphorylating BECN1 at threonine 388. *Autophagy* 12, 1447–1459. doi: 10.1080/15548627.2016.1185576

Zhang, F., Hu, Y., Huang, P., Toleman, C. A., Paterson, A. J., and Kudlow, J. E. (2007). Proteasome function is regulated by cyclic AMP-dependent protein kinase through phosphorylation of Rpt6. *J. Biol. Chem.* 282, 22460–22471. doi: 10.1074/jbc.M702439200

Zhang, H., Pan, B., Wu, P., Parajuli, N., Rekhter, M. D., Goldberg, A. L., et al. (2019). PDE1 inhibition facilitates proteasomal degradation of misfolded proteins and protects against cardiac proteinopathy. *Sci. Adv.* 5:eaaw5870. doi: 10.1126/sciadv.aaw5870

Zhang, M., and Kass, D. A. (2011). Phosphodiesterases and cardiac cGMP: evolving roles and controversies. *Trends Pharmacol. Sci.* 32, 360–365. doi: 10.1016/j.tips.2011.02.019

Zhao, R., Davey, M., Hsu, Y. C., Kaplanek, P., Tong, A., Parsons, A. B., et al. (2005). Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. *Cell* 120, 715–727. doi: 10.1016/j.cell.2004.12.024

Zhao, X. X., Cho, H., Lee, S., Woo, J. S., Song, M. Y., Cheng, X. W., et al. (2020). BAY60-2770 attenuates doxorubicin-induced cardiotoxicity by decreased oxidative stress and enhanced autophagy. *Chem. Biol. Interact.* 328:109190. doi: 10.1016/j.cbi.2020.109190

Zheng, N., and Shabek, N. (2017). Ubiquitin ligases: structure, function, and regulation. *Annu. Rev. Biochem.* 86, 129–157. doi: 10.1146/annurev-biochem-060815-014922

Zheng, Q., Su, H., Ranek, M. J., and Wang, X. (2011). Autophagy and p62 in cardiac proteinopathy. *Circ. Res.* 109, 296–308. doi: 10.1161/CIRCRESAHA.111.244707

Zhou, J., Freeman, T. A., Ahmad, F., Shang, X., Mangano, E., Gao, E., et al. (2013). GSK-3α is a central regulator of age-related pathologies in mice. *J. Clin. Invest.* 123, 1821–1832. doi: 10.1172/JCI64398

Zhou, L., Jiang, Y., Luo, Q., Li, L., and Jia, L. (2019). Neddylation: a novel modulator of the tumor microenvironment. *Mol. Cancer* 18:77. doi: 10.1186/s12943-019-0979-1

Zhu, H., and He, L. (2015). Beclin 1 biology and its role in heart disease. *Curr. Cardiol. Rev.* 11, 229–237. doi: 10.2174/1573403X10666141106104606

Zhu, Y., Bogomolovas, J., Labeit, S., and Granzier, H. (2009). Single molecule force spectroscopy of the cardiac titin N2B element: effects of the molecular chaperone alphaB-crystallin with disease-causing mutations. *J. Biol. Chem.* 284, 13914–13923. doi: 10.1074/jbc.M809743200

Zong, C., Gomes, A. V., Drews, O., Li, X., Young, G. W., Berhane, B., et al. (2006). Regulation of murine cardiac 20S proteasomes: role of associating partners. *Circ. Res.* 99, 372–380. doi: 10.1161/01.RES.0000237389.40000.02

Zong, C., Young, G. W., Wang, Y., Lu, H., Deng, N., Drews, O., et al. (2008). Two-dimensional electrophoresis-based characterization of post-translational modifications of mammalian 20S proteasome complexes. *Proteomics* 8, 5025–5037. doi: 10.1002/pmic.200800387

Zu, L., Bedja, D., Fox-Talbot, K., Gabrielson, K. L., Van Kaer, L., Becker, L. C., et al. (2010). Evidence for a role of immunoproteasomes in regulating cardiac muscle mass in diabetic mice. *J. Mol. Cell Cardiol.* 49, 5–15. doi: 10.1016/j.yjmcc.2010.02.007

Zuo, Y., Chong, B. K., Jiang, K., Finley, D. J., Klenerman, D., and Ye, Y. (2020). A general in vitro assay to study enzymatic activities of the ubiquitin system. *Biochemistry* 59, 851–861. doi: 10.1021/acs.biochem.9b00602

Conflict of Interest: BD-E and MR co-inventors on a patent application (PCT: 448070145WO1) that was filed in July 2018 (provisional filed in June 2017). The patent relates to the use of TSC2 (S1365/S1364) modifications for immunological applications. MR is a co-founder and shareholder of Meta-T Cellular, a start-up company that aims to develop applications of this intellectual property for immune therapy.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 13 May 2021
doi: 10.3389/fphys.2021.673145

Autophagy Controls Nrf2-Mediated Dichotomy in Pressure Overloaded Hearts

Weiwei Wu[1], Qingyun Qin[2], Yan Ding[3], Huimei Zang[1], Dong-Sheng Li[3], Mitzi Nagarkatti[4], Prakash Nagarkatti[4], Wenjuan Wang[5], Xuejun Wang[6] and Taixing Cui[1]**

[1] Department of Cell Biology and Anatomy, School of Medicine, University of South Carolina, Columbia, SC, United States, [2] Department of Cardiology, Tianjing First Central Hospital, Tianjing, China, [3] Hubei Key Laboratory of Embryonic Stem Cell Research, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei, China, [4] Department of Pathology, Microbiology and Immunology, School of Medicine, University of South Carolina, Columbia, SC, United States, [5] Vascular Biology Center and Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta University, Augusta, GA, United States, [6] Division of Basic Biomedical Sciences, Sanford School of Medicine, University of South Dakota, Vermillion, SD, United States

Burgeoning evidence has indicated that normal autophagy is required for nuclear factor erythroid 2-related factor (Nrf2)-mediated cardiac protection whereas autophagy inhibition turns on Nrf2-mediated myocardial damage and dysfunction in a setting of pressure overload (PO). However, such a concept remains to be fully established by a careful genetic interrogation *in vivo*. This study was designed to validate the hypothesis using a mouse model of PO-induced cardiomyopathy and heart failure, in which cardiac autophagy and/or Nrf2 activity are genetically inhibited. Myocardial autophagy inhibition was induced by cardiomyocyte-restricted (CR) knockout (KO) of autophagy related (Atg) 5 (CR-Atg5KO) in adult mice. CR-Atg5KO impaired cardiac adaptations while exacerbating cardiac maladaptive responses in the setting of PO. Notably, it also turned off Nrf2-mediated defense while switching on Nrf2-operated tissue damage in PO hearts. In addition, cardiac autophagy inhibition selectively inactivated extracellular signal regulated kinase (ERK), which coincided with increased nuclear accumulation of Nrf2 and decreased nuclear translocation of activated ERK in cardiomyocytes in PO hearts. Mechanistic investigation revealed that autophagy is required for the activation of ERK, which suppresses Nrf2-driven expression of angiotensinogen in cardiomyocytes. Taken together, these results provide direct evidence consolidating the notion that normal autophagy enables Nrf2-operated adaptation while switching off Nrf2-mediated maladaptive responses in PO hearts partly through suppressing Nrf2-driven angiotensinogen expression in cardiomyocytes.

Keywords: Nrf2, autophagy, ERK, cardiac dysfunction, pressure overload

OPEN ACCESS

Edited by:
Mark J. Ranek, Johns Hopkins University, United States

Reviewed by:
Jun Ren, University of Washington, United States
Subhash K. Das, University of Alberta, Canada

****Correspondence:***
Xuejun Wang
xuejun.wang@usd.edu
Taixing Cui
taixing.cui@uscmed.sc.edu

Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *26 February 2021*
Accepted: *21 April 2021*
Published: *13 May 2021*

Citation:
Wu W, Qin Q, Ding Y, Zang H, Li D-S, Nagarkatti M, Nagarkatti P, Wang W, Wang X and Cui T (2021) Autophagy Controls Nrf2-Mediated Dichotomy in Pressure Overloaded Hearts. Front. Physiol. 12:673145. doi: 10.3389/fphys.2021.673145

INTRODUCTION

Autophagy is an evolutionarily conserved pathway that targets cytoplasmic contents to the lysosome for degradation (Wang and Hill, 2015; Wang and Cui, 2017; Sciarretta et al., 2018). Based on how the target is delivered into lysosomes for final degradation, autophagy in mammals has been classified into three types: (i) macroautophagy, (ii) microautophagy, and (iii) chaperone-mediated autophagy (CMA). The macroautophagy (thereafter referred to as autophagy) is the

best characterized. Autophagy serves as a house keeping process to maintain cardiac integrity and function under baseline conditions. Though, the role of autophagy in stressed hearts remains controversial. Depending on the nature of stress and timing of assessment, activation of cardiac autophagy has been proposed to be either adaptive or maladaptive (Wang and Hill, 2015; Wang and Cui, 2017; Sciarretta et al., 2018). A biphasic modality of cardiac autophagy regulation in obesity has been proposed (Zhang et al., 2018). At an early stage of obesity, cardiac autophagy may be increased as an adaptive response; however, at a later stage of obesity, cardiac autophagy is impaired, which is characterized by increased initiation of autophagy and suppressed degradation of autophagosomes (Zhang et al., 2018). Importantly, most of the recent studies have shown that autophagy activation is likely an adaptive response in the hearts after pressure overload (PO hearts) (Wang and Cui, 2017; Sciarretta et al., 2018). In this regard, we lately demonstrated that PO-induced cardiomyopathy and heart failure are dramatically ameliorated by enhancing myocardial autophagy via cardiomyocyte-restricted (CR)-autophagy related 7 (Atg7) transgenic overexpression (Qi et al., 2020), providing critical evidence to support adaptive nature of autophagy activation in PO hearts. Nevertheless, the precise role of autophagy in stressed hearts remains to be investigated and it may not be fully understood until the signaling network of cardiac autophagy is completely mapped.

Nuclear factor-erythroid factor 2-related factor 2 (Nrf2), a transcription factor, controls the basal and inducible expression of several hundred genes that can be grouped into several categories with different functions including antioxidant defense, detoxification, inflammatory responses, gene transcription, transporters, protein degradation, and metabolism (Cui et al., 2016; Zang et al., 2020a). Thus, the functions of Nrf2 spread rather broadly from antioxidant defense to protein quality control and metabolism regulation. Historically, Nrf2 has been considered as a master regulator of antioxidant defense, thereby providing protection against diverse cardiomyopathies associated with oxidative stress (Li et al., 2009a). However, this notion is challenged by the emerging evidence which revealed a mediator role of Nrf2 in the progression of cardiomyopathies associated with various pathological settings including proteotoxicity associated with aging, myocardial ischemia-reperfusion injury, pressure overload, and type 1 diabetes (Zang et al., 2020a,b). Although the precise mechanisms underlying Nrf2-mediated dichotomy in the heart are poorly understood, we observed that activation of Nrf2 is protective (Li et al., 2009a) when autophagy is intact in PO hearts, whereas it become detrimental to PO hearts when cardiac autophagy is impaired (Qin et al., 2016). In addition, we found that autophagy inhibition via CR-Atg5KO activates Nrf2-driven expression of angiotensinogen in cardiomyocytes, presumably creating a pathological signaling axis in which autophagy inhibition activates Nrf2-driven angiotensinogen expression, which in turn promotes angiotensin II production and subsequent activation of angiotensin II receptor type 1 signaling, thereby contributing to cardiac pathological remodeling and dysfunction (Qin et al., 2016). These results indicate a unique role of autophagy in controlling Nrf2-mediated dichotomy in PO hearts, i.e., normal autophagy is required for Nrf2-mediated cardiac protection whereas autophagy inhibition turns on Nrf2-mediated myocardial damage and dysfunction. Yet, such a concept remains to be fully established by genetic interrogation of an axis of autophagy-Nrf2 activation-cardiac remodeling and dysfunction *in vivo*.

In the present study, we verified the critical role of autophagy in controlling Nrf2-mediated dichotomy in PO hearts using CR-Atg5KO and Nrf2KO mice. We demonstrated that autophagy inhibition via CR-Atg5KO wipes out Nrf2-operated defense while activating Nrf2-mediated injuries in PO hearts toward heart failure. We also found that autophagy is required for maintaining ERK-dependent downregulation of Nrf2-driven angiotensinogen expression in cardiomyocytes. These findings provide direct evidence to consolidate the emerging notion that autophagy controls Nrf2-mediated dichotomy in the heart.

MATERIALS AND METHODS

Animals

All animals were kept on a 12-hour light/dark cycle in a temperature-controlled room with ad libitum access to food and water. All animals were treated in compliance with the USA National Institute of Health Guideline for Care and Use of Laboratory Animals. The use of animals and all animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina, United States. The breeding pairs of Nrf2 heterozygote knockout ($Nrf2^{+/-}$) and floxed Atg5 ($Atg5^{fl/fl}$) mice in a C57BL/6J genetic background were purchased from Riken BioResource Research Center, Tsukuba, Japan. The breeding pairs of a transgenic mouse strain harboring a tamoxifen-inducible Cre-fusion protein (MerCreMer) under the control of cardiomyocyte-specific α-myosin heavy-chain promoter (Myh6 or αMHC) in a C57BL/6J genetic background were purchased from JAX. Littermates of wild type (WT) and $Nrf2^{-/-}$ (Nrf2KO) mice were generated by breeding pairs of $Nrf2^{+/-}$ mice as we previously described (Li et al., 2014). Littermates of $MerCreMer^{+}$, $Atg5^{fl/fl}$, and $MerCreMer^{+}$::$Atg5^{fl/fl}$ mice were produced using the breeding pairs of $MerCreMer^{+}$::$Atg5^{fl/+}$ and $Agt5^{fl/+}$, which were generated by crossing $MerCreMer^{+}$ with $Atg5^{fl/fl}$ mice; and double Nrf2 and CR-Atg5 KO ($Nrf2^{-/-}$::$Atg5^{-/-}$) mice were generated by crossing $Nrf2^{-/-}$ mice with $MerCreMer^{+}$::$Atg5^{fl/fl}$ mice as we previously described (Qin et al., 2016).

Induction and Quantification of Recombination

Tamoxifen (Cat#: T5648, Sigma-Aldrich, St. Louis, MO, United States) was dissolved in warm sunflower seed oil at a concentration of 10 mg/ml and injected intraperitoneally (i.p.) at 20 mg/kg body weight daily (20 mg/kg/d) for 3 weeks to avoid the cardiac Cre toxicity as previously reported (Koitabashi et al., 2009). The induction of recombination with tamoxifen was started in male mice at 6 weeks of age. After a time period of 2 weeks for washing out the tamoxifen,

the efficacy of recombination was determined by Western blot analysis of ATG5 expression in WT, MerCreMer^{+}, MerCreMer^{+}::Atg5$^{fl/+}$, and MerCreMer^{+}::Atg5$^{fl/fl}$ mice which received the tamoxifen induction.

Transverse Aortic Arch Constriction (TAC)

Male littermates of WT and Nrf2$^{-/-}$ (Nrf2KO) mice at 11 weeks of age were subject to sham or TAC operation for 4 or 8 weeks. Male littermates of WT, MerCreMer^{+}, Agt5$^{fl/fl}$, and MerCreMer^{+}::Atg5$^{fl/fl}$ or MerCreMer^{+}, MerCreMer^{+}::Nrf2KO, MerCreMer^{+}Atg5$^{fl/fl}$, and MercreMer^{+}::Atg5$^{fl/fl}$::Nrf2KO mice which received tamoxifen induction as described above were subject to sham or TAC operation at 11–12 weeks of age and euthanized 4 or 6 weeks later. The sham or TAC operation in mice was performed under deep anesthesia as previously described (Li et al., 2009b; Qin et al., 2016). Briefly, mice were anesthetized by i.p. injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). The use of a horizontal incision at the level of the suprasternal notch allows direct visualization of the transverse aorta without entering the pleural space and thus obviates the need for mechanical ventilation. The transverse aorta was banded between the right innominate and left carotid arteries to a 27-gage needle using a 6-0 nylon silk suture. Sham operation on mice were similar but without actual aortic banding and these mice served as a control group for all experimental groups. Cardiac hypertrophy was determined by heart weight–to–tibial length (HW/TIBIA) ratio, heart weight–to–body weight (HW/BW) ratio and expression levels of cardiac hypertrophy marker genes including atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), alpha-myosin heavy chain (α-MHC), beta-myosin heavy chain (β-MHC), sarco-endoplasmic reticulum calcium ATPase2a (SERCA2a).

Echocardiographic Analysis

Echocardiography was performed on anesthetized mice using the Vevo 2100 High-Resolution Imaging System (VisualSonics Inc.) with a 30-MHz high-frequency linear transducer as previously described (Li et al., 2009b, Li et al., 2014; Qin et al., 2016). Briefly, mice were anesthetized with 3% isoflurane and maintained with 1.5% isoflurane in room air supplemented with 100% O_2. After the anterior chest was shaved, the animals were placed on a warming pad to maintain normothermia. The echocardiographic gel was warmed before use to avoid hypothermia. Care was taken to avoid excessive pressure on the thorax, which can induce bradycardia. Two-dimensional (2D) long axis images of the left ventricle (LV) were obtained at the plane of the aortic and mitral valves where the LV cavity is largest, and visualization of the LV apex is adequate; and a short-axis image was recorded at the level of the papillary muscles. A 2D guided M-mode echocardiogram was recorded through the anterior and posterior LV walls at 21 frames/s. Images were obtained at the level of the papillary muscle tips, and measurements were then performed to obtain the LV internal dimension (LVID; in mm), interventricular septum thickness (IVS), and LV posterior wall thickness (LVPW; in mm). LV percent fractional shortening FS (%) was calculated via VisualSonics Measurement Software.

Pathology

Mice were anesthetized and perfused via the LV apex with saline (0.9% NaCl) to wash out the blood from the heart tissue. Then, the hearts were dried on gauze, weighed, dissected, and frozen. Lungs and tibias were also dissected. Lungs were dried on gauze and weighed. The length of the tibia from the condyle to the tip of the medial malleolus was measured by micrometer calipers.

Histological and Immunochemical Analysis

Hearts were cannulated via the LV apex, cleared of blood by perfusion with normal saline at 90 mmHg, arrested in diastole with 60 mM KCl, fixed by perfusion with 4% paraformaldehyde, and embedded in paraffin. Paraffin sections were prepared (5 μm, Leica RM2235, rotary microtome) and stored at room temperature until staining. For LV cardiomyocyte cross-sectional area, coronal sections were deparaffinized and the cardiomyocyte membranes were stained with Alexa Fluor 488 conjugated wheat germ agglutinin (WGA) (Invitrogen Corp., Carlsbad, CA, United States) and observed using a fluorescence microscope (Nikon Eclipse 80i; Nikon Instruments Inc. Tokyo, Japan) at 400× magnification. Twenty fields of each section were randomly photographed using NIS-Elements F 4.0 imaging software (Nikon Instruments Inc. Tokyo, Japan) and cross-sectional areas of 1,000–1,400 circular cardiomyocytes per heart was measured using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, United States). For myocardial fibrosis, coronal sections were stained for collagen with a Masson's Trichrome Kit (Poly Scientific, Bay Shore, NY, United States) according to the protocol provided by the manufacturer. Sections were observed under a light microscope (Nikon Eclipse 80i; Nikon Instruments Inc. Tokyo, Japan) at 200× magnification. Twenty fields of each section were randomly photographed using NIS-Elements F 4.0 imaging software (Nikon Instruments Inc. Tokyo, Japan). The percentage of fibrosis (the blue stained area) was measured by Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, United States). At least two sections of each heart were analyzed for the measurements of cardiomyocyte cross sectional areas and cardiac fibrosis. Sub-cellular locations of p-ERK and Nrf2 were analyzed by immunochemical staining using anti-p-ERK (cat#: 9101, Cell Signaling Technology, Inc., Danvers, MA, United States) and anti-Nrf2 (cat#: sc-722, 1:200, Santa Cruz Biotechnology Inc., Dallas, TX, United States). Two sections from each heart were analyzed.

Cell Cultures, Adenoviral Infection, Oligo and Plasmid Transfection

Rat neonatal cardiac myocytes were isolated and cultured as previously described (Li et al., 2009b; Qin et al., 2016). Adenovirus of control scramble shRNA (Ad-shCtr) and rat Nrf2 shRNA (Ad-shNrf2) were generated as previously reported (Li et al., 2009b). Rat neonatal cardiomyocytes were infected with Ad-shCtr (20 MOI) and Ad-shNrf2 (20 MOI)

in serum-free DMEM for 6 h and the cultured with full growth medium (1 g/L glucose DMEM supplemented with 8% horse serum (HS) and 5% newborn calf serum (NCS) for additional 24 h. The infected cardiomyocytes were further transfected with scramble siRNA against luciferase (siCtr, 5′-CGUACGCGGAAUACUUCGATT-3′, purchased from Invitrogen Corp., Carlsbad, CA, United States), Agt5 siRNA (siAtg5-1: 5′-GACGCUGGUAACUGACAAATT-3′, siAtg5-2: 5′-GUCAGGUGAUCAACGAAAUTT-3′, or siAtg5-3: 5′-CCACAACUGAACGGCCUUUTT-3′, purchased from Ribobio, Guangzhou, China) using Lipofectamine 2000 (Cat#: 12566-014, Thermo Fisher Scientific, Waltham, United States) for 6 h and then cultured with full growth medium aforementioned for 24 h. The transfection was repeated once and then the cells were serum starved for 48 h prior to the treatment with Ang II (1 μM, Cat#: RAB0010, Sigma-Aldrich, St. Louis, MO, United States) and U0126 (1 μM, an ERK inhibitor, Cat#: U120, Sigma-Aldrich, St. Louis, MO, United States) in serum free DMEM. Based on our pilot study that Ang II stimulation induced phosphorylation of ERK at 10 min and upregulation of LC3-II at 24 h while enhancing protein expression of angiotensinogen at 48 h in rat neonatal cardiomyocytes (data not shown), we treated the cardiomyocytes with Ang II for 10 min prior to Western blot analysis of ERK phosphorylation and 24 h prior to Western blot analysis of LC3-II and p62 protein expression, and 48 h prior to Western blot analysis of angiotensinogen protein expression. These experiments were repeated for four times.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Quantitative Real Time (qPCR)

The total RNA from the LV was extracted using RNeasy Fibrous Tissue Mini kit (Qiagen Inc., Valencia, CA, United States), and the reverse transcription reaction was performed with 1 μg of total RNA using a RevertAid^{TM} First Strand cDNA Synthesis Kit (Cat#: K1622, Thermo Scientific). qPCR was carried out using the Bio-Red CFX96^{TM} Real-Time system (C1000^{TM} Thermal Cycler, Bio-Red Laboratories, Inc. Hercules, CA, United States). Expression levels of target genes were normalized by concurrent measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Genomic DNAs were also extracted from mouse tails and subjected to PCR for genotyping of transgene mice. Primers that were used for PCR are summarized in **Supplementary Table S1**.

Western Blot Analysis

Left ventricle or cardiomyocyte whole lysates or nuclear and cytoplasmic fractions of cardiomyocytes extracted with a NE-PER Nuclear and Cytoplasmic Extraction Kit (Cat#: 78833, ThermoFisher Scientific, United States) were subject to Western blot analysis as we previously described.(Li et al., 2009b, Li et al., 2014; Qin et al., 2016) Antibodies used included anti-APG5L/ATG5 monoclonal antibody (Cat#: 3447-1, Abcam, Cambridge, United Kingdom), anti-LC3B polyclonal antibody (Cat#: L7543, Sigma-Aldrich, St. Louis, MO, United States), anti-ERK antibody (Cat#: 9101S, Cell Signaling Technology, United States), anti-angiotensinogen (Cat#: sc-7419, Santa Cruz Biotechnology Inc., Dallas, TX, United States), anti-p62 (Cat#: ab91526, Abcam, Cambridge, United Kingdom), anti-NQO1 monoclonal antibody (Cat#: sc-376023, Santa Cruz Biotechnology, Inc., Dallas, Texas, United States), anti-Nrf2 polyclonal antibody (Cat#: sc-722, Santa Cruz Biotechnology, Inc., Dallas, TX, United States), anti-GAPDH polyclonal antibody (Cat#: G9545, Sigma-Aldrich, St. Louis, MO, United States), peroxidase-conjugated AffiniPure goat anti-Mouse IgG (H+L) (Cat#: ZB2305, ZSGB-BIO, Beijing, China), and peroxidase-conjugated AffiniPure rabbit anti-goat IgG (H+L) (Cat#: ZB2306, ZSGB-BIO, Beijing, China).

Statistics

Data are shown as mean ± SD. Differences between 2 groups were evaluated for statistical significance using the Student t test. When differences among >3 groups were evaluated, results were compared by one-way ANOVA with Bonferroni test for multiple comparisons. Survival rate between experimental groups after TAC was analyzed using Kaplan Meier test. Differences were considered significant at $P < 0.05$.

RESULTS

Cardiac Autophagy Protects Against PO-Induced Cardiomyopathy and Controls Nrf2-Mediated Dichotomy in PO Hearts

To determine a causative role of autophagy in the regulation of cardiomyopathy and Nrf2-mediated dichotomy in PO hearts, we determined the impact of cardiac specific autophagy inhibition via CR-Atg5KO on TAC-induced cardiac remodeling and dysfunction as well as Nrf2-mediated dichotomy (Qin et al., 2016) in adult mice. In our pilot study, we found that the expression of Cre *per se* induced by the nontoxic doses of tamoxifen established by a previous study (Koitabashi et al., 2009) did not have negative impact on the heart or cause any other health issues at the basal conditions, but it did slightly worsen TAC-induced cardiomyopathy (data not shown). To minimize the impact of off-target effects of MerCreMer, we used mice which all carried the MerCreMer transgene and all received the same regime of tamoxifen treatment.

Transverse aortic arch constriction caused around 20% mortality in Control MerCreMer^{+} (Ctl) mice as described elsewhere (Qin et al., 2016) and it was slightly increased by additional Nrf2KO (25%) (**Figure 1A**), suggesting Nrf2-mediated protection. Compared with the Ctl or Nrf2KO groups, CR-Atg5KO (32%) enhanced TAC-induced death; however, the CR-Atg5KO-enhanced death was reversed by additional Nrf2KO (**Figure 1A**), demonstrating autophagy inhibition-dependent activation of Nrf2-mediated detrimental effects. In addition, when cardiac autophagy flux is intact within the first 2 weeks after TAC (Qin et al., 2016), Nrf2KO enhanced TAC-induced cardiac dysfunction (**Figure 1B** and **Supplementary Table S2**) as we previously reported (Li et al., 2009b). When cardiac

autophagy is inhibited at 6 weeks after TAC (Qin et al., 2016), however, Nrf2KO improved TAC-induced cardiac dysfunction (**Figure 1Ca** and **Supplementary Table S3**) and attenuated TAC-induced cardiac hypertrophy and fibrosis (**Figure 2** and **Supplementary Table S3**) as we previously reported (Qin et al., 2016). On the other hand, CR-Atg5KO resulted in declined cardiac function in sham-operated mice and exaggerated TAC-induced cardiac dysfunction at both 2 and 6 weeks (**Figures 1B,Ca,b** and **Supplementary Tables S2, S3**). Also, CR-Atg5KO exacerbated TAC-induced cardiac hypertrophy and fibrosis at 6 weeks (**Figure 2** and **Supplementary Table S3**). These results demonstrate an essential role of cardiac autophagy in maintaining cardiac homeostasis at both basal and PO conditions. Notably, all these adverse impacts of CR-Atg5KO could be rescued by additional Nrf2KO (**Figures 1**, **2** and **Supplementary Tables S2, S3**), demonstrating that autophagy inhibition activates Nrf2-mediated damage in stressed hearts, such as PO hearts. To this end, we demonstrate that Cre (MerCreMer) expression *per se* has less impact on Nrf2-mediated dichotomy in PO hearts. Accordingly, we validate our previous findings (Li et al., 2009b; Qin et al., 2016) but give in addition, direct evidence to consolidate the concept that autophagy controls Nrf2-mediated dichotomy in PO hearts.

Autophagy Inhibition-Induced Inactivation of ERK Is an Upstream Signal to Enhance Nrf2-Operated Upregulation of Angiotensinogen in PO Hearts

We have demonstrated that autophagy inhibition enables Nrf2 to upregulate myocardial expression of angiotensinogen, a primary cause of cardiac pathological remodeling and dysfunction (Wollert and Drexler, 1999), in PO hearts (Qin et al., 2016). At the molecular level, autophagy impairment suppresses activation of Jak2/Fyn pathway which operates Nrf2 nuclear export for degradation in PO hearts (Qin et al., 2016). A previous study showed that the autophagosomal membrane could serve as a platform to regulate intracellular signaling transduction such as the activation of extracellular signal regulated kinase (ERK) (Martinez-Lopez et al., 2013). Given that autophagy are intertwined with multiple kinase pathways (Kroemer et al., 2010) including mitogen activated protein kinases (MAPKs), such as ERK, and protein kinase B (AKT) cascades, all of which regulate Nrf2 activity (Li et al., 2009a; Bryan et al., 2013), we questioned whether these kinases are also involved in the emerging axis of autophagy inhibition-Nrf2-angiotensinogen expression in PO hearts.

In 4-week TAC hearts in which the axis of Nrf2-angiotensinogen activation is boosted by CR-Atg5KO (Qin et al., 2016), we found that such autophagy inhibition minimally regulated the activities of MAPK p38 and JNK or AKT, but strongly suppressed the activities of ERK (**Figure 3A**). On the other hand, Nrf2KO did not affect PO-induced activation of these kinases (**Figure 4**) but downregulated PO-induced upregulation of angiotensinogen expression at 4 weeks (data not shown) when cardiac autophagy flux declines as we previously observed (Qin et al., 2016). These results indicate that proper initiation of autophagy, e.g., autophagosome formation, is critical for signal-induced activation of ERK in cardiomyocytes as observed in the other cell types (Martinez-Lopez et al., 2013). The autophagy-operating ERK activity is likely an upstream event of Nrf2-mediated regulation of angiotensinogen expression in the heart.

To further establish a pathophysiological relevance of the autophagy inhibition-ERK-Nrf2 axis, we studied the nuclear location of ERK and Nrf2 in PO hearts. Immunochemical staining showed that there is no detectable level of nuclear phosphorylated ERK (p-ERK) or Nrf2 in 4-week sham hearts of $Atg5^{fl/fl}$, $MerCreMer^{+}$ and CR-Atg5KO mice (data not shown). However, 4-week TAC led to increased levels of nuclear p-ERK in the hearts of control $Atg5^{fl/fl}$ and $MerCreMer^{+}$ mice but not in the hearts of CR-Atg5KO mice (**Figure 3B**). Also, 4-week TAC led to increased levels of nuclear Nrf2 in the hearts of control $Agt5^{fl/fl}$ and $MerCreMer^{+}$ mice and it was more dramatic in the hearts of CR-Atg5KO mice (data not shown) as we previously observed (Qin et al., 2016). Taken together, this reciprocal relationship between nuclear p-ERK and Nrf2 in PO hearts, as well as the enhanced nuclear Nrf2 levels in autophagy deficient cardiomyocytes suggest that not only inactivation of Jak/Fyn pathway (Qin et al., 2016) but also ERK is responsible for the nuclear accumulation of Nrf2 leading to upregulation of angiotensinogen in autophagy insufficient myocardium.

Autophagy Inhibition Suppresses ERK-Mediated Nrf2-Driven Angiotensinogen Expression in Cardiomyocytes

To establish the signaling axis of autophagy inhibition-ERK inactivation-Nrf2 activation-angiotensinogen expression in PO hearts, we determined the effect of angiotensin II (Ang II) on ERK activation, Nrf2 activity and angiotensinogen expression in primary culture of neonatal rat ventricular myocytes (NRVMs) with a combination of Atg5 and/or Nrf2 knockdown and/or ERK inhibition. As shown in **Figure 5A**, Ang II induced phosphorylation of ERK along with increased LC3 and angiotensinogen expression (data not shown) as we previously observed (Qin et al., 2016) in the control group. These results indicate that Ang II-induced upregulation of angiotensinogen associates with the activation of autophagy and ERK in cardiomyocytes. Knockdown of ATG5 alone resulted in downregulation of LC3-II and upregulation of p62 (data not show) as we previously observed (Qin et al., 2016), indicating impaired autophagy in cardiomyocytes. The ATG5 knockdown-induced autophagy inhibition blocked the Ang II-induced activation of ERK whereas dramatically enhancing the Ang II-induced angiotensinogen expression (**Figures 5A,B**), demonstrating that autophagy inhibition suppresses activation of ERK while facilitating upregulation of angiotensinogen in cardiomyocytes as observed in autophagy-impaired PO hearts (Qin et al., 2016). Knockdown of Nrf2 alone had minimal impact on the Ang II-induced activation of ERK but suppressed the Ang II-induced upregulation of angiotensinogen (**Figures 5A,B**).

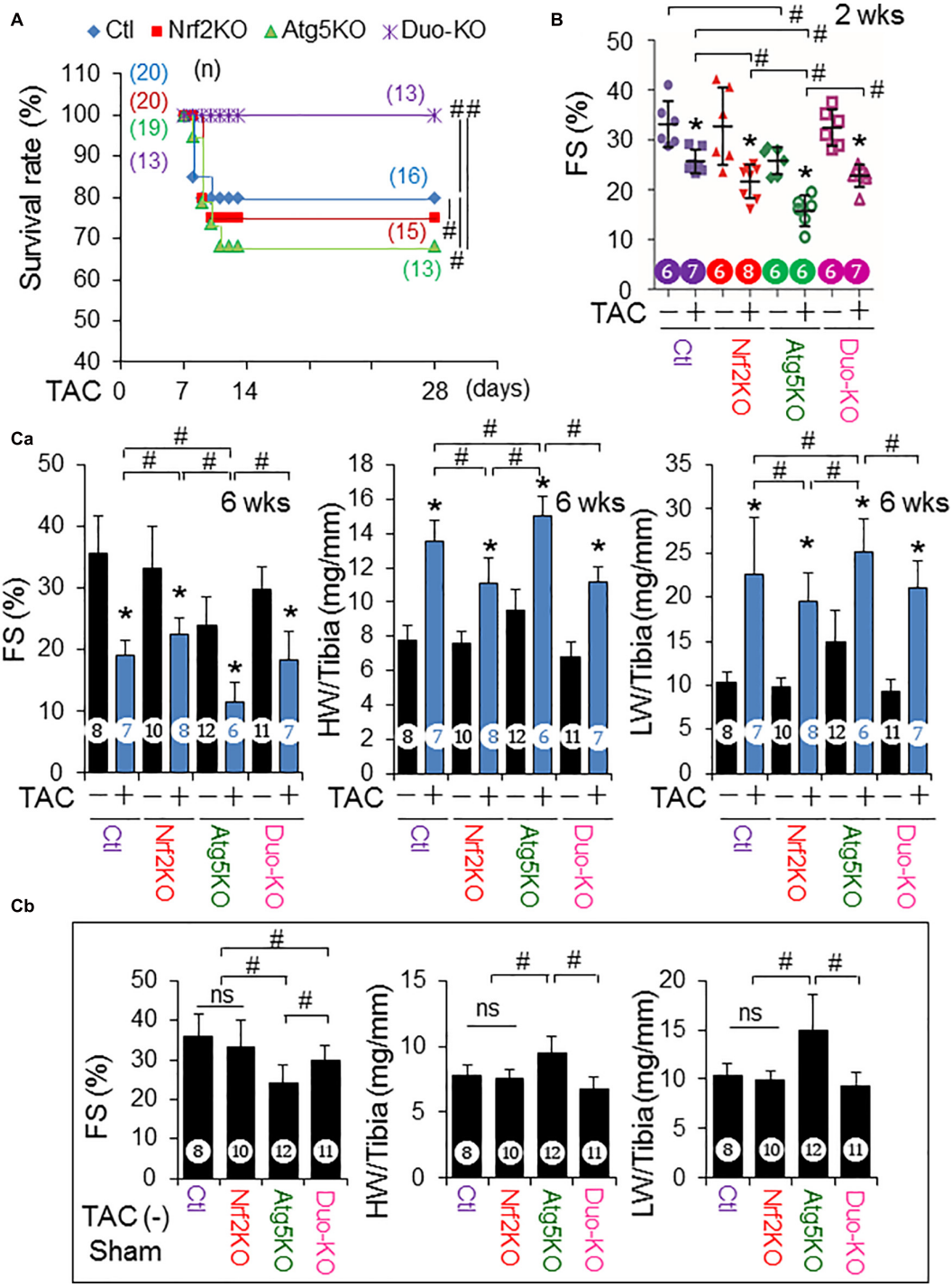


FIGURE 1 | Nrf2-mediated dichotomy in mice in response to pressure overload. The mice with genotypes of MerCreMer^{+} (Ctl), MerCreMer^{+}::Nrf2 KO (Nrf2KO), MerCreMer^{+}::Atg5$^{fl/fl}$ (Atg5KO), and MerCreMer^{+}::Atg5$^{fl/fl}$::Nrf2KO (Duo-KO) received tamoxifen treatment as described in the section "Materials and Methods" and then were subject to sham and TAC operation for 6 weeks. **(A)** Survival rate at 4 weeks after TAC. $^{\#}p < 0.05$ by Kaplan Meier test. **(B)** FS (%) at 2 weeks after TAC. $^{*}p < 0.05$ vs TAC (–) in the same groups using the Student *t* test; $^{\#}p < 0.05$ between indicated groups using one-way ANOVA followed by Bonferroni test. **(C)** FS (%), HW/Tibia ratio and LW/Tibia ratio at 6 weeks after TAC. $^{*}p < 0.05$ vs TAC (–) in the same groups using the Student *t* test; $^{\#}p < 0.05$ between indicated groups using one-way ANOVA followed by Bonferroni test. (a) Statistic analysis of both sham and TAC groups; (b) Statistic analysis of sham groups alone. Animal number for each group is indicated in each figure. The efficacy of Cre and LoxP recombination which ablates myocardial Atg5 expression in Atg5KO and Duo-KO mice was confirmed by Western blot analysis.

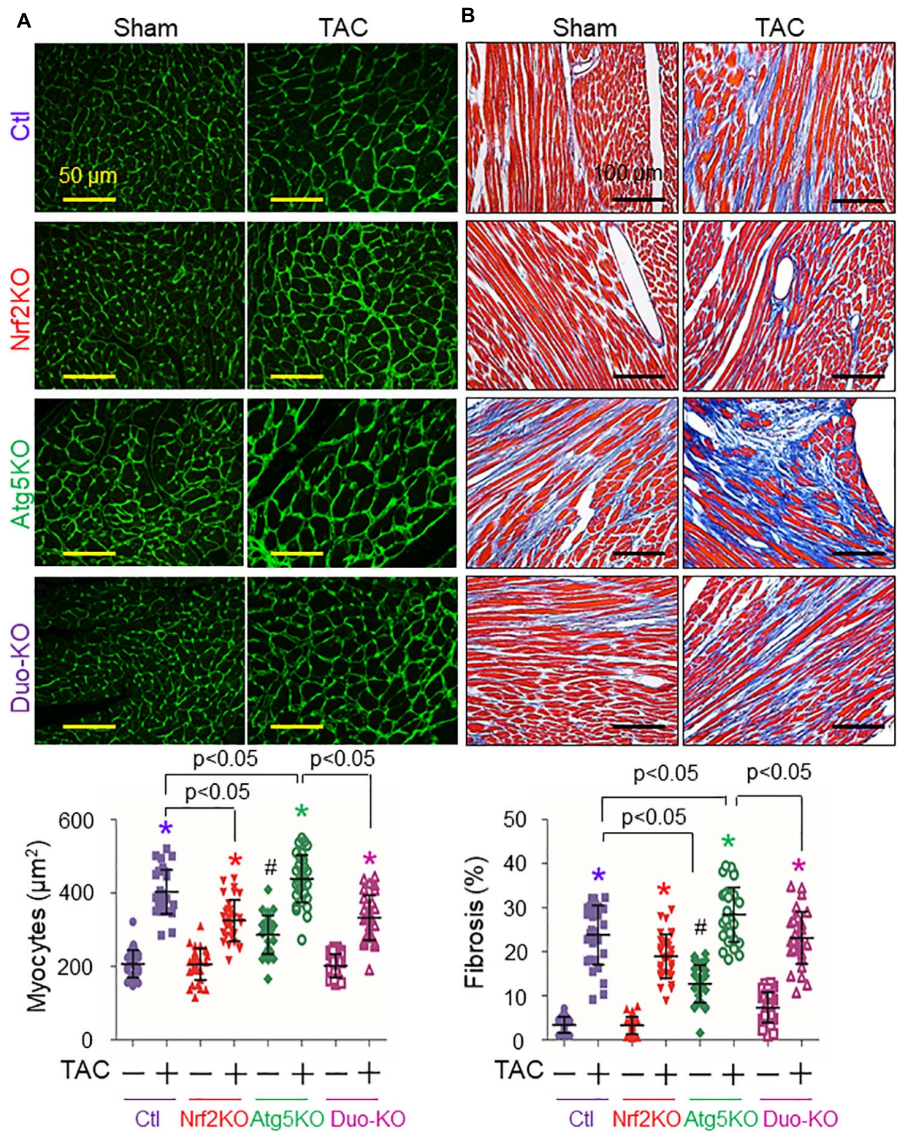


FIGURE 2 | Nrf2-mediated cardiac pathological remodeling in mice in response to pressure overload. The mice with indicated genotypes received tamoxifen treatment as described in the section "Materials and Methods" and then were subject to sham and TAC operation for 6 weeks. **(A)** Cardiomyocyte sizes. **(B)** Cardiac fibrosis. Animal number for each group is 3~4. $^{*}p < 0.05$ vs the sham, TAC (–) in the same groups using the Student *t* test; $^{\#}p < 0.05$ vs TAC (–) in all groups using the Student *t* test; $p < 0.05$ between indicated groups using one-way ANOVA followed by Bonferroni test.

Although knockdown of Nrf2 did not affect the ATG5 deficiency-induced inactivation of ERK, it blocked the ATG5 deficiency-induced enhancement of angiotensinogen expression (**Figures 5A,B**). These results demonstrate that Nrf2-mediated angiotensinogen expression in cardiomyocytes (Qin et al., 2016) is negatively regulated in part by autophagy-dependent ERK activation, supporting that ERK signaling plays an important role in the control of Nrf2-mediated upregulation of angiotensinogen in autophagy-impaired hearts.

To establish a causative link between ERK, Nrf2 activation, and angiotensinogen expression in cardiomyocytes, we used an ERK inhibitor, U0126 in the primary culture of NRVMs. U0126 enhanced Ang II-induced upregulation of NAD(P)H quinone dehydrogenase 1 (Nqo1), a typical Nrf2 target gene and angiotensinogen mRNA levels in control group (**Figure 5B**). These results reveal that ERK serves a negative regulator of Nrf2 activation and angiotensinogen expression when autophagy is intact. Notably, knockdown of ATG5 dramatically enhanced Ang II-induced expression of Nqo1 and angiotensinogen mRNAs and this enhancement could not be further increased by U0126; whereas, both U0126-potentiated and ATG5 deficiency-augmented angiotensinogen expression were blocked by knockdown of Nrf2 (**Figure 5B**). These results indicate that ERK suppresses angiotensinogen expression by inactivating Nrf2, serving as a negative feedback mechanism for the control of angiotensinogen expression in autophagy intact

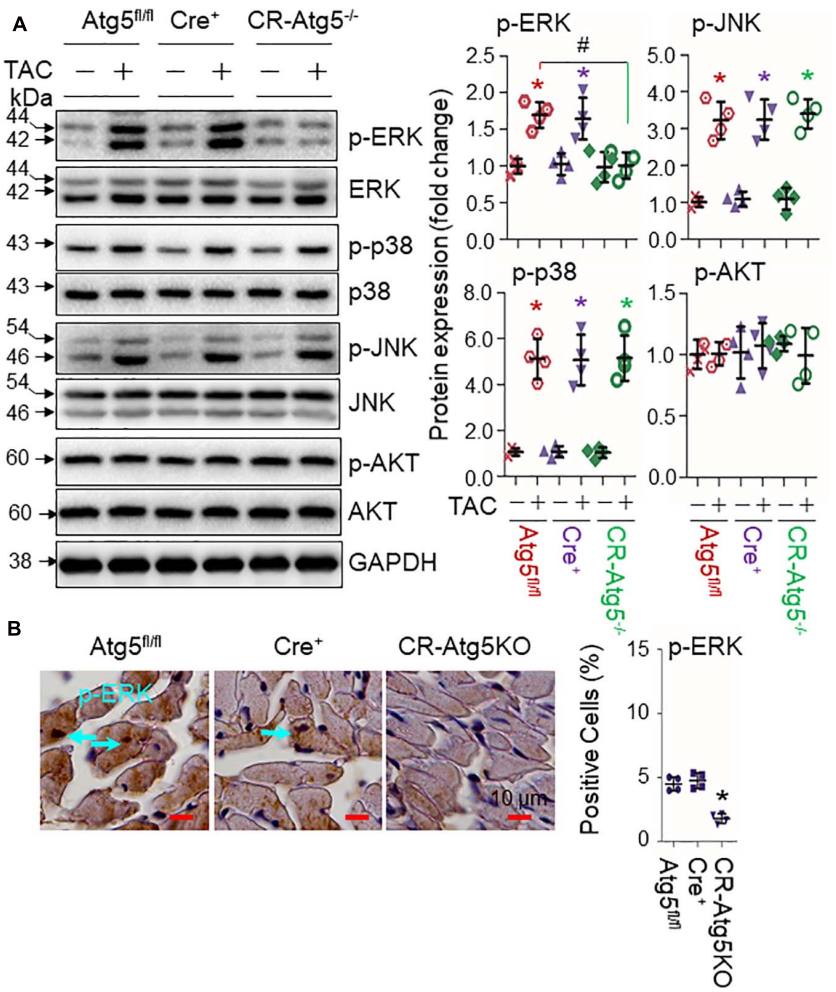


FIGURE 3 | The impact of cardiomyocyte-restricted knockout of Atg5 on pressure overload-induced activation of MAPK and expression of angiotensinogen (Agt) in the heart. Male floxed Atg5 (Atg5$^{fl/fl}$), MerCreMer^{+} (Cre^{+}), and MerCreMer^{+}::Atg5$^{fl/fl}$ (CR-Atg5$^{-/-}$ or CR-Atg5KO) after tamoxifen-induced ablation of Atg5 were subject to sham or TAC operations for 4 weeks. **(A)** Western blot analysis of LV tissues ($n = 4$). The right panel is the semi-quantified densitometry analyses. $^{*}p < 0.05$ vs TAC (–) in the same groups using the Student t test; $^{\#}p < 0.05$ between the indicated groups using one-way ANOVA followed by Bonferroni test. **(B)** Immunohistochemical staining of phosphorylated ERK and Nrf2 in pressure overloaded hearts of Atg5$^{fl/fl}$, MerCreMer^{+} (Cre^{+}) and CR-Atg5KO mice. LV tissue sections of mice with indicated genotypes were stained with antibodies for phosphorylated ERK (p-ERK) and Nrf2 (data not shown).

cardiomyocytes; however, autophagy inhibition turns off the negative feedback regulation thus exaggerating angiotensinogen expression in cardiomyocytes.

DISCUSSION

In the present study, we provided a few pieces of evidence which help clarify the contradictory roles of autophagy and Nrf2 in cardiac remodeling and dysfunction, thereby consolidating the notions that (1) autophagy activation is cardioprotective; (2) autophagy is essential for Nrf2-mediated cardiac protection; (3) autophagy inhibition or impairment switches on Nrf2-operated myocardial damage; and (4) autophagy inhibition activates Nrf2-driven transcription of angiotensinogen in cardiomyocytes, thereby contributing to the pathological activation of Ang II-Ang II receptor type 1 signaling axis in the heart (Wollert and Drexler, 1999) (**Figure 5C**).

Does Autophagy Activation Protect Against PO-Induced Cardiomyopathy?

Although not entirely conclusive, most of the recent studies have revealed that autophagy activation is most likely an adaptive response in PO hearts (Wang and Cui, 2017; Sciarretta et al., 2018). Of note, we lately provided direct evidence to support this notion, i.e., that enhancement of myocardial autophagy via CR-Atg7 transgenic overexpression dramatically ameliorated PO-induced cardiomyopathy and heart failure (Qi et al., 2020). However, the adaptive nature of autophagy activation in PO hearts may not be completely addressed without a CR autophagy inhibition approach. This is likely due to experimental weaknesses of the original study which demonstrated a

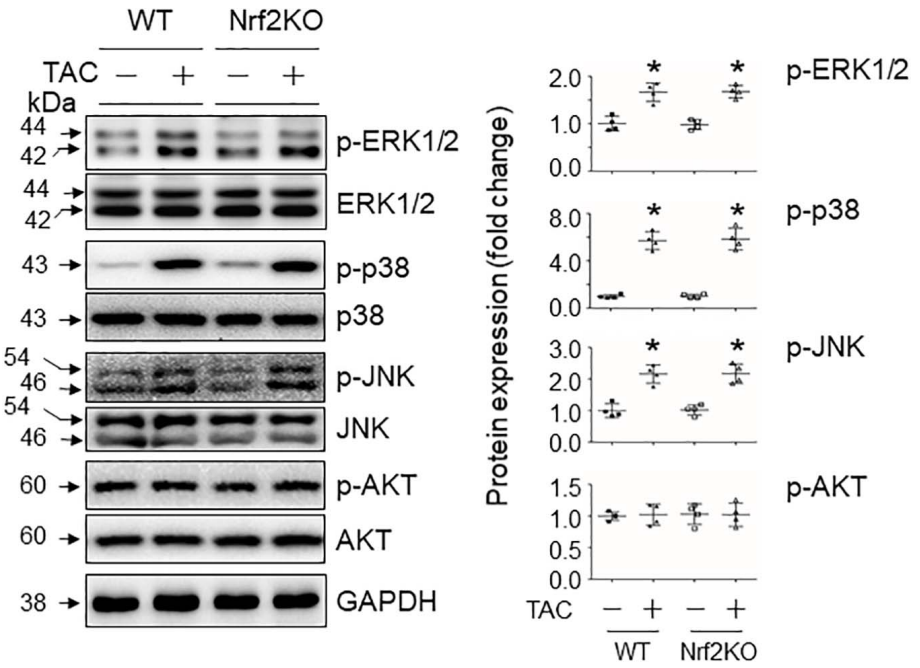


FIGURE 4 | The impact of Nrf2 knockout on pressure overload-induced activation of MAPKs and AKT in the heart. Male WT and Nrf2 knockout (Nrf2KO) mice at age of 9∼10 weeks were subject to sham or TAC operations for 4 weeks and then left ventricles of these mice were harvested for Western blot analyses of MAPKs and AKT activities ($n = 4$). *$p < 0.05$ vs TAC (–) in the same groups using the Student t test; $p < 0.01$ between the indicated groups using one-way ANOVA followed by Bonferroni test.

cardioprotective role of autophagy using MerCreMer^{+}::Atg5$^{fl/fl}$ mice after tamoxifen induction (CR-Atg5KO) in adult (Nakai et al., 2007). In this pioneer study, it is highly possible that the observed cardiac hypertrophy and dysfunction of CR-Atg5KO mice at the basal line are caused by Cre-mediated cardiotoxicity (Koitabashi et al., 2009) due to the high dose injection of tamoxifen (80 μg per g body weight, daily, for one week) (Nakai et al., 2007). In addition, Atg5$^{fl/fl}$, but not MerCreMer^{+} mice were used as the control (Nakai et al., 2007), which may cover the Cre-mediated myocardial damage while amplifying the CR-Atg5KO-dependent adverse phenotypes. In the present study, however, we addressed all of these concerns by using mice that all carried MerCreMer transgene and all received the same regime of tamoxifen treatment which has minimal impact on the heart at least at basal line condition (Koitabashi et al., 2009) to minimize the known and yet unknown off-target effects of MerCreMer on the heart. Importantly, compared with such rigorous control of MerCreMer^{+} mice, we found that CR-Atg5KO mice developed more severe cardiac hypertrophy and dysfunction at both 2 and 6 weeks after TAC, demonstrating a cardioprotective role of autophagy in PO hearts. Even in sham operated mice, we observed that CR-Atg5KO led to cardiac hypertrophy and dysfunction, indicating a housekeeping role of autophagy in suppressing cardiac maladaptive remodeling and dysfunction. Taken together, our results consolidate the notions that autophagy plays a critical role in maintaining cardiac homeostasis and autophagy activation is an adaptive response in PO hearts.

Does Autophagy Controls Nrf2-Mediated Dichotomy in PO Hearts?

Nrf2 has been considered as a hermetic factor (Maher and Yamamoto, 2010). However, such a theory that either too much or too little of Nrf2 can lead to pathological endpoints cannot explain Nrf2-mediated cardiac phenotypes (Zang et al., 2020a). For example, CR-Nrf2 Tg mice is perfectly normal under physiological conditions, and sustained PO does not result in super-activation of Nrf2 in the heart although Nrf2 activation promotes the PO-induced cardiomyopathy (Zang et al., 2020a). In the present study, we showed that cardiac specific autophagy inhibition via CR-Atg5KO wiped out Nrf2-mediated cardiac protection while turning on Nrf2-mediated pathological cardiac remodeling and dysfunction, providing direct evidence to demonstrate a critical role autophagy in the control of Nrf2-mediated dichotomy in PO hearts. These findings also indicate that Nrf2 does not act as a hermetic factor and Nrf2-mediated dichotomous effects are coupled with specific cellular processes, such as autophagy, in the heart.

How Does Autophagy Control Nrf2-Mediated Dichotomy in PO Hearts?

Our previous study have shown that autophagy inhibition inactivates Jak/Fyn signaling for Nrf2 nuclear export and degradation, leading to nuclear accumulation of Nrf2 to drive angiotensinogen expression in cardiomyocytes, which inevitably activates pathological Ang II-Ang II receptor type 1 (AT1R)

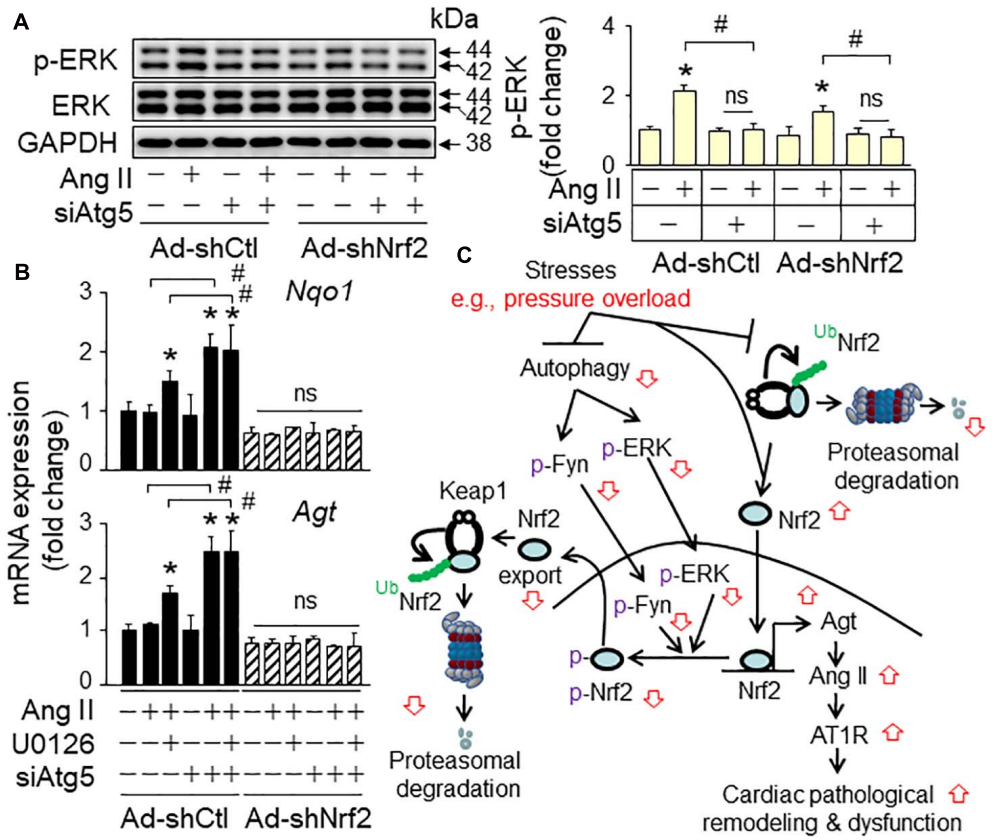


FIGURE 5 | The impact of autophagy inhibition on Ang II-induced ERK activation, Nrf2 activity and angiotensinogen (Agt) expression in the primary culture of neonatal rat ventricular myocytes (NRVMs). NRVMs were infected with adenovirus of scramble (Ad-shCtl) or Nrf2 shRNA (Ad-shNrf2) and then transfected with the control siLuc (siCtl) or Atg5 siRNA (siAtg5) prior to the treatment of Ang II (1 μM) and U0126 (1 μM) as described in the section "Materials and Methods." **(A)** Representative immunoblots and densitometric analysis ($n = 4$). $^{*}p < 0.05$ vs the vehicle control (–) treated Ad-shCtl + siCtl cells using the Student *t* test; $^{\#}p < 0.05$ between indicated groups using one-way ANOVA followed by Bonferroni test. ns, non-significant. **(B)** qPCR analysis of Nqo1 and angiotensinogen (Agt) expression ($n = 4$). $^{*}p < 0.05$ vs the vehicle control (–) group using the Student *t* test; $^{\#}p < 0.05$ between indicated groups using one-way ANOVA followed by Bonferroni test. **(C)** A working hypothesis. Sustained pressure overload interrupts Keap1-mediated Nrf2 ubiquitination for proteasomal degradation, thus increasing free Nrf2 which is translocated into nuclei and activates its target gene expression. On the other hand, it also induces autophagy inhibition to switch off Jak/Fyn and ERK-mediated phosphorylation of nuclear Nrf2 for nuclear export and degradation, which in turn results in enhancement of Nrf2-driven expression of Agt, leading to pathological activation of Ang II-AT1R axis in the heart. Accordingly, sustained pressure overload causes autophagy impairment, which in turn intensifies Nrf2-mediated cardiac pathological remodeling and dysfunction toward heart failure. Ub, ubiquitin; up arrow, increased activity; down arrow, decreased activity.

signaling axis in the heart (Wollert and Drexler, 1999). In this study, we further demonstrated that autophagy inhibition inactivated ERK, thereby activating the Nrf2-angiotensinogen axis in cardiomyocytes. These results not only validate our prior findings, but also give more mechanistic insights into autophagy-dependent control of Nrf2 signaling in PO hearts. However, how exactly autophagy controls Jak/Fyn and/or ERK signaling for the negative control of Nrf2-operated angiotensinogen expression in PO hearts has not been completely delineated. The critical downstream effectors of Nrf2-mediated myocardial damage and dysfunction in autophagy-impaired PO hearts remain to be determined. Recently, we demonstrated that CR-Atg5KO exacerbates Nrf2-mediated cardiac pathological remodeling and dysfunction and intensifies Nrf2-driven transcription of a subset of genes, such as acyl-CoA synthetase long-chain family 4 (Acsl4), cluster of differentiation 36 (Cd36), angiotensinogen (Agt), and Kruppel-like factor 9 (Klf9), while suppressing Nrf2-operated transcription of cellular defense genes including glutathione peroxidase 4 (Gpx4) and ferroptosis suppressor protein 1 (Fsp1), also known as apoptosis-inducing factor mitochondria 2 (Aifm2), thereby exaggerating the progression of cardiomyopathy associated with type 1 diabetes (Zang et al., 2020b). Whether such autophagy-governed coordination of Nrf2 signaling pathways is also disturbed in PO hearts deserves investigations, thus providing mechanistic insights into the molecular network by which autophagy controls Nrf2-mediated dichotomy in the heart.

What Are Clinical Relevance and Limitations of This Study?

The enthusiasm for activating Nrf2 as a novel approach to treat human diseases, at least non-cardiac disease, remains very high; a number of clinical trials of various phases on Nrf2

activators for treating diseases such as diabetic complications and cancers are still actively ongoing (Al-Sawaf et al., 2015). This study clearly demonstrated autophagy inhibition-dependent activation of Nrf2-mediated cardiac damage and dysfunction. Accordingly, it raises a concern regarding the 'dark' side of Nrf2 in these clinical therapies, especially when the treated subjects are compounded with conditions such as hypertensive, ischemic and diabetic cardiomyopathies, all of which likely have myocardial autophagy inhibition (Wang and Cui, 2017; Zang et al., 2020a). The therapeutic efficacy of targeting Nrf2 for these diseases may not be achieved without shutting down Nrf2-mediated adverse signaling in the heart. These issues further create the need for better understanding of the molecular mechanisms underlying such unusual coupling between autophagy inhibition and detrimental activation of Nrf2 in stressed hearts, which have not been completely dissected in the present study. Whether such coupling contributes to PO-induced cardiac diastolic dysfunction has also remained to be addressed. These limitations need further investigation, thereby uncovering the nature of the unique coupling between autophagy function and Nrf2 signaling in cardiac pathological remodeling and dysfunction. As a result, the outcome may provide new insights into the development of a novel therapeutic approach which could simultaneously activate autophagy and Nrf2 for treatment of chronic heart diseases with cardiac autophagy inhibition.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Kenneth Walsh, IACUC of University of South Carolina.

AUTHOR CONTRIBUTIONS

XW and TC conception, design, and grant support of this research. MN and PN grant support of this research. WWu, QQ, YD, HZ, D-SL, and WWa performed experiments and analyzed data. WWu, QQ, YD, and TC interpreted results of experiments and prepared figures. WWu, XW, MN, PN, and TC wrote the manuscript. All authors approved the final version of the manuscript.

FUNDING

This study was supported by the National Institutes of Health [National Center for Complementary and Alternative Medicine (P01 AT003961), National Heart, Lung, and Blood Institute (R01HL131667)] and American Diabetes Association (1-16-IBS-059).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.673145/full#supplementary-material

REFERENCES

Al-Sawaf, O., Clarner, T., Fragoulis, A., Kan, Y. W., Pufe, T., Streetz, K., et al. (2015). Nrf2 in health and disease: current and future clinical implications. *Clin. Sci. (Lond)* 129, 989–999. doi: 10.1042/cs20150436

Bryan, H. K., Olayanju, A., Goldring, C. E., and Park, B. K. (2013). The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. *Biochem. pharmacol.* 85, 705–717. doi: 10.1016/j.bcp.2012.11.016

Cui, T., Lai, Y., Janicki, J. S., and Wang, X. (2016). Nuclear factor erythroid-2 related factor 2 (Nrf2)-mediated protein quality control in cardiomyocytes. *Front. Biosci. (Landmark Ed)* 21:192–202. doi: 10.2741/4384

Koitabashi, N., Bedja, D., Zaiman, A. L., Pinto, Y. M., Zhang, M., Gabrielson, K. L., et al. (2009). Avoidance of transient cardiomyopathy in cardiomyocyte-targeted tamoxifen-induced MerCreMer gene deletion models. *Circ. Res.* 105, 12–15. doi: 10.1161/circresaha.109.198416

Kroemer, G., Marino, G., and Levine, B. (2010). Autophagy and the integrated stress response. *Mol. Cell* 40, 280–293. doi: 10.1016/j.molcel.2010.09.023

Li, J., Ichikawa, T., Janicki, J. S., and Cui, T. (2009a). Targeting the Nrf2 pathway against cardiovascular disease. *Expert Opin. Ther. Targets* 13, 785–794. doi: 10.1517/14728220903025762

Li, J., Ichikawa, T., Villacorta, L., Janicki, J. S., Brower, G. L., Yamamoto, M., et al. (2009b). Nrf2 protects against maladaptive cardiac responses to hemodynamic stress. *Arterioscler. Thromb. Vasc. Biol.* 29, 1843–1850. doi: 10.1161/atvbaha.109.189480

Li, S., Wang, W., Niu, T., Wang, H., Li, B., Shao, L., et al. (2014). Nrf2 deficiency exaggerates doxorubicin-induced cardiotoxicity and cardiac dysfunction. *Oxid. Med. Cell Longev.* 2014:748524.

Maher, J., and Yamamoto, M. (2010). The rise of antioxidant signaling–the evolution and hormetic actions of Nrf2. *Toxicol. Appl. Pharmacol.* 244, 4–15. doi: 10.1016/j.taap.2010.01.011

Martinez-Lopez, N., Athonvarangkul, D., Mishall, P., Sahu, S., and Singh, R. (2013). Autophagy proteins regulate ERK phosphorylation. *Nat. Commun.* 4:2799.

Nakai, A., Yamaguchi, O., Takeda, T., Higuchi, Y., Hikoso, S., Taniike, M., et al. (2007). The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. *Nat. Med.* 13, 619–624. doi: 10.1038/nm1574

Qi, L., Zang, H., Wu, W., Nagarkatti, P., Nagarkatti, M., Liu, Q., et al. (2020). CYLD exaggerates pressure overload-induced cardiomyopathy via suppressing autolysosome efflux in cardiomyocytes. *J. Mol. Cell Cardiol.* 145, 59–73. doi: 10.1016/j.yjmcc.2020.06.004

Qin, Q., Qu, C., Niu, T., Zang, H., Qi, L., Lyu, L., et al. (2016). Nrf2-Mediated cardiac maladaptive remodeling and dysfunction in a setting of autophagy insufficiency. *Hypertension* 67, 107–117. doi: 10.1161/hypertensionaha.115.06062

Sciarretta, S., Maejima, Y., Zablocki, D., and Sadoshima, J. (2018). The role of autophagy in the heart. *Annu. Rev. Physiol.* 80, 1–26.

Wang, X., and Cui, T. (2017). Autophagy modulation: a potential therapeutic approach in cardiac hypertrophy. *Am. J. Physiol. Heart Circ. Physiol.* 313, H304–H319.

Wang, Z. V., and Hill, J. A. (2015). Protein quality control and metabolism: bidirectional control in the heart. *Cell Metab.* 21, 215–226. doi: 10.1016/j.cmet.2015.01.016

Wollert, K. C., and Drexler, H. (1999). The renin-angiotensin system and experimental heart failure. *Cardiovasc. Res.* 43, 838–849. doi: 10.1016/s0008-6363(99)00145-5

Zang, H., Mathew, R. O., and Cui, T. (2020a). The dark side of Nrf2 in the heart. *Front. Physiol.* 11:722.

Zang, H., Wu, W., Qi, L., Tan, W., Nagarkatti, P., Nagarkatti, M., et al. (2020b). Autophagy inhibition enables Nrf2 to exaggerate the progression of diabetic cardiomyopathy in mice. *Diabetes* 69, 2720–2734. doi: 10.2337/db19-1176

Zhang, Y., Sowers, J. R., and Ren, J. (2018). Targeting autophagy in obesity: from pathophysiology to management. *Nat. Rev. Endocrinol.* 14, 356–376. doi: 10.1038/s41574-018-0009-1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

SYSTEMATIC REVIEW
published: 28 June 2021
doi: 10.3389/fphys.2021.690423

Cullin Deneddylation Suppresses the Necroptotic Pathway in Cardiomyocytes

Megan T. Lewno[1], *Taixing Cui*[2] *and Xuejun Wang*[1]*

[1] Division of Basic Biomedical Sciences, The University of South Dakota Sanford School of Medicine, Vermillion, SD, United States, [2] Department of Cell Biology and Anatomy, University of South Carolina School of Medicine, Columbia, SC, United States

Cardiomyocyte death in the form of apoptosis and necrosis represents a major cellular mechanism underlying cardiac pathogenesis. Recent advances in cell death research reveal that not all necrosis is accidental, but rather there are multiple forms of necrosis that are regulated. Necroptosis, the earliest identified regulated necrosis, is perhaps the most studied thus far, and potential links between necroptosis and Cullin-RING ligases (CRLs), the largest family of ubiquitin E3 ligases, have been postulated. Cullin neddylation activates the catalytic dynamic of CRLs; the reverse process, Cullin deneddylation, is performed by the COP9 signalosome holocomplex (CSN) that is formed by eight unique protein subunits, COPS1/CNS1 through COPS8/CNS8. As revealed by cardiomyocyte-restricted knockout of *Cops8* (Cops8-cko) in mice, perturbation of Cullin deneddylation in cardiomyocytes impairs not only the functioning of the ubiquitin–proteasome system (UPS) but also the autophagic–lysosomal pathway (ALP). Similar cardiac abnormalities are also observed in Cops6-cko mice; and importantly, loss of the desmosome targeting of COPS6 is recently implicated as a pathogenic factor in arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C). Cops8-cko causes massive cardiomyocyte death in the form of necrosis rather than apoptosis and rapidly leads to a progressive dilated cardiomyopathy phenotype as well as drastically shortened lifespan in mice. Even a moderate downregulation of Cullin deneddylation as seen in mice with Cops8 hypomorphism exacerbates cardiac proteotoxicity induced by overexpression of misfolded proteins. More recently, it was further demonstrated that cardiomyocyte necrosis caused by Cops8-cko belongs to necroptosis and is mediated by the RIPK1–RIPK3 pathway. This article reviews these recent advances and discusses the potential links between Cullin deneddylation and the necroptotic pathways in hopes of identifying potentially new therapeutic targets for the prevention of cardiomyocyte death.

Keywords: Cullin, COP9 signalosome, deneddylation, necroptosis, cardiomyocyte, mice, RIPK1 signal pathway, RIPK3

OPEN ACCESS

Edited by:
Aldrin V. Gomes, University of California, Davis, United States

Reviewed by:
Paul Goldspink, University of Illinois at Chicago, United States
Manish Gupta, University of Central Florida, United States

***Correspondence:**
Xuejun Wang
xuejun.wang@usd.edu

Specialty section:
This article was submitted to Striated Muscle Physiology, a section of the journal Frontiers in Physiology

Received: *02 April 2021*
Accepted: *24 May 2021*
Published: *28 June 2021*

Citation:
Lewno MT, Cui T and Wang X (2021) Cullin Deneddylation Suppresses the Necroptotic Pathway in Cardiomyocytes. Front. Physiol. 12:690423. doi: 10.3389/fphys.2021.690423

INTRODUCTION

The ubiquitin–proteasome system (UPS) mediates the degradation of most cellular proteins that are either native or misfolded; hence, the proper functioning of the UPS is pivotal to both protein homeostasis (proteostasis) and the regulation of nearly all cellular functions. There is a large and growing body of evidence that UPS dysfunction plays a major

role in cardiac pathogenesis, including the progression from a large subset of heart disease to heart failure (Wang and Wang, 2020). The latter is the leading cause of morbidity and mortality in humans. Thus, targeting UPS dysfunction is a conceivable strategy in treating heart disease, but such a strategy has not been applied clinically yet. To facilitate the development of such a new strategy, exciting progress has been made lately in research areas such as the cellular and molecular mechanisms by which cardiac UPS is regulated (Ranek et al., 2013, 2014, 2020), how to alter the regulation when needed (Zhang et al., 2019; Oeing et al., 2020; Wang and Wang, 2020), and how UPS malfunction causes cardiac injury (Tian et al., 2012; Ranek et al., 2015; Su et al., 2015), although answers to most of these questions remain incomplete.

Degradation of a substrate protein by the UPS requires polyubiquitination of the substrate protein, but the substrate specificity is conferred by ubiquitin ligases. The largest family of ubiquitin ligases is Cullin-RING ligases (CRLs), where Cullin serves as the scaffold for a RING protein and a substrate receptor module to assemble into a multiprotein complex to act as a ubiquitin ligase (Rao et al., 2020). Covalent attachment of a ubiquitin-like protein NEDD8 (Neural precursor cell Expressed Developmentally Downregulated 8) to a lysine residue of Cullin *via* a ubiquitination-like post-translational modification process known as neddylation is essential for the activation of CRLs (Li J. et al., 2020; Zhang et al., 2020). The reverse process of Cullin neddylation, referred to as Cullin deneddylation, is catalyzed by the COP9 (constitutive photomorphogenesis 9) signalosome (CSN), an evolutionally conserved protease complex (Wei and Deng, 2003; Lingaraju et al., 2014). Countering Cullin neddylation which is essential to the assembly and activation of CRLs, Cullin deneddylation triggers a timely disassembly of an active CRL that has completed ubiquitination of its specific substrate so that key components of the CRL can be recycled and used for the formation of new and different CRLs. Hence, Cullin deneddylation is equally important to the assembly/disassembly dynamic and thereby helps maintain the proper functioning of CRLs (Rao et al., 2020). Indeed, *in vivo* and genetic studies reveal that loss of function of the CSN also suppresses the overall ubiquitination activity of CRLs, although earlier *in vitro* biochemical studies suggested an inhibiting effect of Cullin deneddylation on CRLs (Wei and Deng, 2003). Mice with cardiomyocyte-restricted knockout of a canonical subunit of the CSN develop cardiomyopathy and display significantly shortened lifespans associated with massive cardiomyocyte necrosis and impairment of both the autophagic–lysosomal pathway (ALP) and UPS proteolytic function (Su et al., 2011a,b). While the CSN is ubiquitously expressed in all tissues, there seem to be tissue-specific functional roles; this review will focus on current studies on the CSN and its biological function in maintaining the survival of cardiomyocytes. Most recently, an elegant study by Liang et al. (2021) unveiled that COPS6 is a resident protein of myocardial desmosomes, a main mechanical junction of the intercalated disk. They also demonstrated that the interaction between the MPN domain of COPS6 and the spectrin repeats in the N-terminus of desmoplakin (DSP) mediates the localization of COPS6 to the desmosome. Moreover, they discovered that COPS6 protein enrichment in the intercalated disk is remarkably lost or attenuated in the myocardial biopsies from human patients with arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C), a *bona fide* disease of desmosomes. Furthermore, they showed strong evidence that human ARVD/C-associated mutations in DSP and plakophilin-2 (PKP2) diminish or even abrogate the desmosome targeting of COPS6, arguing strongly for a pathogenic role of the loss of junctional COPS6 in ARVD/C; indeed, they showed that Cops6-cko recapitulates many aspects of the ARVD/C phenotype (Liang et al., 2021). This represents an important advance in cardiac CSN research because it provides not only new mechanistic insight into CSN biology but also direct evidence for the clinical relevance of investigating the CSN in the heart.

Loss of cardiomyocytes in the form of either apoptosis or necrosis can be the tipping point during the progression from various forms of primary heart disease to heart failure. Recent advances in the research into the mechanisms of cell death have unveiled that necrosis includes not only accidental but also regulated forms as well (Galluzzi et al., 2018). Depending on the primary underlying cause and thereby pathways taken, regulated necrosis can further be categorized into many forms, such as necroptosis, ferroptosis, and pyroptosis, to name a few (Galluzzi et al., 2018). Tumor necrosis factor α (TNFα) induces apoptosis in most cells; however, TNFα was found to induce necrosis in cells deficient of caspase-8 or when there was inhibition of caspases (Hitomi et al., 2008). The term necroptosis was coined to refer to this type of necrosis (Degterev et al., 2005). Hence, necroptosis is probably the earliest form of regulated necrosis described. Subsequent studies have established that the canonical pathway for death receptor activation to induce necroptosis involves the activation of the receptor-interacting protein kinase 1 (RIPK1), RIPK3, and mixed-lineage kinase domain-like pseudokinase (MLKL) (Galluzzi et al., 2018). Emerging evidence not only indicates that cardiomyocyte necroptosis plays an important role in cardiac pathogenesis but also begins unveiling the molecular mechanisms that govern cardiomyocyte necroptosis (Del Re et al., 2019). This article will review recent advances in elucidating the link between dysregulated proteostasis and necroptosis in cardiomyocytes, leveraging on the massive cardiomyocyte necrosis induced by the genetic perturbation of CSN-mediated Cullin deneddylation.

CULLIN DENEDDYLATION BY THE CSN

Initially discovered in *Arabidopsis thaliana*, a small flowering plant (Wei et al., 1994), the CSN quickly has been found to be highly conserved in all eukaryotes, from fungi to humans (Lingaraju et al., 2014). It is composed of eight canonical subunits, which are eight unique proteins named CSN1 through CSN8 or, more officially, COPS1 through COPS8, according to the descending order of molecular weights (Kwok et al., 1998). Depletion of any of the canonical subunits impairs Cullin deneddylation, demonstrating that the deneddylase activity of the CSN requires the formation of the CSN holocomplex by the eight subunits (**Figure 1**). The deneddylase of the CSN resides in COPS5, but COPS5 exerts Cullin deneddylation activity only

when it is situated in the fully assembled CSN holocomplex consisting of all eight subunits, which is why the loss of any of the eight CSN subunits abolishes Cullin deneddylation in the cell (Wei and Deng, 2003). A recent report claims the discovery of the ninth subunit of the CSN, a small protein with the molecular weight of 6.5 kDa, referred to as CSN acidic protein (CSNAP), but deletion of CSNAP does not alter Cullin deneddylation (Rozen et al., 2015), indicating that CSNAP is not essential to the Cullin deneddylation by the CSN. This newly discovered CSNAP might function to enhance the interaction of the CSN with CRLs (Rozen et al., 2015).

In a CRL complex, Cullin serves as a scaffold; the substrate receptor module binds to its N-terminal segment, responsible for the recruitment of a specific substrate protein for ubiquitination; and the RING protein bound to the C-terminal domain recruits the ubiquitin-charged E2. In the absence of Cullin neddylation, the substrate and the ubiquitin associated with the two arms of the CRL are too far apart for ubiquitin transfer to take place. Cullin neddylation, however, changes the conformation of the CRL so that the N- and C-terminal arms of the Cullin bend toward each other, rendering the substrate and ubiquitin-charged E2 in close proximity to each other, allowing the ubiquitin to be efficiently transferred from the E2 to the substrate protein (Rao et al., 2020). CRLs represent the largest family of E3 ubiquitin ligases. Over 700 currently known ubiquitin ligase complexes in humans belong to CRLs, responsible for the ubiquitin-dependent degradation of ∼20% of cellular proteins (Dubiel et al., 2020). CRLs are crucial for UPS-mediated proteolysis of regulatory proteins and, as such, participate in the regulation of a vast array of cellular pathways and processes, such as the progression of the cell cycle. Blondelle et al. (2020) comprehensively reviewed the role of CRLs in the development, physiology, and pathology of striated muscle, including cardiac muscle.

As described earlier, neddylation is crucial for the ubiquitin ligase activity of CRLs, and disruption of neddylation *via*, for example, inhibition of NEDD8 activating enzyme 1 (NAE1) has emerged as a potential new therapeutic strategy for tumor suppression (Soucy et al., 2009; Nawrocki et al., 2013). Phase III clinical trials on neddylation inhibitor MLN4924 (also known as pevonedistat) to treat hemopoietic malignancies (NCT03268954) are ongoing. Mice with cardiomyocyte-restricted knockout of NAE1 driven by the *Myh6-Cre* displayed perinatal lethality due to myocardial hypoplasia, ventricular non-compaction, and heart failure at late gestation, which is associated with defective Hippo-YAP signaling (Zou et al., 2018). By virtue of Cullin deneddylation, the CSN modulates the assembly and activity of CRLs. Without timely Cullin deneddylation as seen in depletion of any of the canonical CSN subunits, a CRL complex cannot correctly disassemble but rather stays in its active form and continues ubiquitinating the current substrate it has bound, as well as auto-ubiquitinating its own components such as its substrate receptor, leading to self-destruction (Wee et al., 2005). Thus, loss of function of the CSN can impair the overall ubiquitination by CRLs by hindering the exchange of substrate receptors in CRLs (Lydeard et al., 2013; Rao et al., 2020). A small molecule inhibitor of the CSN (CSN5i-3) has been reported to impair Cullin deneddylation and shows great promise in the experimental treatment of cancer (Schlierf et al., 2016).

PATHWAYS TO CARDIOMYOCYTE NECROPTOSIS

Necroptosis is a form of caspase-independent regulated cell death that shares the morphologic characteristic of necrosis. The prototype of necroptosis is the necrosis induced by stimulation of the death receptor family in cells deficient of intracellular apoptotic signaling (Degterev et al., 2005). It is characterized by the lack of the typical nuclear condensation and internucleosomal DNA fragmentation that are characteristic of apoptosis and by the presence of enlarged and swollen organelles and early cell plasma membrane perforation and resultant release of inflammatory damage-associated molecular patterns (DAMPs), which initiate innate immune responses, resulting in an inflammatory response (Zhou and Yuan, 2014; Pasparakis and Vandenabeele, 2015; Wegner et al., 2017; Samson et al., 2021). Consequently, necroptosis has been widely implicated in innate immunity and inflammatory diseases (Zhou and Yuan, 2014; Pasparakis and Vandenabeele, 2015; Wegner et al., 2017; Molnar et al., 2019).

For detection of apoptosis, a well-defined set of assays have been developed on the basis of its unique biochemical and morphological features, but the current situation for the identification of necroptosis or any forms of regulated necrosis is quite different because all regulated necrosis [e.g., necroptosis, ferroptosis, pyroptosis, and MPT (mitochondrial permeability transition)] and even incidental necrosis share virtually the same morphological changes although the molecular pathways to the various forms of regulated necrosis differ (Del Re et al., 2019). Thus, the detection of these forms of necrosis must rely on a combination of assessments that identify the co-existence of necrosis with the activation of the mechanistic pathway characteristic of the respective forms of regulated necrosis (Mishra et al., 2019). For cardiomyocyte necroptosis, the following pathways have been documented.

The RIPK1–RIPK3–MLKL Pathway

At the molecular level, necroptosis can be induced by activation of death receptors upon engagement of their respective ligands, which includes binding of TNFα to tumor necrosis factor receptor 1 (TNFR1), first apoptotic signal ligand (FasL) to Fas, TNF-related apoptosis-inducing ligand (TRAIL) to TRAIL receptor 1/2 (TRAIL-R1/2), interferons (INFs) to INF receptors (INFRs), Toll-like receptor (TLR) ligands to TLR3/4, or by binding of virus Z-form DNA or RNA to the cytosolic nucleic acid sensor, Z-DNA binding protein 1 (ZBP1) (Zhou and Yuan, 2014; Pasparakis and Vandenabeele, 2015; Wegner et al., 2017; Samson et al., 2021). Necroptosis triggered by TNFα ligation of TNFR1 is best understood (**Figure 2**), thus being considered as the prototypical form (Zhou and Yuan, 2014). In this model, the binding of TNFα to TNFR1 induces the recruitment of various proteins to the cytoplasmic tail of TNFR1, including TNFR-associated death domain (TRADD), RIPK1,

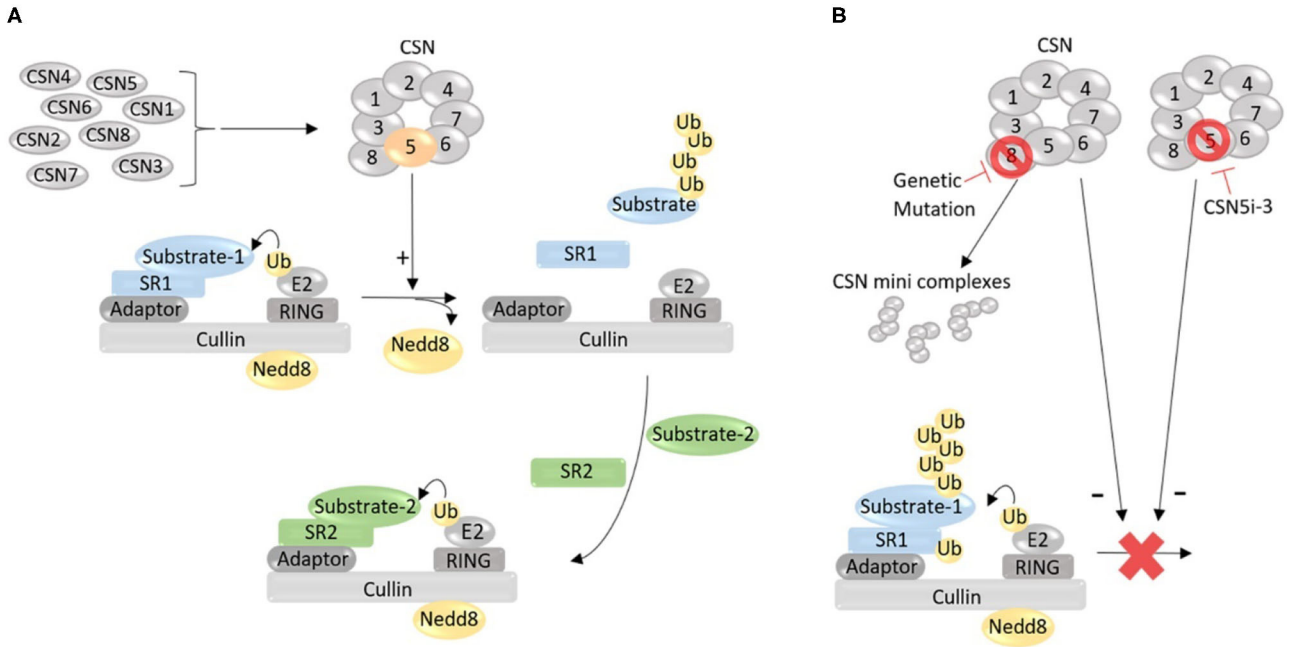


FIGURE 1 | A working model for the role of the COP9 signalosome (CSN) in the regulation of the catalytic dynamic of Cullin-RING ligases (CRLs). **(A)** Under normal condition, the CSN holocomplex formed by eight unique protein subunits (CSN1 through CSN8) removes the Nedd8 from the neddylated Cullin, thereby inactivates and dissembles the CRL that has completed ubiquitinating a substrate protein (Substrate-1) recruited by substrate receptor 1 (SR1), allowing for the formation of a new CRL with a new SR (SR2) to recruit a new substrate (Substrate-2) for ubiquitination. **(B)** Defect in the formation of the CSN holocomplex, such as depletion of a subunit due to genetic mutation, may increase the abundance of certain species of minicomplexes composed of some of the CSN subunits but reduces or loses the deneddylase activity, impairing Cullin deneddylation; Cullin deneddylation by the CSN can also be inhibited by small molecules (e.g., CSN5i-3). When Cullin deneddylation is lost, the exchange of SRs in CRLs will be compromised, and as a result, the catalytic dynamic of CRLs will be stalled, leading to autoubiquitination and destruction of CRL components (e.g., SR).

TNFR-associated factor 2 (TRAF2), linear ubiquitin chain assembly complex (LUBAC), cellular inhibitor of apoptosis-1 (cIAP1), and cIAP2, forming a membrane-bound assembly known as Complex I (Zhou and Yuan, 2014; Pasparakis and Vandenabeele, 2015; Wegner et al., 2017; Samson et al., 2021). At this point, LUBAC adds N-terminal methionine (Met1)-linked linear ubiquitin chains to RIPK1 and other proteins, whereas cIAP1/2 catalyze the addition of K63-linked polyubiquitin chains to RIPK1 and other proteins within Complex I. The ubiquitinated RIPK1 functions as a scaffold to recruit nuclear factor κB (NFκB) essential modulator (NEMO, also known as an inhibitor of NFκB kinase subunit gamma, IKKγ) and transforming growth factor beta-activating kinase 1 (TAK1), leading to the activation of NFκB and mitogen-activated protein kinase (MAPK) pathways, respectively (**Figure 2**). Activation of these pathways promotes the synthesis of proinflammatory cytokines and cell survival. Of note, RIPK1 mediates NFκB activation independent of its kinase activity. However, Complex I can be transformed into the secondary cytosolic complex, Complex II, which mediates cell death. This transition requires deubiquitination of Complex I and association with Fas-associated protein with death domain (FADD) and caspase-8. Complex II may contain different components and promote cell death in a manner that is either independent or dependent of the kinase activity of RIPK1. The canonical RIPK1 kinase-independent proapoptotic caspase-8 complex is termed Complex IIa (also known as ripoptosome), whereas RIPK1 kinase-dependent complexes are named Complex IIb (also named necrosome). Complex IIa may be composed of TRADD, FADD, RIPK1, or RIPK3, while Complex IIb consists of RIPK1, RIPK3, MLKL, FADD, and inactive caspase-8, although the complete composition of these complexes is not yet established. Caspase-8 activity in Complex IIa determines downstream signaling toward an apoptotic outcome by activating Bid and caspase-3 and simultaneously prevents necroptosis by cleaving RIPK1 or RIPK3. Inactivation of caspase-8 in Complex IIb promotes necroptosis *via* a core signaling axis of RIPK1–RIPK3–MLKL, which is called canonical necroptosis. In the canonical pathway, blockade of caspase activity triggers autophosphorylation of RIPK1 on Ser166 (Degterev et al., 2008), which in turn assembles with RIPK3 into hetero-amyloid filaments, resulting in RIPK3 phosphorylation on Ser232 as well as subsequent recruitment of downstream necroptosis effector, MLKL, and phosphorylating MLKL on Thr357/Ser358 sites (Sun et al., 2012). This RIPK3-mediated phosphorylation of MLKL triggers a conformational change in MLKL, leading to its translocation to the plasma membrane, where it oligomerizes and forms pores that mediate necroptosis by lysing the membrane (Dondelinger et al., 2014;

Wang et al., 2014). Necroptotic signaling triggered by the activation of other receptors upon engagement of their ligands may be different at the upstream, but all converge with RIPK3-dependent phosphorylation and activation of MLKL toward plasma membrane lysis (Pasparakis and Vandenabeele, 2015; Wegner et al., 2017; Samson et al., 2021).

It should be noted that signaling mechanisms underlying necroptosis act in a context-, cell type-, and species-dependent manner and are still far from a comprehensive understanding (Wegner et al., 2017; Samson et al., 2021). Physiologically, the essential regulators which determine activation of distinct death pathways remain unclear. In addition, the endogenous factors which determine the formation of Complex IIb (necrosome) are not well-understood. Intriguingly, a few deubiquitinating enzymes (DUBs), such as cylindromatosis (CYLD), A20 (Tnfaip3), and OTULIN (ovarian tumor deubiquitinase with linear linkage specificity), appear to regulate necrosome formation and outcome. CYLD deubiquitinates RIPK1 to decrease the interaction of RIPK1 with NEMO, thus destabilizing Complex I, whereas it also deubiquitinates RIPK1 in TNFα-induced necrosome to facilitate kinase activation and necroptosis (O'Donnell et al., 2011; Moquin et al., 2013). CYLD is negatively regulated by caspase-8-mediated cleavage, protecting against TLR-mediated necroptosis (Legarda et al., 2016). The ubiquitin-chain editing function of A20 can replace K63 polyubiquitin chains from RIPK1 with K48 polyubiquitin chains, leading to its proteasomal degradation (Wertz et al., 2004), while restricting K63-ubiquitination of RIPK3 at K5 and reducing its binding to RIPK1, thus suppressing necroptosis (Onizawa et al., 2015). Mutations causing hypomorphic A20 expression and function are linked to a wide range of human inflammatory and autoimmune diseases (Martens and van Loo, 2020), and as necroptosis is intimately involved in inflammation, a better understanding of how A20 regulates necroptosis is expected to advance the investigation into the pathogenesis of human inflammatory and autoimmune disease. In addition, OTULIN inhibits necroptosis *via* downregulation of necroptotic RIPK1 ubiquitination and activation (Douglas and Saleh, 2019). Cell-type-specific knockout approaches demonstrate non-redundant functions of these DUBs, which are presumably explained by their differential specificity for different types of ubiquitin chains (Lork et al., 2017). However, the molecular mechanisms underlying specific functions of CYLD, A20, and OUTLIN as well as their crosstalk with each other or with other DUBs remain poorly understood.

The complexity of necroptosis regulation is further intensified by emerging crosstalk between necroptosis and other types of necrotic cell death, including pyroptosis, ferroptosis, and mitochondrial permeability transition pore (MPT)-mediated necrosis. Unlike the reciprocally negative regulation between apoptosis and necroptosis, these types of necrotic cell death usually help each other in facilitating necrotic processes *via* yet unknown mechanisms (Kist and Vucic, 2021). In addition, necroptosis is usually associated with the accumulation of autophagosomes, double membrane-enclosed vesicles that package cytoplasmic components and deliver the cargo to lysosomes for degradation (Shen and Codogno, 2012). As discussed specifically in the *Interactions between macroautophagy and the RIPK1–RIPK3–MLKL pathways* section, autophagy may function as a prosurvival mechanism *via* suppression of necroptosis.

The RIPK3–CaMK2–MPT Pathway

An early study showed that RIPK3 was upregulated in ischemic myocardium, but the infarct size of a myocardial infarction (MI) model induced by a comparable permanent ligation of the coronary artery did not seem to differ between wild-type (WT) and RIPK3$^{-/-}$ mice (Luedde et al., 2014). Nevertheless, this study has provided the first experimental evidence that RIPK3, a critical kinase regulating necroptosis, plays a mediator role in post-MI maladaptive remodeling. This is because this study revealed that myocardial reactive oxygen species (ROS) levels examined at 24 h post-MI, the CD3-positive cell infiltration examined at 4 days post-MI, and the maladaptive cardiac remodeling observed at 30 days post-MI were all significantly attenuated in the RIPK3$^{-/-}$ mice compared with WT mice (Luedde et al., 2014). Furthermore, data collected from neonatal rat ventricular myocyte (NRVM) cultures did support the requirement of RIPK3 and potentially its interaction with RIPK1 for the induction of cardiomyocyte necrosis by treatment of TNFα combined with a broad-spectrum caspase inhibitor (zVAD-fmk) (Luedde et al., 2014), but no direct evidence was provided for the role of cardiomyocyte necroptosis in post-MI remodeling. This is because RIPK3 was ablated in all cells in RIPK3$^{-/-}$ mice, and the protection of RIPK3 deficiency could have come from the reduced inflammatory responses resulting from the loss of RIPK3 in inflammatory cells.

A subsequent study using myocardial ischemia–reperfusion (I-R) injury and doxorubicin (Dox)-induced acute cardiotoxicity models showed that cardiomyocyte necrosis was significantly reduced in RIPK3$^{-/-}$ mice compared with WT mice (Zhang et al., 2016), indicating that RIPK3 activation plays an essential role in I-R injury and Dox cardiotoxicity. Moreover, these authors presented evidence that RIPK3 does so through activation of calcium-calmodulin-dependent protein kinase II (CaMKII) rather than through the well-established partners RIPK1 and MLKL (Zhang et al., 2016). They showed that siRNA-mediated knockdown of either RIPK1 or MLKL yielded no significant effects on the leakage of LDH and the reduction of cell viability induced by adenovirus-mediated RIPK3 overexpression in cultured NRVMs. The increases of myocardial Thr287-phosphorylated CaMKII in response to I-R injury or Dox treatment were attenuated or abolished by RIPK3 deficiency in mice, and RIPK3 was found to bind to and, *via* both phosphorylation and oxidation, activate CaMKII in cultured NRVMs (Zhang et al., 2016). It is well-known that CaMKII is activated and plays a mediating role in myocardial cell death and I-R injury (Bell et al., 2014). Hence, it is not surprising that treatment of a CaMKII inhibitor (KN-93) was found to effectively reduce the infarct size and serum LDH elevation induced by myocardial I-R and similarly attenuate the Dox-induced cardiac damage and malfunction in WT mice (Zhang et al., 2016). Importantly, adenoviral overexpression of a dominant-negative CaMKII was shown to reduce the cytotoxicity induced by not

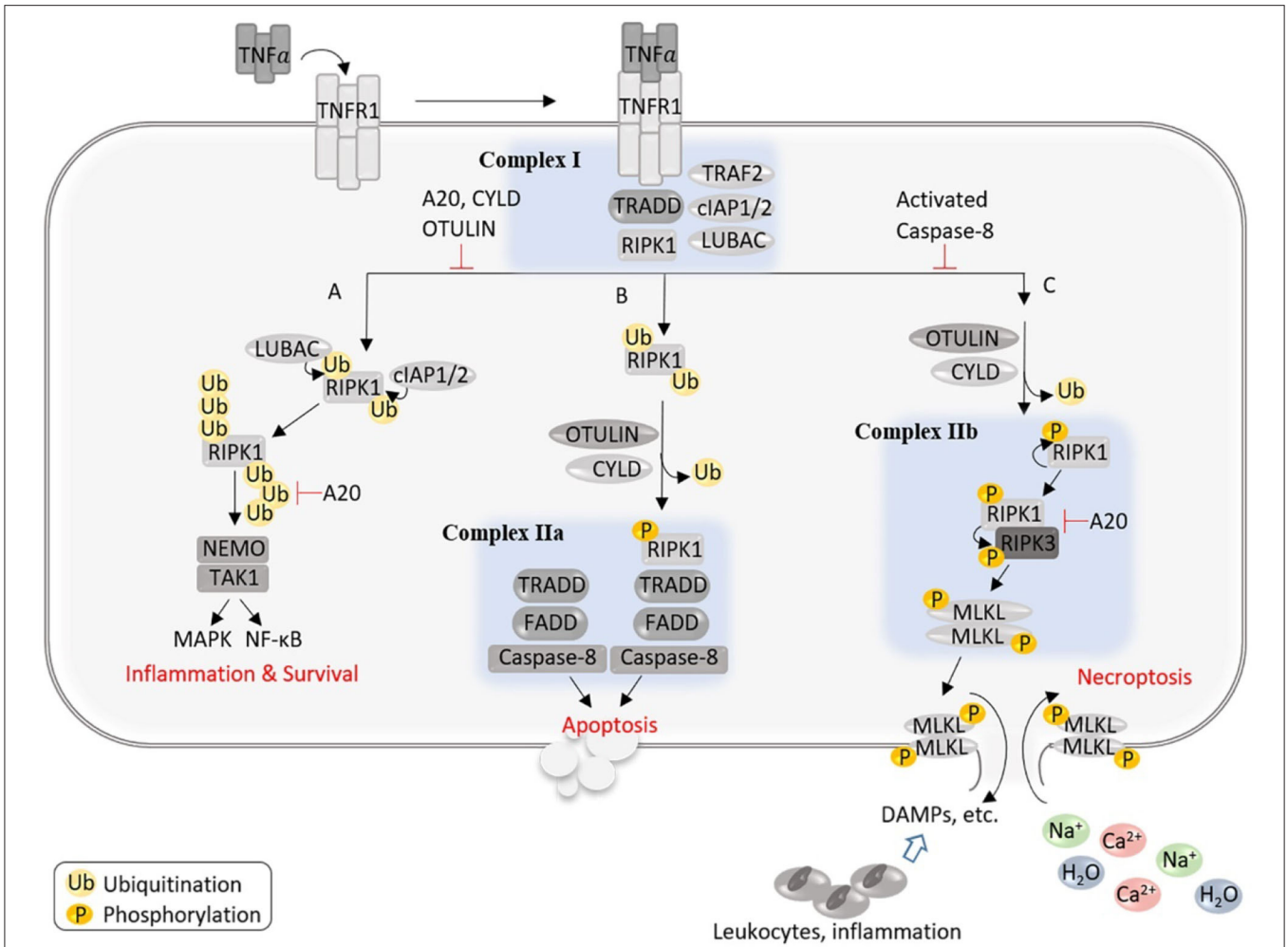


FIGURE 2 | An illustration of the potential signaling events and outcomes after the activation of TNFR1. The binding of TNFα to TNFR1 induces the formation of a membrane-bound assembly known as Complex I, which is associated with the cytoplasmic tail of TNFR1 and contains TRADD, RIPK1, TRAF2, LUBAC, and cIAP1/2, where LUBAC adds linear ubiquitin chains to RIPK1 and other proteins, whereas cIAP1/2 catalyze the addition of K63-linked polyubiquitin chains to RIPK1 and other proteins within Complex I. The ubiquitinated RIPK1 functions as a scaffold to recruit NEMO and TAK1, leading to the activation of NFκB and MAPK pathways, respectively, and promotes cell survival and inflammation **(A)**. Complex I can be transformed into the secondary cytosolic Complex IIa and IIb to signal apoptosis **(B)** or necroptosis **(C)**, respectively. **(B)** Caspase-8 activity in Complex IIa determines downstream signaling toward an apoptotic outcome and simultaneously prevents necroptosis by cleaving RIPK1 or RIPK3. **(C)** In Complex IIb, the absence or inactivation of caspase-8 triggers autophosphorylation of RIPK1, which in turn binds and phosphorylates RIPK3; phosphorylated RIPK3 recruits and phosphorylates MLKL, leading to MLKL's translocation to the plasma membrane where MLKL oligomerizes and forms pores, allowing an influx of extracellular ions and water into the cell that causes cell swelling and rupture, and the release of cellular contents triggers proinflammatory responses.

only Dox treatment or hypoxia/reoxygenation (H/R) but also RIPK3 overexpression in NRVM cultures (Zhang et al., 2016). It should be noted that this study has also unveiled that RIPK3 contributes to the induction of apoptosis by myocardial I-R, and CaMKII plays a mediating role in the caspase activation by RIPK3 overexpression in cultured NRVMs. Hence, the RIPK3–CaMKII pathway appears to be responsible for both necroptosis and apoptosis in I-R injury and acute Dox cardiotoxicity.

Since the activation of CaMKII was known to cause cardiomyocyte death *via* opening MPT pores (Joiner et al., 2012), Zhang et al. further tested whether MPT is the downstream executor of the RIPK3–CaMKII pathway in NRVM cultures. They found that siRNA-mediated downregulation of cyclophilin D, a key regulator of MPT, moderately but statistically significantly alleviated RIPK3 overexpression-induced cell death as assessed by LDH release and cell viability assays (Zhang et al., 2016). It was also found that RIPK3 deficiency reduced the induction of the depolarization of the mitochondrial membrane potential ($\Delta\Psi_m$) by I-R or Dox treatment, RIPK3 overexpression induced $\Delta\Psi_m$ depolarization in a cyclophilin D-dependent manner, and inhibition of CaMKII prevented RIPK3 from inducing $\Delta\Psi_m$ depolarization (Zhang et al., 2016). Taken together, these *in vivo* and *in vitro* findings support a RIPK3–CaMKII–MPT pathway to cardiomyocyte necroptosis

(**Figure 3**). This blurs the boundary between necroptosis and the MPT-driven necrosis (Galluzzi et al., 2018); the latter can be differentiated from other types of regulated necrosis by its dependency on cyclophilin D (Baines et al., 2005), according to a recent classification scheme (Galluzzi et al., 2018). The RIPK3–CamKII–MPT pathway may be unique to cardiomyocytes because a prior study demonstrated that cells depleted of mitochondria can still undergo necroptosis (Tait et al., 2013), arguing against an obligatory role of MPT opening in necroptosis. The RIPK1/RIPK3-mediated necroptosis and MPT-driven necrosis have been shown to contribute uniquely to renal I-R injury (Linkermann et al., 2013), whereas RIPK1 and MPT were implicated to work in the same pathway in myocardial I-R injury (Lim et al., 2007).

The defining evidence provided by Zhang et al. (2016) to support the role of MPT opening in the RIPK3–CaMKII necroptotic pathway was collected primarily from cell cultures, which may represent a caveat. This is because in the cell culture setting, a cell undergoing apoptosis may also show loss of its membrane integrity (the characteristic of necrosis), although this does not occur *in vivo* (Del Re et al., 2019). Therefore, decreased cell viability and increased LDH leakage induced by RIPK3 overexpression in cultured NRVMs might not necessarily result from necroptosis, especially when RIPK3 can mediate both necroptosis and apoptosis as shown by this and other prior studies. It also should be pointed out that evidence for ruling out the involvement of MLKL or RIPK1 also seems to be quite weak, and further studies to gather more comprehensive and especially *in vivo* genetic evidence are warranted. MLKL knockout mice have been reported and used to test the involvement of MLKL in necroptosis and pathogenesis (Wu et al., 2013); it will be very interesting and important to test whether MLKL null mice show resistance to I-R injury and Dox cardiotoxicity. As highlighted in an earlier section, the requirement of RIPK1 in cardiomyocyte necroptosis induced by at least TNFR1 signaling has been well-demonstrated.

INTERACTIONS BETWEEN MACROAUTOPHAGY AND THE RIPK1–RIPK3–MLKL PATHWAYS

Emerging evidence increasingly suggests that the processes of autophagy and necroptosis interact at multiple levels in both non-myocyte cells and cardiomyocytes. For example, lysosome inhibition with bafilomycin A1 is capable of accumulating RIPK1 and RIPK3, and conversely, rapamycin-activated autophagy protects against necroptosis in rat pheochromocytoma PC12 cells (Liu et al., 2018). ATG16L-dependent autophagy has been shown to clear RIPK1, PIPK3, TRIF, and ZBP1 and attenuate necroptosis induced by TNFα and TLR ligands in macrophages (Lim et al., 2019). This is further complicated by the observations that MLKL suppresses autophagy, most likely at the level of autolysosome efflux, while the protein turnover of MLKL is indeed controlled by autophagy in hepatocytes under physiological conditions (Wu et al., 2020b). Lipid overloading intensifies MLKL-mediated autophagy inhibition and necroptosis in hepatocytes (Wu et al., 2020b). In addition, although MLKL attenuates autophagy characterized by autophagosome and autolysosome dysfunction in immortalized mouse dermal fibroblasts and HT-29 colorectal cancer cells treated with TSI (TNF, the SMAC mimetic Compound A, and caspase inhibitor IDN-6556), suppression of autophagosome formation *via* CRISPR/Cas9-mediated knockout of autophagy-related gene 5 (Atg5) or of Atg7 does not affect necroptosis in these cells (Frank et al., 2019). Taken together, these findings from non-cardiomyocytes seem to indicate that defective removal of autophagosomes resulting from decreased autophagic flux may contribute to necroptosis.

At the molecular level, autophagy-initiating kinase ULK1 has been shown to phosphorylate RIPK1 at Ser357, thereby inhibiting TNF-induced cell death (Wu et al., 2020a). Activation of mTOR is known to suppress autophagy. A recent study showed that mTOR hyperactivation by either Western diet or genetic inhibition of Tsc1 led to necroptosis of gut epithelia, contributing to inflammatory bowel disease; mechanistically, mTOR suppresses TRIMM11-mediated ubiquitination of RIPK3, and the latter targets RIPK3 for autophagic degradation (Xie et al., 2020). A different group subsequently reported that during necroptosis, RIPK3 reduces autophagosome–lysosome fusion and thereby decreasing autophagic flux in intestine epithelia (Otsubo et al., 2020). MLKL-dependent but RIPK3-independent signaling was shown to suppress autophagy in hepatocytes, contributing to Western diet-induced liver injury in mice (Wu et al., 2020b). In other cells undergoing necroptosis, the association of active MLKL with cell membrane was shown to suppress autophagosome removal (Frank et al., 2019). Conversely, the phosphorylation of ULK1 at Ser746 by RIPK3 was shown to be required for the induction of alternative autophagy by genotoxic stress (Torii et al., 2020).

It appears that impaired autophagic flux contributes to necroptosis in cardiomyocytes. Two recent reports showed evidence collected from cultured H9c2 cells suggesting a major contribution of impaired autophagy to the induction of necroptosis by TNFα (Ogasawara et al., 2017; Abe et al., 2019). According to these reports, autophagic flux was suppressed as the RIPK1–RIPK3 interaction and necroptosis were induced by the combined treatment with TNFα and a broad-spectrum caspase inhibitor (Ogasawara et al., 2017); improving autophagic flux through inhibition of mTORC1 was able to attenuate the necroptosis in an autophagy- and transcription factor EB (TFEB; a master regulator of the ALP)-dependent fashion (Ogasawara et al., 2017; Abe et al., 2019); and MPT was not important in the execution of necroptosis (Ogasawara et al., 2017). More recently, Li C. et al. (2020) reported that aging-associated impairment of autophagy promoted myocardial I-R injury, the protection of metformin against such injury was associated with improving autophagic flux, and the upregulation of p62 resulting from decreased autophagy promoted the interaction of RIPK1 and RIPK3, a key step for the activation of the RIPK1–RIPK3-mediated necroptotic pathway. Given that both necroptosis and autophagic impairment have been shown as mediators in cardiac pathogenesis, it will be extremely important to improve our understanding of the interaction between autophagy and necroptosis in cardiomyocytes as the

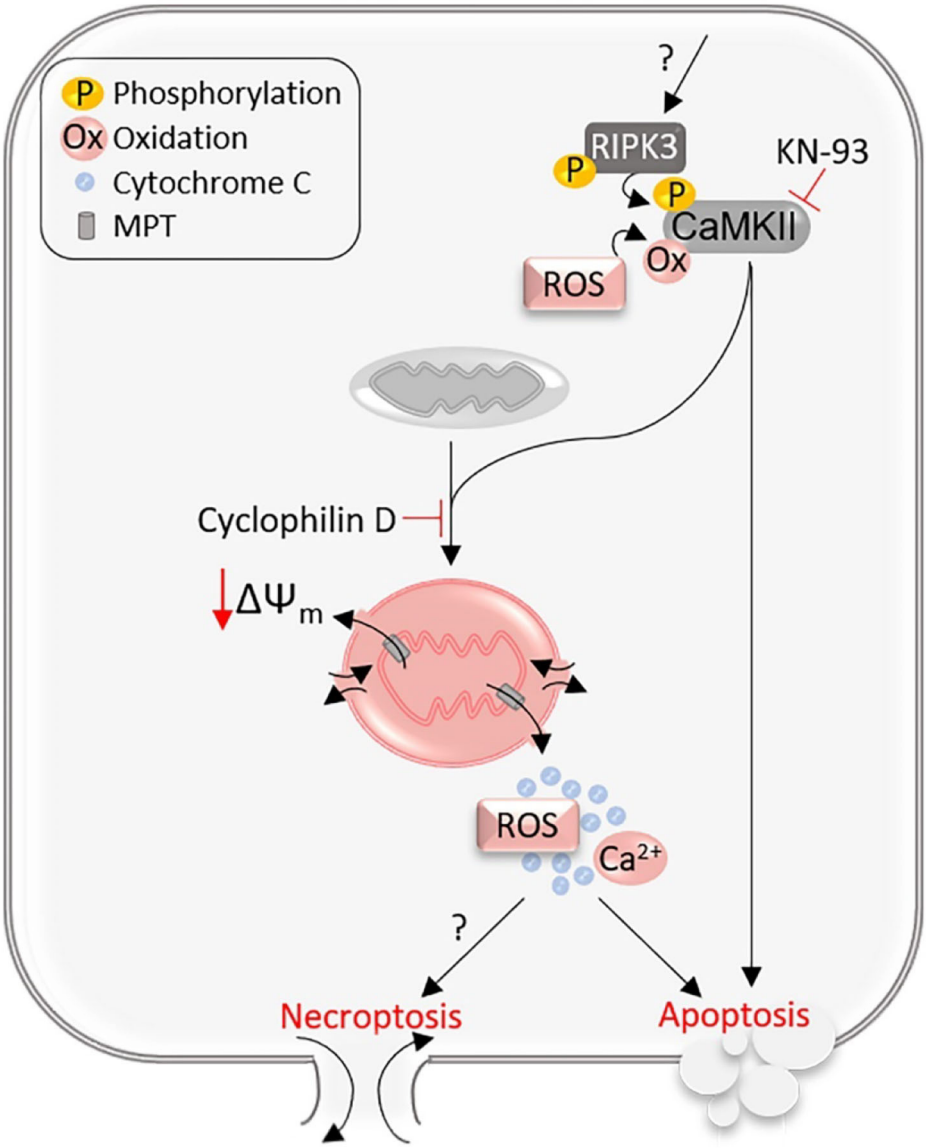


FIGURE 3 | The RIPK3–CaMKII–MPT pathway. RIPK3 phosphorylated and activated through an unknown mechanism binds to and phosphorylates CaMKII. Both phosphorylation and oxidation on CaMKII can activate CaMKII, which opens the mitochondrial membrane permeability transition (MPT) pores, inducing mitochondrial membrane depolarization ($\Delta\Psi_m$) and release of mitochondrial contents, and thereby the RIPK3–CaMKII–MPT pathway mediates both necroptosis and apoptosis in cardiomyocytes.

resultant mechanistic insight is expected to help identify potentially new therapeutic strategies to treat a large subset of heart disease.

CARDIAC Cops8 DEFICIENCY CAUSES MASSIVE CARDIOMYOCYTE NECROSIS AND HEART FAILURE IN MICE

The cardiac physiological significance of the CSN has been studied using mice with cardiomyocyte-restricted Cops8 knockout (Cops8-cko) (Su et al., 2011a,b). In mice with Cops8-cko produced by coupling a Cops8-floxed allele with a transgenic *Cre* driven by the mouse α myosin heavy chain (*Myh6*) promoter, the depletion of Cops8 protein in cardiomyocytes was found to take place during the perinatal period; hence, this Cops8-cko is referred to as perinatal Cops8-cko. The perinatal Cops8-cko did not appear to affect prenatal cardiac development but remarkably affected post-natal cardiac development and functioning. In these mice, pathological cardiac hypertrophy was evident at 2 weeks post-natal, left ventricular (LV) chamber dilatation and malfunction became detectable by 3 weeks, and decompensated left heart failure (as reflected by increased lung

weight to body weight ratios) as well as overt development retardation (as reflected by lack of body weight gain between 3 and 4 weeks) was discerned between 3 and 4 weeks of age. Mice with perinatal Cops8-cko all died by post-natal 52 days, with a median lifespan of ∼32 days (Su et al., 2011b). These findings demonstrate that Cops8 is indispensable for post-natal cardiac development and cardiac function. Since the Cops8-cko mice display remarkably reduced myocardial protein levels of virtually all other CSN subunits and increases in neddylated Cullins as expected, the abnormal phenotype observed in the Cops8-cko mice is conceivably attributable to perturbation of the CSN and of Cullin deneddylation although this remains to be confirmed by conditional knockout of at least another CSN subunit.

Biochemical and histopathological examination by Su et al. (2011b) revealed that cardiomyocyte apoptosis in Cops8-cko mice was not increased until 4 weeks of age when overt heart failure had occurred; moreover, cardiac overexpression of anti-apoptotic protein BCL2 failed to delay the premature death of Cops8-cko mice. These findings compellingly indicate that apoptosis is not a primary cause of the cardiac pathology induced by Cops8-cko. Further examination revealed that massive cardiomyocyte necrosis took place in the Cops8-cko mice as early as 3 weeks of age, as evidenced by increased *in vivo* Evans blue dye (EBD) uptake by cardiomyocytes, increased leukocyte infiltration, and necrotic morphology detected by transmission electron microscopy (Su et al., 2011a,b). Furthermore, massive cardiomyocyte necrosis, dilated cardiomyopathy, rapidly progressed heart failure, and an overwhelmingly increased mortality were subsequently observed in Cops8-cko initiated in adult mice as well (Su et al., 2013), indicating that post-natal cardiac development-associated cardiomyocyte proliferation and hypertrophy are not a required compounding factor for the induction of cardiomyocyte necrosis by Cops8 deficiency. Notably, apoptosis appears to be the primary mode of cell death observed in mice with conditional deletion of the Cops8 in peripheral T lymphocytes and in the liver of mice with the hepatocyte-restricted ablation of *Cops8* (Menon et al., 2007; Lei et al., 2011, 2013); hence, the mode of cell death induced by Cops8/CSN deficiency may be tissue- or cell-type specific. It will be interesting to dissect the mechanism governing this specificity.

MECHANISMS UNDERLYING CARDIOMYOCYTE NECROSIS INDUCED BY Cops8 DEFICIENCY

The Cardiomyocyte Necrosis in Cops8-cko Mice Is Primarily RIPK1–RIPK3-Mediated Necroptosis

To determine the nature of the cardiomyocyte necrosis induced by Cops8-cko, Xiao et al. (2020) examined the potential involvement of the RIPK1–RIPK3 pathway. They found that myocardial protein levels of major known players of the canonic necroptotic pathway (RIPK1, RIPK3, RIPK1-bound RIPK3, and MIKL) were all markedly increased in the face of significantly increased myocardial Bcl2 and decreased cleavage of caspase-8 in mice with perinatal Cops8-cko (Xiao et al., 2020). These findings are indicative of suppressed apoptotic pathways but an activation of the RIPK1–RIPK3–MLKL pathway in Cops8-cko mouse hearts. This is because defective caspase-8 or suppression of the apoptotic pathway has been shown as the prerequisite for death receptor activation to induce necroptosis, and the increased binding of RIPK3 with RIPK1 is a hallmark of the activation of the necroptotic pathway by death receptor stimulation (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). To further establish the role of the RIPK1–RIPK3 pathway, Xiao et al. (2020) tested the effects of necrostatin-1 (Nec-1, a specific kinase inhibitor of RIPK1) and of germline RIPK3 knockout on the cardiomyocyte necrosis and mouse premature death induced by Cops8-cko. The osmotic minipump-mediated treatment of the Cops8-cko mice with Nec-1 initiated at 2 weeks of age achieved a nearly complete blockade of cardiomyocyte necrosis examined at 3 weeks of age and significantly delayed mouse premature death, demonstrating that kinase activity of RIPK1 is required for the induction of cardiomyocyte necrosis by Cops8-cko. Moreover, heterozygous knockout of *Ripk3* also significantly attenuated cardiomyocyte necrosis and elongated the lifespan of mice with Cops8-cko (Xiao et al., 2020). Taken together, these experimental findings provide unequivocal evidence that cardiomyocyte necrosis induced by Cops8-cko is primarily necroptosis mediated by the RIPK1–RIPK3 pathway. The upregulation of myocardial MLKL proteins in Cops8-cko mice suggests that MLKL is probably the downstream effector for this pathway, but this remains to be established because neither the phosphorylation status of MLKL nor the requirement of MLKL in the increased necrosis has been examined in these mice.

Cardiomyocyte Necroptosis Induced by Cops8-cko Is Independent of MPT

As discussed in the *Pathways to cardiomyocyte necroptosis* section, MPT-dependent necrosis by itself can be an independent form of regulated necrosis (Galluzzi et al., 2018); more recently, a RIPK3–CamKII–MPT pathway to cardiomyocyte necroptosis was reported to play an important role in myocardial I-R injury and acute Dox cardiotoxicity (Zhang et al., 2016). Cyclophilin D is a mitochondrial peptidyl-prolyl cis-trans isomerase, a genetically confirmed activator of MPT, and is required for MPT (Baines et al., 2005). Hence, the golden standard for the identification of MPT-dependent necrosis is to show that the necrosis can be blocked by depletion of cyclophilin D (Galluzzi et al., 2018). Neither homozygous nor heterozygous knockout of cyclophilin D was able to attenuate cardiomyocyte necrosis or premature death of Cops8-cko mice (Xiao et al., 2020), demonstrating that cardiomyocyte necrosis induced by Cops8-cko is independent of MPT. Intriguingly, depletion of cyclophilin D *via* homozygous knockout exacerbated cardiomyocyte necrosis and shortened the lifespan of Cops8-cko mice, suggesting that homeostatic levels of cyclophilin D are essential for cardiomyocyte survival and heart function under the stress condition created by Cops8-cko. This is in agreement with a prior report showing that cyclophilin D

knockout increases the propensity for heart failure in mice (Elrod et al., 2010).

How Could Cops8 Deficiency Activate the Cardiac RIPK1–RIPK3 Necroptotic Pathway?

Although the link between Cops8/CSN deficiency and activation of the RIPK1–RIPK3 necroptotic pathway has not been delineated, existing literature has offered possible candidates. First of all, increases of p62 and LC3-II proteins and decreased autophagic flux due to impaired autophagosome–lysosome fusion occur in the cardiomyocytes of mice with perinatal Cops8-cko before 2 weeks of age, which is clearly before cardiomyocyte necrosis becomes detectable (Su et al., 2011a); hence, autophagic impairment, especially combined with the UPS dysfunction that results directly from Cops8 deficiency (Su et al., 2015) and indirectly from autophagic impairment (Tian et al., 2014; Wang and Wang, 2015), could have served as an underlying cause of the cardiomyocyte necrosis *via* a RIPK1–RIPK3-dependent or -independent pathway. This is because it has been shown that duo inhibition of the proteasome and autophagy is sufficient to cause cardiomyocyte necrosis in mice (Su et al., 2011a) and, as discussed in the *Interactions between macroautophagy and the RIPK1–RIPK3–MLKL pathways* section, accumulated p62 may serve as a scaffold to promote the interaction between RIPK1 and RIPK3 and thereby activation of the RIPK1–RIPK3 pathway (Li C. et al., 2020).

Second, the release of DAMPs from the necrotic cardiomyocytes can conceivably further induce inflammatory responses that lead to increased secretion of inflammatory cytokines including TNFα, which in turn could trigger the death receptor signaling of the cardiomyocytes *via* endocrinal, paracrinal, or autocrinal modes. As elaborated below, the malfunction of CRLs intrinsic to Cops8 deficiency due to loss of Cullin deneddylation can affect multiple pathways in such a manner that the TNFα-triggered signaling is ultimately steered to the RIPK1–RIPK3 necroptotic pathway in Cops8-deficient cardiomyocytes (**Figure 4**). As illustrated in **Figure 2**, the engagement of TNFR1 by TNFα can potentially trigger at least three downstream events: (1) formation of the membrane-associated Complex I where RIPK1 and its ubiquitinated forms serve as a scaffold in a manner independent of its kinase activity, which produces survival signals through activation of NFκB pathway and MAPKs; (2) formation of Complex IIa, which leads to apoptosis *via* caspase-8 and downstream events in the so-called extrinsic apoptotic pathway; and (3) formation of Complex IIb (i.e., the RIPK1–RIPK3–MLKL), thus induction of necroptosis when caspase-8 is absent or suppressed (Del Re et al., 2019). The kinase activity of RIPK1 is indispensable for RIPK1 to mediate programmed cell death in Complex IIa. UPS-dependent degradation of IκBα is an essential step for TNFα to activate NFκB where the K48-linked ubiquitination of IκBα is catalyzed by Skp1-Cul1-β-TrCP ($SCF^{\beta-TrCP}$) (Kanarek and Ben-Neriah, 2012), a member of the CRL1 family of ubiquitin ligases. Since the catalytic dynamics of CRLs is perturbed by Cops8-cko, it is very likely that the NFκB-centered survival signaling in cardiomyocytes is impaired in Cops8-cko mice. Myocardial F-box protein β-TrCP protein levels were found to be lower in Cops8-cko mice compared with control mice (Su et al., 2011b), arguing further for a predicted reduction of $SCF^{\beta-TrCP}$ ligase activities. Therefore, there is a great possibility that Cops8 deficiency steers TNFR1 signaling toward cell death direction. It is not known yet whether serum or myocardial TNFα is increased in Cops8-cko mice, and the impact of Cops8 deficiency on the NFκB signaling in cardiomyocytes remains to be determined.

Third, why cardiomyocyte necroptosis rather than apoptosis takes place in Cops8-cko mice? The induction of the necroptotic pathway by death receptor activation has two prerequisites: a) the so-called Complex IIb containing RIPK1 and RIPK3 is formed and b) caspase-8 cannot be activated (Del Re et al., 2019). The study by Xiao et al. (2020) has shown that both prerequisites are met in the $Cops8^{CKO}$ hearts. Myocardial RIPK1, RIPK3, and MLKL protein levels as well as RIPK1-bound RIPK3 were all significantly increased in Cops8-cko mice; and importantly, impaired caspase-8 activation as reflected by markedly decreases in the cleaved form of caspase-8 and in caspase-8 activity were observed in mice with perinatal $Cops8^{CKO}$ compared with mice with control genotypes (Xiao et al., 2020). This caspase-8 impairment is likely due to the loss of Cullin deneddylation because Cul3-RBX1-mediated polyubiquitination of caspase-8 is essential for further processing and activation of caspase-8 and for the progression of the extrinsic apoptotic pathway (Jin et al., 2009). Both neddylation and deneddylation of Cullins are indispensable for the assembly and disassembly of CRLs; hence, the ubiquitination of caspase-8 by Cul3-RBX1 is conceivably compromised by Cops8 deficiency. This postulate is further supported by that inhibition of neddylation with a NEDD8 activation enzyme inhibitor MLN4924 makes monocytes more susceptible to necroptosis *in vitro* (El-Mesery et al., 2015). As discussed in section *The RIPK1–RIPK3–MLKL pathway*, the deubiquitinating enzyme CYLD deubiquitinates RIPK1 to decrease its interaction with NEMO, thus destabilizing Complex I, whereas it also deubiquitinates RIPK1 in TNFα-induced necrosomes to facilitate kinase activation and necroptosis (O'Donnell et al., 2011; Moquin et al., 2013). CYLD was shown to be negatively regulated by caspase-8-mediated cleavage, protecting against TLR-mediated necroptosis (Legarda et al., 2016). Therefore, it is possible that the reduced caspase-8 activation in Cops8-cko hearts promotes cardiomyocyte necroptosis *via* accumulation of CYLD. It will be very interesting to examine whether CYLD is increased in Cops8-deficient cardiomyocytes and whether CYLD plays a critical role in cardiomyocyte cell death caused by Cops8/CSN deficiency.

Not only the extrinsic pathway (as discussed above) but also the intrinsic pathway of apoptosis is likely suppressed in Cops8-cko cardiomyocytes because the powerful anti-apoptotic factor Bcl2 was significantly increased (Su et al., 2011b; Xiao et al., 2020). Increased ROS has been shown to play a role in RIPK3-mediated necroptosis in cultured cells (Zhang et al., 2009; Schenk and Fulda, 2015). In the induction of necroptosis by TNFα, the RIPK3-centered necrosome stimulates aerobic metabolism and thereby increases ROS production; it appears that RIPK3

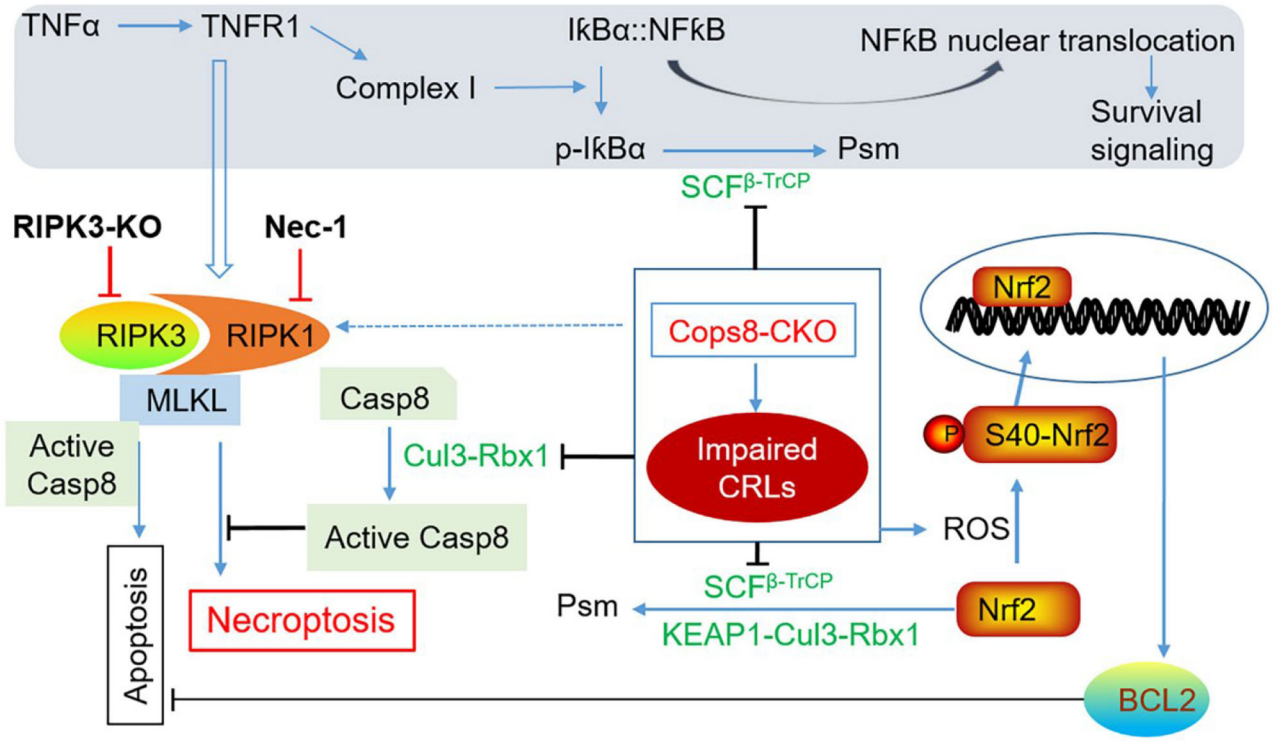


FIGURE 4 | A working model for induction of cardiomyocyte necroptosis by Cops8 deficiency. Loss of function of Cops8/CSN is expected to suppress the CRLs shown in green font, which in turn disrupts the respective ubiquitination events and thereby blocks NFκB-mediated survival signaling and inhibits the apoptotic pathway *via* increasing BCL2 and hindering the activation of caspase-8 (Casp8), steering the death receptor-triggered pathway to the RIPK1–RIPK3-mediated necroptosis. The pathways in the shaded zone are likely involved but not directly examined in Cops8-deficient mice. It is hypothesized that TFNα comes from autocrinal or paracrinal routes from the myocardium with cardiomyocyte-restricted *Cops8* knockout (Cops8-CKO). The interrogations that have been completed are marked with bold black font. Dotted line denotes a potential link that has not been tested yet. Nec-1, necrostatin-1; p-IκBα, phosphorylated IκBα; Psm, proteasomal degradation; RIPK3-KO, global knockout of RIPK3; ROS, reactive oxygen species [adopted from Xiao et al. (2020)].

does so through activation of key metabolic enzymes such as glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), glutamate dehydrogenase 1 (GLUD1) (Zhang et al., 2009), and more recently pyruvate dehydrogenase (PDH), a rate-limiting enzyme linking glycolysis to aerobic respiration (Yang et al., 2018). The elevated ROS further facilitates the formation of necrosomes and increases cytotoxicity during necroptosis (Schenk and Fulda, 2015). Elevated levels of ROS or oxidative stress are indeed associated with cardiomyocyte necroptosis in $Cops8^{CKO}$ mice, as evidenced by increased levels of protein carbonyls and superoxide anion (O_2^-) in $Cops8^{CKO}$ hearts (Xiao et al., 2020). As revealed by the transcriptome analysis (Abdullah et al., 2017), the increased oxidative stress apparently induced the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, the master regulator of antioxidant and defensive responses, in $Cops8^{CKO}$ hearts even before cardiomyocyte necrosis was discernible (Abdullah et al., 2017). The activation of cardiac Nrf2 by ROS is expected to be more robust in Cops8-cko mice than in WT mice as the UPS-mediated degradation of Nrf2 is likely impaired by Cops8 deficiency. This is because $SCF^{\beta-TrCP}$ and KEAP1-Cul3-Rbx1, the two known ubiquitin ligases for Nrf2 ubiquitination, are CRLs and expected to be impaired by Cops8/CSN deficiency. The increases in myocardial mRNA levels of Bcl2 (Xiao et al., 2020), a known target gene of Nrf2 (Niture and Jaiswal, 2012), further attest Nrf2 activation in Cops8-cko hearts. Nevertheless, the role of an altered redox state in cardiomyocyte necroptosis induced by Cops8/CSN deficiency has not been established.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In conclusion, COPS8 and very likely the CSN suppress cardiac RIPK1–RIPK3 necroptotic pathway *in vivo*. The underlying mechanism remains unclear, but the regulatory roles of the COPS8/CSN in both autophagosome maturation and supporting the catalytic dynamics of CRLs *via* Cullin deneddylation are conceivably involved. To establish these conceived molecular links, many questions remain to be addressed. For example, to confirm Cullin deneddylation is required, it will be essential to test whether ablation of other CSN subunits in cardiomyocytes yields the same phenotype as Cops8-cko. The phenotype reported for Cops6-cko shares a lot of similarities with that

of Cops8-cko (Liang et al., 2021), but it remains unclear whether cardiomyocyte necrosis occurs in Cops6-cko mice. COPS8/CSN is required for autophagosome maturation in at least cardiomyocytes (Su et al., 2011a), but the molecular underpinning for this requirement remains unknown; it is even unclear whether Cullin deneddylation is a mediating mechanism. To establish the role of decreased autophagic flux or of p62 upregulation will require testing if improving autophagic flux or prevention of p62 accumulation, respectively, can attenuate cardiomyocyte necroptosis in Cops8-cko mice. The regulation of death receptor signaling involves arguably the most diverse forms of ubiquitination (e.g., K48, K63, and linear ubiquitination). They signal for either proteolytic or non-proteolytic fates and are regulated by many ubiquitin ligases (of which some are CRLs), ubiquitin-editing enzymes (e.g., A20), and DUBs (e.g., CYLD, OTULIN); hence, it will be extremely important to understand how COPS8/CSN regulates these regulators and thereby the death receptor signaling. Although the *bona fide* biochemical activity of the CSN is Cullin deneddylation, COPS subunits or minicomplexes of them may also exert deneddylase-independent function. For example, in Cops8 hypomorphic mouse embryonic fibroblasts, the free Cops5 or the Cops5-containing minicomplex may promote the cell cycle transition from G0/G1 to S phase *via* facilitation of nuclear exclusion of p27 and thereby promoting cell proliferation (Liu et al., 2013). Hence, it will also be interesting to investigate the potential involvement of the minicomplex in cardiomyocyte survival. Since both the CSN/Cullin deneddylation and necroptosis have been implicated to play a significant role in cardiac pathogenesis, more intensive effort on deciphering the molecular mechanisms that govern these processes is expected to facilitate the search for new measures to prevent or more effectively treat heart disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

ML preparing the first draft of ∼15% of the manuscript and preparing **Figures 1–3** and their legends under the guidance of XW. TC preparing the first draft of ∼10% of the manuscript. XW planning and organizing the overall manuscript, writing ∼75% of the manuscript, incorporating various parts into the manuscript, polishing, and corresponding to the editors. All authors have read and approved the manuscript.

FUNDING

This work was supported in part by NIH Grants HL072166, HL085629, and HL153614 (to XW), as well as HL131667 (to XW and TC), and an American Heart Association Grant 20TPA35490091 (to XW).

REFERENCES

Abdullah, A., Eyster, K. M., Bjordahl, T., Xiao, P., Zeng, E., and Wang, X. (2017). Murine myocardial transcriptome analysis reveals a critical role of COPS8 in the gene expression of cullin-RING ligase substrate receptors and redox and vesicle trafficking pathways. *Front. Physiol.* 8:594. doi: 10.3389/fphys.2017.00594

Abe, K., Yano, T., Tanno, M., Miki, T., Kuno, A., Sato, T., et al. (2019). mTORC1 inhibition attenuates necroptosis through RIP1 inhibition-mediated TFEB activation. *Biochim. Biophys. Acta Mol. Basis Dis.* 1865:165552. doi: 10.1016/j.bbadis.2019.165552

Baines, C. P., Kaiser, R. A., Purcell, N. H., Blair, N. S., Osinska, H., Hambleton, M. A., et al. (2005). Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 434, 658–662. doi: 10.1038/nature03434

Bell, J. R., Vila-Petroff, M., and Delbridge, L. M. (2014). CaMKII-dependent responses to ischemia and reperfusion challenges in the heart. *Front. Pharmacol.* 5:96. doi: 10.3389/fphar.2014.00096

Blondelle, J., Biju, A., and Lange, S. (2020). The role of cullin-RING ligases in striated muscle development, function, and disease. *Int. J. Mol. Sci.* 21:7936. doi: 10.3390/ijms21217936

Cho, Y. S., Challa, S., Moquin, D., Genga, R., Ray, T. D., Guildford, M., et al. (2009). Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 137, 1112–1123. doi: 10.1016/j.cell.2009.05.037

Degterev, A., Hitomi, J., Germscheid, M., Ch'en, I. L., Korkina, O., Teng, X., et al. (2008). Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat. Chem. Biol.* 4, 313–321. doi: 10.1038/nchembio.83

Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., et al. (2005). Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat. Chem. Biol.* 1, 112–119. doi: 10.1038/nchembio711

Del Re, D. P., Amgalan, D., Linkermann, A., Liu, Q., and Kitsis, R. N. (2019). Fundamental mechanisms of regulated cell death and implications for heart disease. *Physiol. Rev.* 99, 1765–1817. doi: 10.1152/physrev.00022.2018

Dondelinger, Y., Declercq, W., Montessuit, S., Roelandt, R., Goncalves, A., Bruggeman, I., et al. (2014). MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. *Cell Rep.* 7, 971–981. doi: 10.1016/j.celrep.2014.04.026

Douglas, T., and Saleh, M. (2019). Post-translational modification of OTULIN regulates ubiquitin dynamics and cell death. *Cell Rep.* 29, 3652–3663.e3655. doi: 10.1016/j.celrep.2019.11.014

Dubiel, W., Chaithongyot, S., Dubiel, D., and Naumann, M. (2020). The COP9 signalosome: a multi-DUB complex. *Biomolecules* 10:1082. doi: 10.3390/biom10071082

El-Mesery, M., Seher, A., Stuhmer, T., Siegmund, D., and Wajant, H. (2015). MLN4924 sensitizes monocytes and maturing dendritic cells for TNF-dependent and -independent necroptosis. *Br. J. Pharmacol.* 172, 1222–1236. doi: 10.1111/bph.12998

Elrod, J.W., Wong, R., Mishra, S., Vagnozzi, R.J., Sakthievel, B., Goonasekera, S.A., et al. (2010). Cyclophilin D controls mitochondrial pore-dependent Ca(2+) exchange, metabolic flexibility, and propensity for heart failure in mice. *J. Clin. Invest.* 120, 3680–3687. doi: 10.1172/JCI43171

Frank, D., Vaux, D. L., Murphy, J. M., Vince, J. E., and Lindqvist, L. M. (2019). Activated MLKL attenuates autophagy following its translocation to intracellular membranes. *J. Cell Sci.* 132:jcs220996. doi: 10.1242/jcs.220996

Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., et al. (2018). Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018. *Cell Death Differ.* 25, 486–541. doi: 10.1038/s41418-017-0012-4

He, S., Wang, L., Miao, L., Wang, T., Du, F., Zhao, L., et al. (2009). Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* 137, 1100–1111. doi: 10.1016/j.cell.2009.05.021

Hitomi, J., Christofferson, D. E., Ng, A., Yao, J., Degterev, A., Xavier, R. J., et al. (2008). Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell* 135, 1311–1323. doi: 10.1016/j.cell.2008.10.044

Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V. C., Lill, J. R., et al. (2009). Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell* 137, 721–735. doi: 10.1016/j.cell.2009.03.015

Joiner, M. L., Koval, O. M., Li, J., He, B. J., Allamargot, C., Gao, Z., et al. (2012). CaMKII determines mitochondrial stress responses in heart. *Nature* 491, 269–273. doi: 10.1038/nature11444

Kanarek, N., and Ben-Neriah, Y. (2012). Regulation of NF-kappaB by ubiquitination and degradation of the IkappaBs. *Immunol. Rev.* 246, 77–94. doi: 10.1111/j.1600-065X.2012.01098.x

Kist, M., and Vucic, D. (2021). Cell death pathways: intricate connections and disease implications. *EMBO J.* 40:e106700. doi: 10.15252/embj.2020106700

Kwok, S. F., Solano, R., Tsuge, T., Chamovitz, D. A., Ecker, J. R., Matsui, M., et al. (1998). Arabidopsis homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic cop/det/fus mutations. *Plant Cell* 10, 1779–1790. doi: 10.1105/tpc.10.11.1779

Legarda, D., Justus, S. J., Ang, R. L., Rikhi, N., Li, W., Moran, T. M., et al. (2016). CYLD Proteolysis protects macrophages from TNF-mediated auto-necroptosis induced by LPS and licensed by type I IFN. *Cell Rep.* 15, 2449–2461. doi: 10.1016/j.celrep.2016.05.032

Lei, D., Li, F., Su, H., Liu, J., Wei, N., and Wang, X. (2013). Hepatic deficiency of COP9 signalosome subunit 8 induces ubiquitin-proteasome system impairment and Bim-mediated apoptosis in murine livers. *PLoS ONE* 8:e67793. doi: 10.1371/journal.pone.0067793

Lei, D., Li, F., Su, H., Tian, Z., Ye, B., Wei, N., et al. (2011). COP9 signalosome subunit 8 is required for postnatal hepatocyte survival and effective proliferation. *Cell Death Differ.* 18, 259–270. doi: 10.1038/cdd.2010.98

Li, C., Mu, N., Gu, C., Liu, M., Yang, Z., Yin, Y., et al. (2020). Metformin mediates cardioprotection against aging-induced ischemic necroptosis. *Aging Cell* 19:e13096. doi: 10.1111/acel.13096

Li, J., Zou, J., Littlejohn, R., Liu, J., and Su, H. (2020). Neddylation, an emerging mechanism regulating cardiac development and function. *Front. Physiol.* 11:612927. doi: 10.3389/fphys.2020.612927

Liang, Y., Lyon, R. C., Pellman, J., Bradford, W. H., Lange, S., Bogomolovas, J., et al. (2021). Desmosomal COP9 regulates proteome degradation in arrhythmogenic right ventricular dysplasia/cardiomyopathy. *J. Clin. Invest.* 131:e137689. doi: 10.1172/JCI137689

Lim, J., Park, H., Heisler, J., Maculins, T., Roose-Girma, M., Xu, M., et al. (2019). Autophagy regulates inflammatory programmed cell death via turnover of RHIM-domain proteins. *Elife* 8:36. doi: 10.7554/eLife.44452.036

Lim, S. Y., Davidson, S. M., Mocanu, M. M., Yellon, D. M., and Smith, C. C. (2007). The cardioprotective effect of necrostatin requires the cyclophilin-D component of the mitochondrial permeability transition pore. *Cardiovasc. Drugs Ther.* 21, 467–469. doi: 10.1007/s10557-007-6067-6

Lingaraju, G. M., Bunker, R. D., Cavadini, S., Hess, D., Hassiepen, U., Renatus, M., et al. (2014). Crystal structure of the human COP9 signalosome. *Nature* 512, 161–165. doi: 10.1038/nature13566

Linkermann, A., Brasen, J. H., Darding, M., Jin, M. K., Sanz, A. B., Heller, J. O., et al. (2013). Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury. *Proc. Natl. Acad. Sci. U.S.A.* 110, 12024–12029. doi: 10.1073/pnas.1305538110

Liu, C., Guo, L. Q., Menon, S., Jin, D., Pick, E., Wang, X., et al. (2013). COP9 signalosome subunit Csn8 is involved in maintaining proper duration of the G1 phase. *J. Biol. Chem.* 288, 20443–20452. doi: 10.1074/jbc.M113.468959

Liu, S., Li, Y., Choi, H. M. C., Sarkar, C., Koh, E. Y., Wu, J., et al. (2018). Lysosomal damage after spinal cord injury causes accumulation of RIPK1 and RIPK3 proteins and potentiation of necroptosis. *Cell Death Dis.* 9:476. doi: 10.1038/s41419-018-0469-1

Lork, M., Verhelst, K., and Beyaert, R. (2017). CYLD, A20 and OTULIN deubiquitinases in NF-kappaB signaling and cell death: so similar, yet so different. *Cell Death Differ.* 24, 1172–1183. doi: 10.1038/cdd.2017.46

Luedde, M., Lutz, M., Carter, N., Sosna, J., Jacoby, C., Vucur, M., et al. (2014). RIP3, a kinase promoting necroptotic cell death, mediates adverse remodelling after myocardial infarction. *Cardiovasc. Res.* 103, 206–216. doi: 10.1093/cvr/cvu146

Lydeard, J. R., Schulman, B. A., and Harper, J. W. (2013). Building and remodelling Cullin-RING E3 ubiquitin ligases. *EMBO Rep.* 14, 1050–1061. doi: 10.1038/embor.2013.173

Martens, A., and van Loo, G. (2020). A20 at the crossroads of cell death, inflammation, and autoimmunity. *Cold Spring Harb. Perspect. Biol.* 12:a036418. doi: 10.1101/cshperspect.a036418

Menon, S., Chi, H., Zhang, H., Deng, X. W., Flavell, R. A., and Wei, N. (2007). COP9 signalosome subunit 8 is essential for peripheral T cell homeostasis and antigen receptor-induced entry into the cell cycle from quiescence. *Nat. Immunol.* 8, 1236–1245. doi: 10.1038/ni1514

Mishra, P. K., Adameova, A., Hill, J. A., Baines, C. P., Kang, P. M., Downey, J. M., et al. (2019). Guidelines for evaluating myocardial cell death. *Am. J. Physiol. Heart Circ. Physiol.* 317, H891–H922. doi: 10.1152/ajpheart.00259.2019

Molnar, T., Mazlo, A., Tslaf, V., Szollosi, A. G., Emri, G., and Koncz, G. (2019). Current translational potential and underlying molecular mechanisms of necroptosis. *Cell Death Dis.* 10:860. doi: 10.1038/s41419-019-2094-z

Moquin, D. M., McQuade, T., and Chan, F. K. (2013). CYLD deubiquitinates RIP1 in the TNFalpha-induced necrosome to facilitate kinase activation and programmed necrosis. *PLoS ONE.* 8:e76841. doi: 10.1371/journal.pone.0076841

Nawrocki, S. T., Kelly, K. R., Smith, P. G., Espitia, C. M., Possemato, A., Beausoleil, S. A., et al. (2013). Disrupting protein NEDDylation with MLN4924 is a novel strategy to target cisplatin resistance in ovarian cancer. *Clin. Cancer Res.* 19, 3577–3590. doi: 10.1158/1078-0432.CCR-12-3212

Niture, S. K., and Jaiswal, A. K. (2012). Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. *J. Biol. Chem.* 287, 9873–9886. doi: 10.1074/jbc.M111.312694

O'Donnell, M. A., Perez-Jimenez, E., Oberst, A., Ng, A., Massoumi, R., Xavier, R., et al. (2011). Caspase 8 inhibits programmed necrosis by processing CYLD. *Nat. Cell Biol.* 13, 1437–1442. doi: 10.1038/ncb2362

Oeing, C. U., Mishra, S., Dunkerly-Eyring, B. L., and Ranek, M. J. (2020). Targeting protein kinase G to treat cardiac proteotoxicity. *Front. Physiol.* 11:858. doi: 10.3389/fphys.2020.00858

Ogasawara, M., Yano, T., Tanno, M., Abe, K., Ishikawa, S., Miki, T., et al. (2017). Suppression of autophagic flux contributes to cardiomyocyte death by activation of necroptotic pathways. *J. Mol. Cell. Cardiol.* 108, 203–213. doi: 10.1016/j.yjmcc.2017.06.008

Onizawa, M., Oshima, S., Schulze-Topphoff, U., Oses-Prieto, J. A., Lu, T., Tavares, R., et al. (2015). The ubiquitin-modifying enzyme A20 restricts ubiquitination of the kinase RIPK3 and protects cells from necroptosis. *Nat. Immunol.* 16, 618–627. doi: 10.1038/ni.3172

Otsubo, K., Maeyashiki, C., Nibe, Y., Tamura, A., Aonuma, E., Matsuda, H., et al. (2020). Receptor-interacting protein kinase 3 (RIPK3) inhibits autophagic flux during necroptosis in intestinal epithelial cells. *FEBS Lett.* 594, 1586–1595. doi: 10.1002/1873-3468.13748

Pasparakis, M., and Vandenabeele, P. (2015). Necroptosis and its role in inflammation. *Nature* 517, 311–320. doi: 10.1038/nature14191

Ranek, M. J., Kost, C. K. Jr., Hu, C., Martin, D. S., and Wang, X. (2014). Muscarinic 2 receptors modulate cardiac proteasome function in a protein kinase G-dependent manner. *J. Mol. Cell. Cardiol.* 69, 43–51. doi: 10.1016/j.yjmcc.2014.01.017

Ranek, M. J., Oeing, C., Sanchez-Hodge, R., Kokkonen-Simon, K. M., Dillard, D., Aslam, M. I., et al. (2020). CHIP phosphorylation by protein kinase G enhances protein quality control and attenuates cardiac ischemic injury. *Nat. Commun.* 11:5237. doi: 10.1038/s41467-020-18980-x

Ranek, M. J., Terpstra, E. J., Li, J., Kass, D. A., and Wang, X. (2013). Protein kinase g positively regulates proteasome-mediated degradation of misfolded proteins. *Circulation* 128, 365–376. doi: 10.1161/CIRCULATIONAHA.113.001971

Ranek, M. J., Zheng, H., Huang, W., Kumarapeli, A. R., Li, J., Liu, J., et al. (2015). Genetically induced moderate inhibition of 20S proteasomes in cardiomyocytes facilitates heart failure in mice during systolic overload. *J. Mol. Cell. Cardiol.* 85, 273–281. doi: 10.1016/j.yjmcc.2015.06.014

Rao, F., Lin, H., and Su, Y. (2020). Cullin-RING ligase regulation by the COP9 signalosome: structural mechanisms and new physiologic players. *Adv. Exp. Med. Biol.* 1217, 47–60. doi: 10.1007/978-981-15-1025-0_4

Rozen, S., Fuzesi-Levi, M. G., Ben-Nissan, G., Mizrachi, L., Gabashvili, A., Levin, Y., et al. (2015). CSNAP Is a Stoichiometric Subunit of the COP9 Signalosome. *Cell Rep.* 13, 585–598. doi: 10.1016/j.celrep.2015.09.021

Samson, A. L., Garnish, S. E., Hildebrand, J. M., and Murphy, J. M. (2021). Location, location, location: a compartmentalized view of TNF-induced necroptotic signaling. *Sci. Signal.* 14:eabc6178. doi: 10.1126/scisignal.abc6178

Schenk, B., and Fulda, S. (2015). Reactive oxygen species regulate Smac mimetic/TNFalpha-induced necroptotic signaling and cell death. *Oncogene* 34, 5796–5806. doi: 10.1038/onc.2015.35

Schlierf, A., Altmann, E., Quancard, J., Jefferson, A. B., Assenberg, R., Renatus, M., et al. (2016). Targeted inhibition of the COP9 signalosome for treatment of cancer. *Nat. Commun.* 7:13166. doi: 10.1038/ncomms13166

Shen, H. M., and Codogno, P. (2012). Autophagy is a survival force via suppression of necrotic cell death. *Exp. Cell Res.* 318, 1304–1308. doi: 10.1016/j.yexcr.2012.02.006

Soucy, T. A., Smith, P. G., Milhollen, M. A., Berger, A. J., Gavin, J. M., Adhikari, S., et al. (2009). An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. *Nature* 458, 732–736. doi: 10.1038/nature07884

Su, H., Li, F., Ranek, M. J., Wei, N., and Wang, X. (2011a). COP9 signalosome regulates autophagosome maturation. *Circulation* 124, 2117–2128. doi: 10.1161/CIRCULATIONAHA.111.048934

Su, H., Li, J., Menon, S., Liu, J., Kumarapeli, A. R., Wei, N., et al. (2011b). Perturbation of cullin deneddylation via conditional Csn8 ablation impairs the ubiquitin-proteasome system and causes cardiomyocyte necrosis and dilated cardiomyopathy in mice. *Circ. Res.* 108, 40–50. doi: 10.1161/CIRCRESAHA.110.230607

Su, H., Li, J., Osinska, H., Li, F., Robbins, J., Liu, J., et al. (2013). The COP9 signalosome is required for autophagy, proteasome-mediated proteolysis, and cardiomyocyte survival in adult mice. *Circ. Heart Fail.* 6, 1049–1057. doi: 10.1161/CIRCHEARTFAILURE.113.000338

Su, H., Li, J., Zhang, H., Ma, W., Wei, N., Liu, J., et al. (2015). COP9 signalosome controls the degradation of cytosolic misfolded proteins and protects against cardiac proteotoxicity. *Circ. Res.* 117, 956–966. doi: 10.1161/CIRCRESAHA.115.306783

Sun, L., Wang, H., Wang, Z., He, S., Chen, S., Liao, D., et al. (2012). Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 148, 213–227. doi: 10.1016/j.cell.2011.11.031

Tait, S. W., Oberst, A., Quarato, G., Milasta, S., Haller, M., Wang, R., et al. (2013). Widespread mitochondrial depletion via mitophagy does not compromise necroptosis. *Cell Rep.* 5, 878–885. doi: 10.1016/j.celrep.2013.10.034

Tian, Z., Wang, C., Hu, C., Tian, Y., Liu, J., and Wang, X. (2014). Autophagic-lysosomal inhibition compromises ubiquitin-proteasome system performance in a p62 dependent manner in cardiomyocytes. *PLoS ONE.* 9:e100715. doi: 10.1371/journal.pone.0100715

Tian, Z., Zheng, H., Li, J., Li, Y., Su, H., and Wang, X. (2012). Genetically induced moderate inhibition of the proteasome in cardiomyocytes exacerbates myocardial ischemia-reperfusion injury in mice. *Circ. Res.* 111, 532–542. doi: 10.1161/CIRCRESAHA.112.270983

Torii, S., Yamaguchi, H., Nakanishi, A., Arakawa, S., Honda, S., Moriwaki, K., et al. (2020). Identification of a phosphorylation site on Ulk1 required for genotoxic stress-induced alternative autophagy. *Nat. Commun.* 11:1754. doi: 10.1038/s41467-020-15577-2

Wang, C., and Wang, X. (2015). The interplay between autophagy and the ubiquitin-proteasome system in cardiac proteotoxicity. *Biochim. Biophys. Acta.* 1852, 188–194. doi: 10.1016/j.bbadis.2014.07.028

Wang, H., Sun, L., Su, L., Rizo, J., Liu, L., Wang, L. F., et al. (2014). Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. *Mol. Cell* 54, 133–146. doi: 10.1016/j.molcel.2014.03.003

Wang, X., and Wang, H. (2020). Priming the proteasome to protect against proteotoxicity. *Trends Mol. Med.* 26, 639–648. doi: 10.1016/j.molmed.2020.02.007

Wee, S., Geyer, R. K., Toda, T., and Wolf, D. A. (2005). CSN facilitates Cullin-RING ubiquitin ligase function by counteracting autocatalytic adapter instability. *Nat. Cell Biol.* 7, 387–391. doi: 10.1038/ncb1241

Wegner, K. W., Saleh, D., and Degterev, A. (2017). Complex pathologic roles of RIPK1 and RIPK3: moving beyond necroptosis. *Trends Pharmacol. Sci.* 38, 202–225. doi: 10.1016/j.tips.2016.12.005

Wei, N., Chamovitz, D. A., and Deng, X.-W. (1994). Arabidopsis COP9 is a component of a novel signaling complex mediating light control of development. *Cell* 78, 117–124. doi: 10.1016/0092-8674(94)90578-9

Wei, N., and Deng, X. W. (2003). The COP9 signalosome. *Annu. Rev. Cell Dev. Biol.* 19, 261–286. doi: 10.1146/annurev.cellbio.19.111301.112449

Wertz, I. E., O'Rourke, K. M., Zhou, H., Eby, M., Aravind, L., Seshagiri, S., et al. (2004). De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. *Nature* 430, 694–699. doi: 10.1038/nature02794

Wu, J., Huang, Z., Ren, J., Zhang, Z., He, P., Li, Y., et al. (2013). Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. *Cell Res.* 23, 994–1006. doi: 10.1038/cr.2013.91

Wu, W., Wang, X., Berleth, N., Deitersen, J., Wallot-Hieke, N., Bohler, P., et al. (2020a). The autophagy-initiating kinase ULK1 controls RIPK1-mediated cell death. *Cell Rep.* 31:107547. doi: 10.1016/j.celrep.2020.107547

Wu, X., Poulsen, K. L., Sanz-Garcia, C., Huang, E., McMullen, M. R., Roychowdhury, S., et al. (2020b). MLKL-dependent signaling regulates autophagic flux in a murine model of non-alcohol-associated fatty liver and steatohepatitis. *J. Hepatol.* 73, 616–627. doi: 10.1016/j.jhep.2020.03.023

Xiao, P., Wang, C., Li, J., Su, H., Yang, L., Wu, P., et al. (2020). COP9 Signalosome suppresses RIPK1-RIPK3-mediated cardiomyocyte necroptosis in mice. *Circ. Heart Fail.* 13:e006996. doi: 10.1161/CIRCHEARTFAILURE.120.006996

Xie, Y., Zhao, Y., Shi, L., Li, W., Chen, K., Li, M., et al. (2020). Gut epithelial TSC1/mTOR controls RIPK3-dependent necroptosis in intestinal inflammation and cancer. *J. Clin. Invest.* 130, 2111–2128. doi: 10.1172/JCI133264

Yang, Z., Wang, Y., Zhang, Y., He, X., Zhong, C. Q., Ni, H., et al. (2018). RIP3 targets pyruvate dehydrogenase complex to increase aerobic respiration in TNF-induced necroptosis. *Nat. Cell Biol.* 20, 186–197. doi: 10.1038/s41556-017-0022-y

Zhang, D. W., Shao, J., Lin, J., Zhang, N., Lu, B. J., Lin, S. C., et al. (2009). RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science* 325, 332–336. doi: 10.1126/science.1172308

Zhang, H., Pan, B., Wu, P., Parajuli, N., Rekhter, M. D., Goldberg, A. L., et al. (2019). PDE1 inhibition facilitates proteasomal degradation of misfolded proteins and protects against cardiac proteinopathy. *Sci. Adv.* 5:eaaw5870. doi: 10.1126/sciadv.aaw5870

Zhang, L., Jing, H., Li, H., Chen, W., Luo, B., Zhang, H., et al. (2020). Neddylation is critical to cortical development by regulating Wnt/beta-catenin signaling. *Proc. Natl. Acad. Sci. U.S.A.* 117, 26448–26459. doi: 10.1073/pnas.2005395117

Zhang, T., Zhang, Y., Cui, M., Jin, L., Wang, Y., Lv, F., et al. (2016). CaMKII is a RIP3 substrate mediating ischemia- and oxidative stress-induced myocardial necroptosis. *Nat. Med.* 22, 175–182. doi: 10.1038/nm.4017

Zhou, W., and Yuan, J. (2014). Necroptosis in health and diseases. *Semin. Cell Dev. Biol.* 35, 14–23. doi: 10.1016/j.semcdb.2014.07.013

Zou, J., Ma, W., Li, J., Littlejohn, R., Zhou, H., Kim, I. M., et al. (2018). Neddylation mediates ventricular chamber maturation through repression of Hippo signaling. *Proc. Natl. Acad. Sci. U.S.A.* 115, E4101–E4110. doi: 10.1073/pnas.1719309115

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership