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Insights in aging, metabolism and redox biology: 2021/2022

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Editorial: Insights in aging, metabolism and redox biology

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Editorial on the Research Topic
Insights in Aging, Metabolism and Redox Biology

Contribution to the field

In this Insights in Aging, Metabolism and Redox Biology Research Topic, we summarized the cutting-edge aging research that provide insights into where we are and where we want to be in Aging research with 9 articles.

Zhang discusses the significant intersections between aging, metabolism, and redox biology. The article describes the critical role of metabolic and redox processes in aging, with focus on mitochondrial function and metabolic pathways (*e.g.*, autophagy and mitophagy), suggesting that understanding these can lead to strategies for extending healthspan and lifespan. The paper discusses the "Mitochondrial dysfunction theory of aging" and the "Hormesis theory of aging," proposing that while mitochondrial dysfunction can contribute to aging, there are beneficial aspects to mitochondrial stress that can extend lifespan. The paper also touches on the importance of diet and lifestyle changes in modulating aging processes, highlighting the potential of interventions like dietary restriction, exercise, and pharmacological agents that target key metabolic pathways to promote healthier aging. Additionally, the article delves into the evolving understanding of redox biology, particularly the dual roles of reactive oxygen species (ROS) as both damaging agents and signaling molecules. This nuanced view challenges the traditional "Free Radical Theory of Aging" and suggests a more complex relationship between redox states and aging. Overall, the paper advocates for a more integrated view of metabolism, redox biology, and aging, calling for continued multidisciplinary research to uncover the molecular mechanisms that link these processes to age-related changes and diseases.

Zhang and colleagues explore the modification of proteins by O-GlcNAcylation in the context of neurodegenerative diseases and aging. O-GlcNAcylation, a post-translational modification, plays a critical role in cellular processes by responding to nutrient availability and stress. This study particularly investigates how this modification may influence neurodegenerative disorders like Alzheimer's disease (AD) and Parkinson's disease (PD) through changes in the proteome of the mouse cortex. The authors employed mass spectrometry to identify proteins that undergo O-GlcNAcylation, finding significant alterations in proteins involved in synaptic function and trafficking—areas crucial to neurodegenerative disease mechanisms. They discovered that increasing O-GlcNAc

levels through the inhibitor Thiamet G (TG) affects the phosphorylation of tau, a protein associated with AD, suggesting therapeutic potential. However, the study also notes that elevated O-GlcNAc levels can impair cognitive functions, highlighting a complex balance between beneficial and detrimental effects. Key findings include the identification of specific proteins such as DNAJC6 and PICALM, which are known risk factors for PD and AD, respectively. These proteins showed changes in their O-GlcNAcylation status, linking this modification to the pathology of these diseases. The study suggests that modulating O-GlcNAcylation could be a strategy for targeting molecular pathways implicated in aging and neurodegenerative diseases by linking metabolic state (through O-GlcNAcylation) to neurodegenerative changes.

Fernandez-Abascal and Artal-Sanz explore the role of prohibitins (PHB) in neurodegeneration and mitochondrial homeostasis, focusing on their significance in age-related neurodegenerative disorders. They discuss the increased incidence of such disorders with rising life expectancy and the challenge of diagnosing them at an advanced stage. Prohibitins, evolutionary conserved within the mitochondrial PHB complex, are highlighted for their regulatory functions in aging, metabolism, and association with neurodegenerative diseases, like Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis through unclear mechanisms. The review consolidates current research on the involvement of PHB in synaptic functions and their protective *versus* toxic effects under different conditions. It emphasizes mitochondrial stress as a common starting point for neurodegeneration, proposing PHB and mitochondrial pathways as potential targets for early diagnosis and treatment.

Chatham and colleagues expertly explore the intricacies of calcium (Ca2+) signaling modulation by STIM (Stromal Interaction Molecule) and Orai proteins, emphasizing their significance in the physiology of aging and age-associated diseases. The tight regulation of intracellular Ca2+ concentrations is crucial for numerous cellular functions, including cell survival, metabolism, and transcription. STIM and Orai proteins, being highly conserved and central to mammalian Ca2+ signaling systems, play pivotal roles in Store-Operated Calcium Entry (SOCE), directly impacting cellular homeostasis. The structural and functional nuances of STIM1, STIM2, Orai1, Orai2, and Orai3 proteins are also outlined, illustrating how their interactions mediate calcium influx into cells. STIM and Orai dysregulation are implicated in various aging-related conditions, notably cardiovascular diseases and neurodegeneration. This process is particularly significant in the context of neurodegeneration, cardiovascular diseases, and cellular aging, where calcium dysregulation is a common theme. The authors emphasize the therapeutic potential of targeting these proteins to ameliorate age-related pathologies, highlighting the need for further research to fully understand their roles in aging and disease progression.

Zaburdaev and colleagues reported their mathematical modeling study that if ethanol is supplied periodically, with certain frequency and concentration, *C. elegans* dauer can live toward an unlimited lifespan. The authors recently have published studies that show that *C. elegans* dauer can use ethanol as an external energy source, and upregulate metabolic enzymes including SODH-1 and ALH-1, that catalyze the conversion from ethanol to acetaldehydrate and then to acetate. As mitochondrial deterioration was found to precede the death of worms, the authors make the assumption that if mitochondria can regenerate and detoxification can happen, then worms would have a longer lifespan. Then periodic ethanol with optimal feeding period was shown advantageous over constant ethanol in the modeled lifespan. Overall a provocative idea.

Chen and Dodson provided critical review and literature, and insights into Nrf2 in aging, metabolism and redox biology. Nrf2 regulates the expression of key genes involved in redox regulation, and there are much evidence that its loss of function significantly exacerbates many disease phenotypes. This article highlighted its involvement in multiple neurodegenerative diseases. Furthermore, the authors reviewed approaches with gene delivery, antisense oligos, pharmacological compounds, and those targeting upstream co-activators and downstream effector have been tested to target Nrf2 with the aim to protect against age related pathologies. As accumulation of cellular damage has been shown a critical hallmark of aging, further understand the role of Nrf2 and its regulation is essential for developing a better approach to promote healthy aging.

Gupta and colleagues reviewed the interaction of BAG5 with heat shock proteins, and PINK1, by which BAG5 plays a role in protein quality control, with special emphasis on cardiovascular and neurodegenerative diseases. As BAG5 is one of the key plays in the junction of autophagy, mitophagy, ER-mediated pathways, understanding the molecular mechanisms of how BAG5 interact with other cellular constituents provide a foundation for tackling this regulatory node for therapeutic interventions of age-related chronic diseases.

McKenna and Tong and colleagues reported in a research article that PU.1/Spi1 knockout in the adipocytes resulted in higher energy expenditure in males at 4–5 months of age, and higher insulin sensitivity and lower adiposity at 10–11 months of age. RNAseq analyses showed PU.1 regulated inflammatory and thermogenic programs. This research represents one of the first steps to fully appreciate the role of PU.1 in different cells and tissues as a contributor of metabolic regulation which is essential for health in the process of aging.

Finally, Borras provided considerable thoughts and significant insights on the challenges of unlocking the biological secretes of aging, including the process, the theories, the models and the biomarkers. There is so much that we do not know. First of all, fundamentally what is aging? There is a nearly continuous process of changes in nearly all cells, tissues, and organisms, with time passing by. However, at what point are these changes deleterious? Whether/when/what early changes pre-destine late changes? What regulates this aging process? Is it really irreversible? Associated with unknowns, there are a lot of theories of aging proposed over the years. And there are *in vivo* models investigators use to identify mechanisms and interventions. Dr. Borras provided summary of the theories and the models with strengths and limitations, which is a great service to the field and helpful to trainees in the aging field to gain an overview. Intertwined with the process and the theories of aging are the hallmarks and biomarkers which often reflect but one aspect of the aging processes and are dependent on models and interventions. Because of these challenges, the search remains on and intense in the pursuit to unlock the secrets of aging.

We hope that this Research Topic provides new insights in aging research, in particular metabolism, redox regulation, protein quality control, and current development and challenges.

Author contributions

CL: Writing–original draft, Writing–review and editing. JZ: Writing–original draft, Writing–review and editing.

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The Promise of a Golden Era for Exploring the Frontiers of Aging, Metabolism and Redox Biology

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From ancient times to the modern day extending longevity or even finding the elixir for eternal life has been a motivating quest for many civilizations. There are no shortage of Hollywood films and TV series that feature long-lived creatures: some heroes and others villains. Many of the ancient Greeks have what we would regard as a normal lifespan (Montagu, 1994; Batrinos, 2008). For example, Socrates before his untimely demise was in his 70s. Physicians had been directed to concoct potions to extend the life of emperors and the wealthy. In the Qin Dynasty, the emperor sent 500 young men and 500 young women to find the elixir of life in the legendary Penglai, the miraculous place of the immortals. Detailed descriptions of medicines for immortality were written in the book "Essential Formulas of Danjing Classics". Some of these concoctions we would regard as remarkably toxic as they contain mercury or arsenic. Interestingly, this quest for longevity continues unabated and has now become a central pillar for modern health care. However, the perils persist with the unverified claims of a broad range of supplements or the off-target effects of therapeutics which maybe toxic or otherwise decrease longevity. Clearly, then as now an understanding of the fundamental biology and chemistry of aging is an essential goal for modern scientific research.

Despite the long history of the fascination of a long life, aging research as a systematic scientific effort is a recent affair. In the United States, the Aging Related Unit in the National Institutes of Health was formed in the 1940s, first in the NIH Division of chemotherapy, then moved to Baltimore City Hospital under the direction of Nathan Shock. In 1974 the National Institute of Aging (NIA) became an independent institute with a focus on aging biology and age related diseases. PubMed documents publications on aging as early as in 1925. In 1988, the first genetic locus *age-1* that modulates lifespan was identified in *C. elegans* (Friedman and Johnson, 1988), and 8 years later cloned and found to encode a PI3 kinase (Morris et al., 1996). Now there are a total of ~487,000 articles using the search term "Aging" in PubMed, with ~20,000 articles since 2020.

There has also been a long-standing interest associating aging with metabolism. Searching PubMed with "Aging and Metabolism" results in ~188,685 articles, with 3,219 since 2020. Dietary restriction has been shown to affect longevity and age related illnesses in several organisms and model systems, with the effects on longevity dependent on genetic background (Mair et al., 2003; Liao et al., 2010; Cava and Fontana, 2013). At the molecular level, extended lifespan has been associated with insulin and IGF-1 receptor function, as well as *age-1*/PI3 kinase activity (Kenyon et al., 1993; Kimura et al., 1997). Modulation of sirtuins, which are NAD+ (Nicotinamide adenine dinucleotide) dependent enzymes, was reported to extend lifespan in yeast (Kennedy et al., 1995; Kaeberlein et al., 1999). AMPK (AMP activated protein kinase), a key sensor of metabolism and cellular energy, is required for lifespan extension in *C. elegans* in response to dietary restriction (Greer et al., 2007). Targeting the nutrient sensing pathway, the mTOR (mechanistic target of rapamycin) signaling pathway, using the inhibitor rapamycin, has been used to enhance longevity in several organisms, and shows efficacy when administered to aged mice (Harrison et al., 2009; Miller et al., 2011; Papadopoli et al., 2019). These studies suggest that the aging can be modified by changes in lifestyle or pharmacological intervention.

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Observations that a deficit of mitochondrial function may result in energy shortage and accumulation of reactive species that are damaging to cellular structure and function inspired the "Mitochondrial dysfunction theory of aging" (Lemasters, 2005; Payne and Chinnery, 2015; Kauppila et al., 2017). Paradoxically, there are also observations that inhibition of mitochondrial respiration can extend lifespan. Reconciliation of these observations leads to the concept that the plasticity of metabolic pathways, which has a preprogrammed genetic component, is central in adapting to the environment and in turn impacts longevity (Kayser et al., 2004; Lapointe and Hekimi, 2008; Copeland et al., 2009; Yang and Hekimi, 2010). This is an idea captured in the "Hormesis theory of aging" (Ristow and Schmeisser, 2014; Yun and Finkel, 2014). Clearly, the term "mitochondrial dysfunction" is inadequate to describe the complexity of the adaptive capacity of the age regulated metabolic pathways. Additionally, a judicious inhibition of mitochondrial respiration may be required for activation of survival pathways (Chouchani et al., 2013). A better understanding of the role of metabolism in aging calls for more insights into the specifics of regulation of bioenergetics and metabolism which is now becoming feasible with the advent of sensitive and high precision technologies in these areas of research (Hill et al., 2019).

Although that "the Free Radical Theory of Aging" was proposed in 1956 based on the idea that free radicals can attack cellular constituents and thus may be a direct cause for aging (Harman, 1956), research linking aging to redox modulation is still evolving. With the realization that "free radicals" have a signaling role, it is clear that this basic hypothesis needs refinement to encompass new advances in redox biology and the recognition that all "free radicals" are not the same. Searching "Aging and Free Radical" has total of 13,754 articles, "Aging and oxidative stress" 19,044, "Aging and Redox" 9,139 articles. Many studies have challenged the idea that cellular oxidative damage due to "free radicals" or oxidative stress is a cause of aging. First, reactive oxygen species (or ROS) may be important in modulating aging, but the hypothesis lacks precision since it fails to identify "which species" contribute to aging or the mechanisms involved. This point is sometimes over-looked but similarly if we say "Genes" and "Proteins" are important in modulating aging, most of us will ask which gene(s) and which protein(s). Fortunately, technical advances are overcoming these barriers and allow specific hypotheses to be tested. Over the last 10 years the genetic regulation of redox related networks has also turned out to be remarkably complex. For example, one of the key regulators of redox modulatory proteins is Nrf2, which is a transcription factor that regulates genes encoding a subset of redox regulatory proteins, and is also a downstream target of insulin receptor and involved in lifespan regulation in *C. elegans* (Tullet et al., 2008). What is not predicted from the "Free radical theory of aging" is that increased expression of Nrf2 and its target antioxidant enzymes is detrimental for health and disease (Rajasekaran et al., 2011; Levonen et al., 2014; Dodson et al., 2015; Schmidlin et al., 2019). This key finding indicates that both "oxidative" and "reductive" stress, depending on their specificity, levels and cellular context, may have contrasting effects on aging-dependent processes.

In many aging related phenomena, including cellular senescence and perturbation circadian control, inadequacy in the autophagy and mitophagy pathways, also have strong connection to redox and metabolic regulation (Lopez-Otin et al., 2013). Autophagy is an intracellular degradation process that is highly regulated by a variety of signals including availability of metabolic substrates, cellular and the environmental redox landscape (Zhang, 2015; Klionsky et al., 2016). It is now clear that autophagy is a pathway that may remove and reverse cellular damage caused by oxidative stress and as such it is important to understand whether it is sufficiently active at the right place and at the right time (Lee et al., 2012; Giordano et al., 2014). Because of its central importance in health, disease and aging, the specific autophagic degradation of the mitochondria was identified as a specific process known as mitophagy (Lemasters, 2005; Redmann et al., 2014; Ma et al., 2020). Autophagy and mitophagy then play a key role in the quality control and turnover of lipids, proteins, and organelles, and their regulation modulates the metabolic and redox landscape (Dodson et al., 2013; Redmann et al., 2016). In aging tissues and age related diseases, these processes are unable to clear excess or dysfunctional proteins and organelles (Wong et al., 2020). Damaged organelles including mitochondria together with the accumulation of toxic proteins may further propagate cellular damage and contribute to the progression of age related diseases (Chen et al., 2020).

Aging research has been gaining momentum as better tools are developed and systems biology approaches are adopted. CRISPR/Cas techniques provide enhanced means of determining experimentally the functional consequence of gene disruption or mutation (Ran et al., 2013; Charpentier et al., 2019). These approaches can give insights into the networks that sense environmental signals that change cellular functions and thereby contributing to healthy aging or age related pathologies. Genomics, transcriptomics, proteomics, and metabolomics, some even at the single cell level, will aid in the understanding of the aging process in complex organs including the brain and the immune system (Aon et al., 2020; Zhang et al., 2020). High throughput bioenergetics analyses are now available which use small quantities of materials and even frozen samples and thus greatly extend the current studies of metabolism and its connection to aging (Dranka et al., 2011; Hill et al., 2012; Chacko et al., 2014; Redmann et al., 2018; Acin-Perez et al., 2020). For example, recent studies substantiated the connection of mitochondrial function, metabolism inflammation, and aging, and revealed the potential for a better understanding of this integrated regulatory network in attenuating age related diseases and promoting healthy aging (Bernard et al., 2018; Dunham-Snary et al., 2018; Rangarajan et al., 2018). In addition, a better understanding of the fundamentals of redox biology, and the improvement of techniques detecting and scavenging different reactive species are resulting in a rapid evolution of redox biology research (Kalyanaraman et al., 2012; Kalyanaraman, 2013; Afonso and Spickett, 2019). Advanced informatics methods have been developed, including GWAS, NetWAS, the

transcriptome-metabolome-wide association study (TMWAS) and xMWAS platforms. These methods facilitate data integration, network visualization, clustering and differential network analyses of data from two or more omics dataset of genetic phenotypic, biochemical, or cell biological assays, which can reveal the whole organism changes that underlie the biology of aging (Beekman et al., 2013; Go et al., 2018; Uppal et al., 2018; Chacko et al., 2019; Roussarie et al., 2020; Smith et al., 2020). Integration of metabolism, redox biology with aging phenotypes will likely reveal novel nodes of regulation which can then identify new targets for healthspan extension interventions.

It is important to recognize that aging is not a single organ disease, and is highly dependent on the fact that tissues functionally interact and cross modulate. The blood and lymph circulate through the body and transport hormones, nutrients, cytokines, myokines, cell-free mitochondrial DNA, and other cellular metabolic products to other parts of the body (Barron and Pike, 2012; Coelho et al., 2019; Cunnane et al., 2020; Iske et al., 2020). Aging associated accumulation of the propionate metabolism product methylmalonic acid in the serum, may reprogram cancer cells to become more aggressive (Gomes et al., 2020). The microbiome contributes to a large portion of the total DNA/RNA in mammals and controls metabolism through its interaction with the diet and other environmental factors (Bernard et al., 2018; Bana and Cabreiro, 2019; Buford, 2020). Bacterial and viral infection, also alters the biology of the body and impact aging and age related diseases (Szaniawski and Spivak, 2020). Thus understanding the crosstalk between the gut, brain, liver, heart and muscle via the circulation is of critical importance to the understanding of aging biology (Lehallier et al., 2019). Since we cannot view age related pathologies only in isolated cells or tissue, we can also learn by observing nature. The existence of long-lived and short-lived species, for example, the naked mole-rat, different varieties of fish, clams, turtles, rodents, and centenarian humans, surely hold important clues to understanding how longevity and healthy living have been achieved (Austad, 2018).

An important contemporary research goal is to convert what we understand about metabolism and redox regulation in the context of aging into approaches to promote healthy aging. This approach can be surprisingly straight forward. For example, it has been shown that supplementation of mitochondrial TCA cycle metabolites, malate, fumarate, alpha-ketoglutarate, and oxaloacetate, supplementation of NAD+ precursor nicotinamide riboside extended lifespan in *Drosophila* and *C. elegans* (Belenky et al., 2007; Williams et al., 2009; Edwards et al., 2013; Chin et al., 2014; Zhang et al., 2016). Supplementation of alpha-ketoglutarate also decreases inflammation and frailty in mice, even when started at 18 months of age (Asadi Shahmirzadi et al., 2020). Not surprisingly, not all TCA cycle metabolites have the same effects. For example, accumulation of succinate is detrimental in the context of ischemia-reperfusion injuries (Chouchani et al., 2014). Dietary restriction, exercise, and circadian regulation have all been explored both in terms of metabolic mechanisms and with regard to reactive species. The networks involved in modifying lifespan are complex, and may be dependent on genetic, environmental, age, and other as yet unknown factors (Longo and Panda, 2016; Radak et al., 2019; Kepp et al., 2020; Perez-Matos & Mair, 2020). Pharmacological reagents that target autophagy and mitophagy can be tested and optimized against age related diseases and promote healthy aging (Galluzzi et al., 2017; Piskovatska et al., 2019). Compounds targeting the mitochondria, for example, MitoQ, and SS-31 have been explored for their potential for healthspan enhancement (Tate et al., 2019; Young & Franklin, 2019; Whitson et al., 2020). Senolytics have been shown to attenuate cell-free mitochondrial DNA release, which then decreases the detrimental immune responses associated with aging (Iske et al., 2020). Mitochondria not only can be targeted to improve metabolism and healthspan, but also can generate the mitochondrial-derived peptides (MDPs) that can regulate metabolism and health (Reynolds et al., 2020).

Advancement of hygiene, food and environmental safety, health care, and a better understanding of aging biology and aging interventions are at such a stage that it has been suggest that the individuals who will reach the age of 150 are already living (https://www.stevenaustad.com/). It is still not clear why age is the single most important risk factor for many diseases including but not limited to: cancer, cardiovascular and neurodegenerative diseases. This is a new exciting era in which enabling technologies can provide exciting new insights into the mechanisms and biology of aging. It is likely that research linking aging to metabolism and redox regulation will feature prominently in the next decade. The grand challenge is then to understand the networks linking metabolism, and redox biology to cell aging and organismal aging and to target specific metabolic and redox networks for the promotion of a healthy lifespan.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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The Challenge of Unlocking the Biological Secrets of Aging

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LIFESPAN VS. HEALTHSPAN

We are currently facing particular challenges related to demographic change. People are reaching very long lives, and the average lifespan has increased considerably since the mid of the twentieth century. The predictions are that citizens older than 65 will increase from 18% of the current population to 28% in 2060. Moreover, citizens with more than 80 years old will increase from 5 to 12% during the same period, becoming as numerous as the young population in 2016 (Christensen et al., 2009).

However, the increase in lifespan has led to a decrease in healthspan, i.e., the period of life free from serious chronic diseases and disabilities (Christensen et al., 2009; Crimmins, 2015). This suggests a situation characterized by an increase in age-related disability and dependency, which will have an impact not only on the well-being and quality of life of the affected people but also on the sustainability of health systems (Murray and Lopez, 2013).

This scenario constitutes the real challenge: unlocking the biological secrets of aging to understand better this process, that will allow to develop adequate interventions to increase not only life but also healthspan and diminish the medical, economic, and social issues associated with old people.

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THE PROCESS OF AGING

Aging is a very complex process, and therefore there are many definitions to describe it depending on the field involved. From a biological point of view, "aging is a progressive sequence of age-related, widespread, more-or-less common changes observed in every individual of a given species" (Harman, 1988). It is characterized by four postulates (Strehler, 1985; Vina et al., 2007). It is universal: it must occur in all individuals of a species; intrinsic: endogenous factors cause it, although exogenous factors can modulate it; progressive: changes must occur progressively during the lifespan, from early adulthood to the old ages; and deleterious: it arranges negative consequences for the individual.

Aging is not a disease: it is a physiological process that differs from disease because the disease is selective (not universal), intrinsic and extrinsic (not only intrinsic), discontinuous (not progressive), and reversible.

Aging starts early in life, after the development of the organism. It implies that, during many years, many exogenous factors can influence it (accelerators or decelerators of the rate of aging). It may be different in the different individuals, leading to the heterogeneous distinctive of aging. Not all individuals age at the same rate, nor do all organs of the same individual. The complexity of the aging process is the reason for the grand challenge of unlocking its biological secrets.

THEORIES OF AGING

As aging is multifaceted, many theories are trying to explain the fundamental biological processes underneath it. In 1990 Medvedev claimed that there are more than 300 theories of aging, and the number continues to increase (Medvedev, 1990). This is the natural consequence of the very rapid progress in our understanding of biological phenomena and the application to gerontological research of many new approaches and methods. Almost every major discovery in cell and molecular biology has spawned a new family of aging theories or new advanced versions of older theories (Vina et al., 2013). This same author also commented that the task of reviewing theories of aging has become much more difficult and that a large number of these theories are very selective or outdated. On the other hand, Vijg affirms that some old hypotheses from the beginnings of gerontological science made possible the great scientific revolution in the understanding of aging that is now witnessing (Vijg, 2000). The author agrees with these views and Medvedev's conclusion that the expectation that a truly unified or single-cause theory of aging will emerge is unrealistic. And it is generally accepted that all the pieces of the aging puzzle are not yet available (Troen, 2003). However, we believe that it is possible to offer preliminary solutions to this problem by integrating several complementary theories, classical and modern, which offer logical explanations of the changes occurring in the fundamental levels of biological organization (Vina et al., 2007). In fact, many authors have proposed a unified theory of aging (Kelly, 2011; Barja, 2019).

Thus, we can affirm that there are many theories to explain the aging phenomenon, and even today is not known for sure what the main causes underlying aging are.

SEARCHING FOR GOOD MODELS OF AGING

Aging research can be conducted in many *in vivo* models, which have their own benefits and limitations. Hence, the use of yeasts (*Saccharomyces cerevisiae*), nematodes (*Caenorhabditis elegans*), fruit flies (*Drosophila melanogaster*), short-living fishes (*Nothobranchius furzeri*), rodents (mice and rats), dogs, and non-human primates is common in aging studies and the selected model depends on the objective of the study. **Table 1** shows the model's strengths and limitations in aging research (adapted from Folch et al., 2018).

Models shown in **Table 1** are those not genetically modified, but of course, many other models are based on genetic modifications of a specific protein or a protein set that are developed to investigate their role. For example, the Arf/p53 mice model which lives longer demonstrated that p53 is involved in longevity (Matheu et al., 2007). Moreover, there are also models developed for studying aging-related disorders such as cardiovascular, bone, or neurodegenerative disease (Santulli et al., 2015). Finally, some models have been developed to simulate frailty, a clinical syndrome common in the elderly (Howlett and Rockwood, 2014; Santulli et al., 2015), which is based on the decline of their functional capacities with age. One of the best examples of frailty mouse models is the interleukin-10 knockout mouse model since it develops an age-related decline in skeletal muscle strength and similar inflammation and weakness pattern to frailty compared to control mice (Walston et al., 2008).

Animal models have been, are, and will be essential for studying the biological insights of the aging process and developing appropriate interventions. However, successful translation to humans is intricate. It constitutes a challenge and requires several careful considerations, including a proper choice of the animal model, systematic experimental designs, and information integration from bench to bedside.

BIOMARKERS OF AGING

A biomarker of aging is a biological parameter of an organism that either alone or in some multivariate composite will, in the absence of disease, better predict biological age and functional capacity at some late age than will chronological age (Baker, 1988). The requirements that a biomarker of aging should include are to change progressively with age, to refer to parameters relevant to health and longevity, to be minimally invasive, to be relatively easy to determine, and to be reproducible.

Most of the biomarkers are related to processes and pathways associated with the different theories of aging. As pointed out some years ago by Lopez-Otin et al. (2013), parameters related to the nine hallmarks of aging should be good candidates to be biomarkers of aging, if they meet the requirements mentioned before. Those hallmarks are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis (and autophagy), deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. Moreover, oxidative stress-related parameters have been also proposed as good candidates as they meet all the requirements for being aging biomarkers (Borras et al., 2003; Ingles et al., 2014). Other good candidates are those parameters related to the so-called process of inflammaging and the immune system function. It is known to decline with age, and many scientists have proposed them as possible aging biomarkers (Martinez De Toda et al., 2016; Fougere et al., 2017; Franceschi et al., 2018).

Although many processes underlying aging are known, and there are many proposed biomarkers of aging related to these processes, there are no fully reliable aging biomarkers. Probably the best approximation to a trustful aging biomarker is that based on a set of several markers. For example, the "epigenetic clock" based on a DNA methylation dataset has enabled accurate age estimates for any tissue across the entire life course (Horvath and Raj, 2018). Indeed, reprogramming the epigenetic clock resets the aging clock, and the organism rejuvenates (Rando and Chang, 2012).

Certainly, a challenge is developing trustful aging biomarkers because it allows a better knowledge of the aging process, and at the same time, it enables developing appropriate interventions to delay aging and promote successful aging.

TABLE 1 | Animal model's strengths and limitations in aging research.

Models of Aging	Strengths	Limitations
Caenorhabditis elegans	Short lifespan. Fast evaluation of interventions. Low costs.	Invertebrate model. Low translationality to humans.
Drosophila melanogaster	Short lifespan. Fast evaluation of interventions. Low costs.	Invertebrate model. Low translationality to humans.
Saccharomyces cerevisiae	Short lifespan. Fast evaluation of interventions. Low costs.	Invertebrate model. Low translationality to humans.
Nothobranchius furzeri	Appropriate for evaluation of interventions	Organs are quite different from those in humans.
Senescence prone inbred strains	Appropriate for evaluation of interventions	Significant differences at a pharmacokinetic level. Lifespan extension could vary between rodent's genders.
Genetically heterogeneous (HET) mouse model	Developed by the National Institute on Aging interventions testing program as the most adequate mammal mice model in aging	Significant differences at a pharmacokinetic level. Lifespan extension could vary between rodent's genders.
Rodent models of progeria	Reduction in time, labor and costs for lifespan studies, as well as the ability to target accelerated aging to specific organs.	Effects of premature aging, not aging itself. Significant differences at a pharmacokinetic level.
Non-human primate models of aging	Best extrapolation of the results to our species.	Expensive. Long time to obtain results.

CONCLUDING REMARKS

We are currently facing particular challenges related to an increased lifespan. However, the increase in lifespan has led to a decrease in healthspan. This scenario constitutes the real challenge: unlocking the biological secrets of aging to understand better this process, that will allow developing adequate interventions to increase not only life but also healthspan and diminish the medical, economic, and social issues associated with old people.

"The molecular mechanisms of aging" specialty section is delved into the basic mechanisms involved in aging to help better understand the aging process. Molecular mechanisms of aging play an integral and interdisciplinary role in modern science and include significant advances in areas including, but not limited to, biomarkers of aging, senescence, altered proteostasis, autophagy, chromosomal alterations, redox system dysregulation, nutrient sensing modulations, genetic and epigenetic changes, mitochondrial energy collapse, intercellular communication alterations, stem cell function dysregulation, and extracellular vesicles alterations.

AUTHOR CONTRIBUTIONS

CB wrote the sections of the manuscript and contributed to manuscript revision, read, and approved the submitted version.

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frontiers in Aging

BRIEF RESEARCH REPORT
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Defining the Dynamic Regulation of O-GlcNAc Proteome in the Mouse Cortex---the O-GlcNAcylation of Synaptic and Trafficking Proteins Related to Neurodegenerative Diseases

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O-linked conjugation of ß-N-acetyl-glucosamine (O-GlcNAc) to serine and threonine residues is a post-translational modification process that senses nutrient availability and cellular stress and regulates diverse biological processes that are involved in neurodegenerative diseases and provide potential targets for therapeutics development. However, very little is known of the networks involved in the brain that are responsive to changes in the O-GlcNAc proteome. Pharmacological increase of protein O-GlcNAcylation by Thiamet G (TG) has been shown to decrease tau phosphorylation and neurotoxicity, and proposed as a therapy in Alzheimer's disease (AD). However, acute TG exposure impairs learning and memory, and protein O-GlcNAcylation is increased in the aging rat brain and in Parkinson's disease (PD) brains. To define the cortical O-GlcNAc proteome that responds to TG, we injected young adult mice with either saline or TG and performed mass spectrometry analysis for detection of O-GlcNAcylated peptides. This approach identified 506 unique peptides corresponding to 278 proteins that are O-GlcNAcylated. Of the 506 unique peptides, 85 peptides are elevated by > 1.5 fold in O-GlcNAcylation levels in response to TG. Using pathway analyses, we found TG-dependent enrichment of O-GlcNAcylated synaptic proteins, trafficking, Notch/Wnt signaling, HDAC signaling, and circadian clock proteins. Significant changes in the O-GlcNAcylation of DNAJC6/AUXI, and PICALM, proteins that are risk factors for PD and/or AD respectively, were detected. We compared our study with two key prior O-GlcNAc proteome studies using mouse cerebral tissue and human AD brains. Among those identified to be increased by TG, 15 are also identified to be increased in human AD brains compared to control, including those involved in cytoskeleton, autophagy, chromatin organization and mitochondrial dysfunction. These studies provide insights regarding neurodegenerative diseases therapeutic targets.

Keywords: OGA, O-GlcNAc, thiamet G, mass spectrometry, PICALM, DnaJC6

INTRODUCTION

First discovered in the 1980s, the O-GlcNAcylation of proteins is now widely accepted as playing a key role in the regulation of diverse biological processes integrating nutrient availability and cellular stress (Zachara and Hart, 2004; Love and Hanover, 2005; Slawson et al., 2006; Paruchuri and Zachara, 2011; Darley-Usmar et al., 2012; Bond and Hanover, 2013; Alonso et al., 2014; Hardivillé and Hart, 2014; Marsh et al., 2014; Wani et al., 2015). System biology studies implicate perturbation of O-GlcNAc transferase (OGT) as one of the nine genes identified to impact transcriptome regulation in similar fashion as caloric restriction/intermittent fasting (Hou et al., 2016). Changes in O-GlcNAc levels have been linked to aging and a number of neurodegenerative diseases (Zhang, 2020; Mueller et al., 2021). For example, several investigators reported age-dependent changes of overall protein O-GlcNAcylation in animal models (Fülöp et al., 2008; Liu et al., 2012). However, there is substantial controversy as to the outcome and mechanisms involved. In human postmortem brains, total O-GlcNAcylation has been found to increase in Alzheimer's disease (AD) brains in detergent-insoluble fractions (Griffith and Schmitz, 1995). A recent more comprehensive proteomics study has reported dynamic changes in protein O-GlcNAcylation in AD patients with 12 peptides decreased and 119 peptides increased compared to controls (Wang et al., 2017).

In animal models, pharmacological approaches to increase O-GlcNAc levels, such as inhibition of O-GlcNAcase (OGA) by Thiamet G (TG), increased tau O-GlcNAcylation and decreased tau phosphorylation and associated neurodegenerative phenotypes (Yuzwa et al., 2008; Yuzwa et al., 2012; Yuzwa et al., 2014; Zhu et al., 2014; Hastings et al., 2017). These putative beneficial effects of increasing tau O-GlcNAc modification suggest a potential utility of OGA inhibitors such as TG or its derivatives, for treatment of AD. However, we have shown that in aged rats overall brain O-GlcNAc levels were increased compared to younger animals (Fülöp et al., 2008), suggesting a dysregulation in O-GlcNAcylation occurs with aging, which is a well-established risk factor for AD. Moreover, we have demonstrated that increases in O-GlcNAc levels *in vivo* impaired learning and memory (Taylor et al., 2014).

In the context of PD, the impact of O-GlcNAcylation is also complex. For example, recent studies have reported that increased O-GlcNAc modification of α-synuclein blocks its aggregation and toxicity *in vitro* (Marotta et al., 2012; Marotta et al., 2015; Lewis et al., 2017). However, in a *C. elegans* model of neurodegeneration, the OGA inactive mutant, which is associated with increased O-GlcNAc levels, has been shown to increase proteotoxicity (Wang et al., 2012). Interestingly, we found a significant increase in overall protein O-GlcNAcylation levels in postmortem brain specimens of PD patients compared to control (Wani et al., 2017). Our recent studies have shown that in primary neurons, pharmacologically increased neuronal O-GlcNAc levels by TG enhanced MTOR phosphorylation, suppressed autophagy and increased α-synuclein accumulation (Wani et al., 2017). Considering these interesting and complex observations, a better understanding of the O-GlcNAc proteome and the effects of OGA inhibition are needed.

There are several approaches that have been used to catalog the brain O-GlcNAcylated proteins and O-GlcNAc proteomes. For example, wheat germ agglutinin (WGA)-based lectin weak affinity chromatography (LWAC) was used to enrich O-GlcNAc-modified peptides that were then identified by mass spectrometry. The first of these studies revealed 145 unique O-GlcNAcylated peptides in mouse brain postsynaptic preparations (Vosseller et al., 2006). Using the same enrichment approach with mouse synaptic membrane preparations and high pH reverse phase chromatography, one study identified over 1750 O-GlcNAcylation sites in 676 proteins (Trinidad et al., 2012). The O-GlcNAcylation process does not appear to be indiscriminate or always reciprocal to phosphorylation. For example, 46 Ser or Thr phosphorylation sites were identified on the microtubule associated protein tau which is extensively phosphorylated in animal models of AD, yet no tau O-GlcNAcylation was found (Trinidad et al., 2012). In addition, it appears that OGT and kinases do not favor the same amino acid residues and statistically the O-GlcNAcylation and phosphorylation sites are independent of one another. Indeed, there are at least 11-fold more phosphorylation than O-GlcNAcylation sites in synaptosomes (Trinidad et al., 2012). However, in cerebrocortical tissue using a different method (CEPC, see the next paragraph), it was found that O-GlcNAcylation sites were either at or near known phosphorylation sites, suggesting mutual exclusion (at the same site) or synergy (at the same site or those in close proximity) (Alfaro et al., 2012). Another study identified 463 O-glycopeptides corresponding to 122 proteins, though the modifications included both O-GlcNAcylation and other types of glycosylation (Trinidad et al., 2013). A similar approach was utilized in a more recent study, which identified 926 and 919 O-GlcNAcylated proteins in substantia nigra - ventral tegmental area, and the striatum, respectively (Lee et al., 2020). Although without any information regarding the sites of modification, 26 proteins were increased and 11 proteins with decreased O-GlcNAcylation levels in the dopaminergic neuron-specific *Oga* knockout mice compared to control (Lee et al., 2020).

Using a chemoenzymatic approach with an unnatural UDP substrate and Y289L GalT enzyme, coupled with biotinylation and avidin enrichment, an earlier study identified 25 O-GlcNAcylated proteins from the rat forebrain (Khidekel et al., 2004). Later studies used similar chemoenzymatic approaches coupled with photochemical cleavage (CEPC) to enrich O-GlcNAc peptides, which enhances analytical sensitivity by tagging the peptides with a photochemical cleavable-biotin probe and have a low false rate due to the specific enzymatic chemical reactions. One such study discovered 274 O-GlcNAcylated proteins from cerebrocortical tissues of 1 year old normal and 3xTg AD mice (Alfaro et al., 2012). Several synaptic, cytoskeletal, transcriptional regulators, membrane proteins, and notably extracellular domains of membrane proteins, were identified (Alfaro et al., 2012). A comprehensive meta-analysis of 378 human studies including

those focusing on overall protein O-GlcNAcylation with an O-GlcNAc antibody based approach, those using focused approaches on individual proteins, and those with mass spectrometry-based omics approach suggested that the overall human O-GlcNAcome may include more than 5000 proteins and 7,000 sites (Wulff-Fuentes et al., 2021). Due to the nature of this meta-analysis, published articles with false positives were also included (Wang et al., 2014; Ma et al., 2021a). The critically curated O-GlcNAcAtalas (https://oglcnac.org) reports 4554 proteins with unambiguous O-GlcNAc sites in different species.

In this study, we used quantitative isobaric tandem mass tag (TMT) labelling combined with CEPC method to enrich O-GlcNAcylated peptides in the mouse brain coupled to tandem mass spectrometry (LC-MS/MS) to identify proteins that are modified by O-GlcNAcylation in the mouse cortex, and to compare O-GlcNAcylation levels between mice injected with saline and with the OGA inhibitor Thiamet G (TG). We identified 506 unique O-GlcNAc peptides corresponding to 278 O-GlcNAcylated proteins in the mouse cortex from both groups. Of these, 67 were not previously identified in the two most extensive studies of the O-GlcNAcome in the mouse cerebrocortical tissues (Alfaro et al., 2012) and in the human mid frontal gyrus region (Wang et al., 2017). Furthermore, O-GlcNAc levels of 155 out of the 506 unique peptides were significantly changed ($p < 0.05$) by systemic administration of TG. O-GlcNAc modification of 85 peptides corresponding to 65 proteins increased at >1.5-fold in the TG treatment group compared to controls. Network analysis of these changes revealed that pathways involved in synaptic function are enriched.

Of note, we found that O-GlcNAcylation of DNAJC6/AUXI was increased 12-fold in response to OGA inhibition. DNAJC6 has been shown to be important for clathrin uncoating (Edvardson et al., 2012), and recent studies identified mutations in DNAJC6, which appear to be responsible for juvenile and early onset PD (Edvardson et al., 2012; Köroğlu et al., 2013; Elsayed et al., 2016; Olgiati et al., 2016). Of relevance to AD, phosphatidylinositol binding clathrin assembly protein (PICALM) (which is a highly validated risk factor in late-onset AD (Harold et al., 2009)) is O-GlcNAcylated and its O-GlcNAcylation was increased by 2-fold in response to OGA inhibition. These findings suggest that DNAJC6 and PICALM O-GlcNAcylation are potential contributing factors to neurodegenerative diseases.

RESULTS AND DISCUSSION

Quantitative Profiling of O-GlcNAcylated Proteins and Peptides in Mouse Cortex

To detect the proteins undergoing dynamic changes in O-GlcNAcylation we injected mice (i.p.) with saline or the OGA inhibitor TG (10 mg/kg) which inhibits O-GlcNAc removal and elevates overall levels of protein O-GlcNAcylation. Three hours after injection, we harvested half of the cortex for western blot (n = 3 each control versus TG) and half for proteomics analyses to assess the global impact on protein O-GlcNAcylation. Using hemi-cortical extracts, we found that there was a significant increase of the overall protein O-GlcNAcylation as detected by the CTD110.6 antibody (**Figure 1A**). There were limited numbers of bands (<10 bands) that were visible and ~5 bands exhibited higher intensity in the TG group compared to the saline group. The patterns obtained by using western blot methodology for O-GlcNAc proteins are strongly representative of the high abundance of some O-GlcNAcylated proteins and, as is evident from the mass spectrometry analysis, a small subset of the total O-GlcNAc proteome.

Using the chemoenzymatic photocleavage (CEPC) approach coupled with TMT labeling and LC-MS/MS analyses (Alfaro et al., 2012; Wang et al., 2014; Wang et al., 2017), we identified 506 unique O-GlcNAcylated peptides corresponding to 278 proteins in cortex samples from mice administered either saline or TG (**Supplementary Table S1**). **Figure 1B** shows the distribution of the number of O-GlcNAcylated sites among the detected proteins. Some proteins have multiple O-GlcNAcylated sites whereas the majority have only one or two detected sites of modification. Notably, the proteins Bassoon (BSN) and Piccolo (PCLO) have 53 and 43 O-GlcNAcylated sites, respectively (**Figure 1B**).

To assess which biological pathways are affected by protein O-GlcNAcylation, we used ToppGene tools (Shannon et al., 2003) for pathway enrichment analysis and Cytoscape (Asanuma et al., 2006; Guo et al., 2018; Ji et al., 2019) to visualize the genetic network. We found that the identified 278 O-GlcNAcylated proteins are highly enriched for cellular component morphogenesis, cytoskeleton organization, synapse organization, and cell projection morphogenesis (**Figure 1C**). Furthermore, the identified O-GlcNAcylated proteins are associated with key neuronal cellular components including synapse and dendrite.

Synaptic and Trafficking Proteins Are Highly Enriched in the 65 O-GlcNAcylated Proteins Regulated by TG Administration

Compared to the saline group, out of the 506 peptides there were 85 unique peptides (corresponding to 86 O-GlcNAcylation sites) that have O-GlcNAcylation levels of >1.5 fold increase after TG (p-value <0.02, **Supplementary Table S2**). Interestingly, five peptides (corresponding to four proteins) exhibited lower O-GlcNAcylation levels ($p < 0.02$) including peptides from EMAL1, PCLO, HCFC1 and WNK1, and one PCLO and one WNK1 peptide have <50% O-GlcNAcylation after TG compared to saline (**Supplementary Table S1**). These results support the hypothesis that OGA activity is an important regulator of O-GlcNAcylation in the brain.

We next searched on databases to see if the 86 sites that exhibited 1.5-fold increase in O-GlcNAcylation levels have been reported or whether they are near phosphorylation sites. We found that 53 sites are known O-GlcNAcylation sites as reported on curated O-GlcNAcAtlas database (https://oglcnac.org/) (Ma et al., 2021b), 28 are reciprocal phosphorylation sites, 75 have proximal (±10 amino acids) phosphorylation sites, and 14 are not

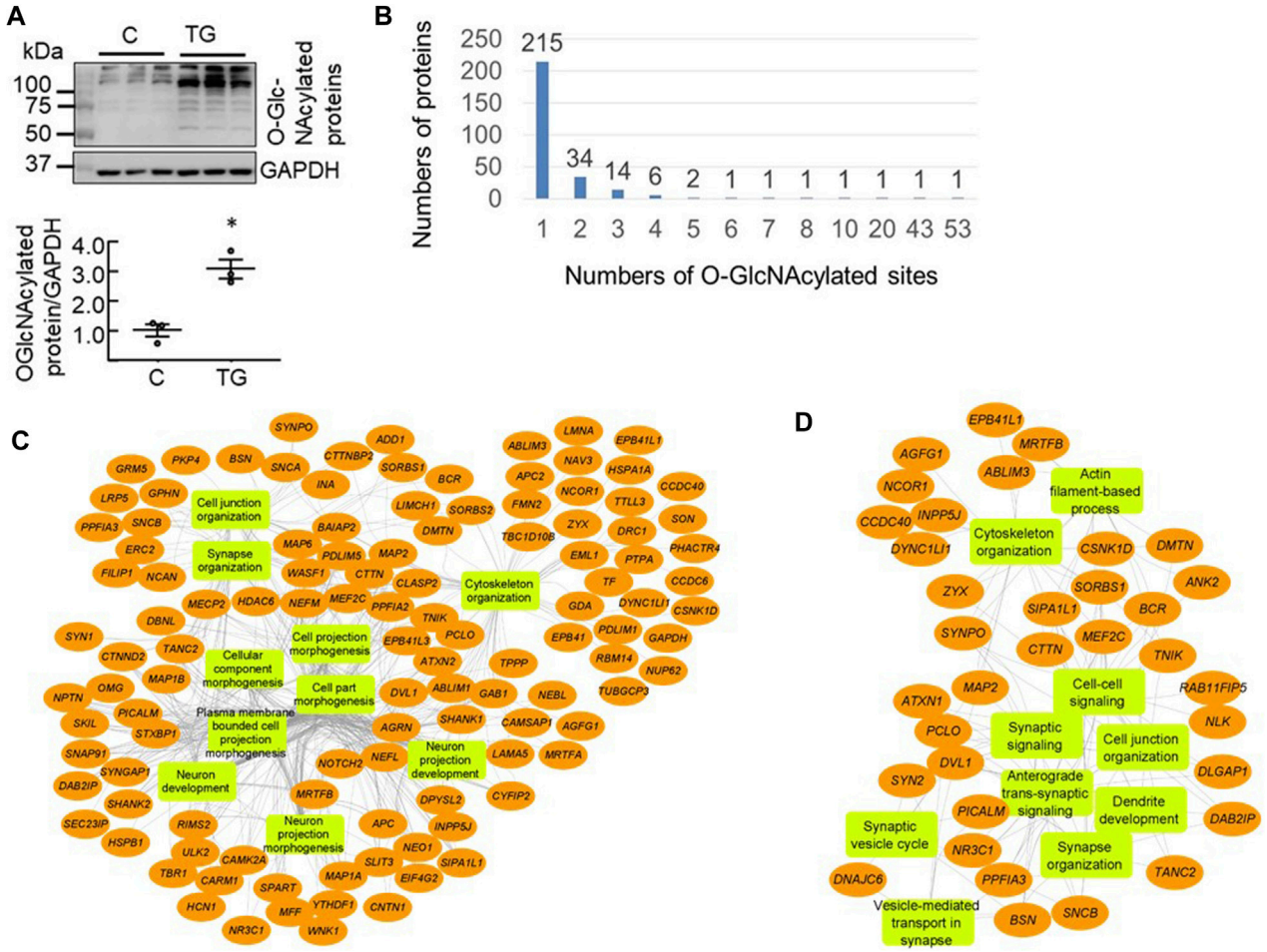

FIGURE 1 | Proteomics analysis of mouse cortical O-GlcNAcome **(A)** Western blot analyses with cortical protein extracts demonstrated that 3 h post i. p. Thiamet G (TG) injection at 10 mg/kg, there was an increase of overall levels of protein O-GlcNAcylation (quantified using the entire lane) as detected by the CTD110.6 antibody ($n = 3$, $^*p < 0.05$ Student t-test) **(B)** Using isobaric tandem mass tag labelling combined with CEPC method we identified 506 peptides corresponding to 278 proteins that were O-GlcNAcylated in the mouse cortex ($n = 3$ for control and $n = 2$ for TG). Of these, 215 proteins have one modification site, 34 proteins have two sites, 14 proteins have 3 sites, six proteins have four sites, two proteins have five sites, and one protein each has 7, 8, 10, 20, 43, and 53 sites **(C, D)** Network visualization of the top 10 Gene Ontology biological process terms enriched for the total 278 O-GlcNAcylated proteins identified in both Saline and TG groups (C), and the 65 proteins that exhibited significant increases in O-GlcNAcylation levels following acute TG treatment **(D)**.

near known phosphorylation sites as reported on PhosphoSitePlus® database (https://www.phosphosite.org/) (Hornbeck et al., 2015) (**Supplementary Table S2**).

As the 85 peptides correspond to 65 proteins, we performed pathway analyses of these 65 proteins that exhibited increased O-GlcNAcylation levels following TG treatment. We found that these proteins are primarily enriched in cytoskeleton organization, vesicle-mediated transport in synapse, and synaptic organization (**Figure 1D**), and that not all pathways that are linked to the 278 O-GlcNAcylated proteins are equally affected by TG.

Using protein-protein interaction data and information from multiple sources including OMIM and specialized databases related to neurodegenerative diseases (AlzGene, PDGene and SZGene) stored in the ToppGene knowledgebase (Shannon et al., 2003), we found that the 65 proteins are highly enriched for synaptic signaling, neuron projection, membrane trafficking, transcription, cytoskeletal protein binding, and circadian regulation (**Figure 2A**, **Supplementary Table S3**). Of the 65 proteins, 10 of them have cytoskeletal protein binding functions, nine proteins are associated with membrane trafficking, 18 proteins are associated with cell projection and neuron development, and 13 proteins are associated with chromatin and transcription factor binding functions.

The 10 O-GlcNAcylated proteins in the group of cytoskeletal protein binding include: MAP2, ABLIM3, SYNPO, TNKS1BP1, RAB11FIP5, RTN3, SNCB, EPB41L1, DMTN and ANK2. Most of these are also in the groups of protein-protein interactions at synapses, and synaptic signaling. The combined group of cytoskeletal protein binding, protein-protein interactions at synapses, and synaptic signaling have ~20 proteins. Microtubule-

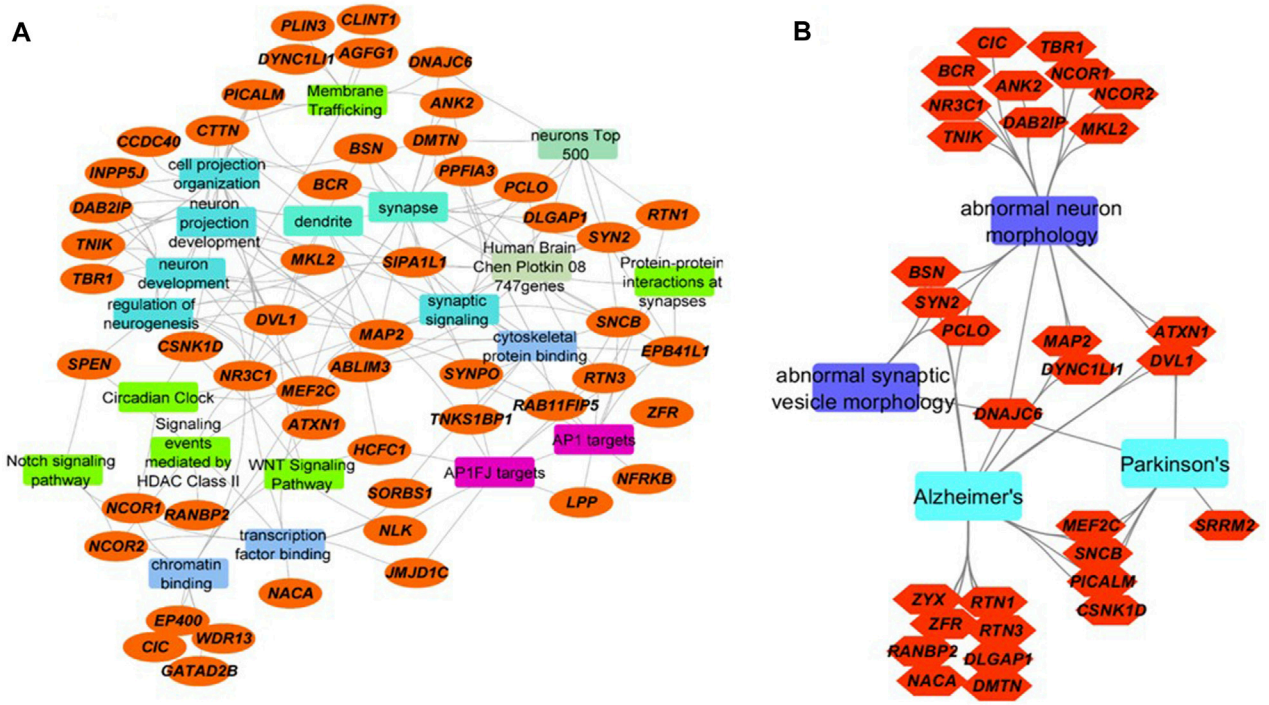

FIGURE 2 | Integrated network visualizations of the involvement of the 65 proteins with significantly increased O-GlcNAcylation levels following TG administration in cellular functions, biological processes, and phenotypes **(A)** We found that 52 of the 65 proteins (orange ellipses) are involved in key pathways (green rectangles), biological processes (aqua blue rectangles), cellular functions (blue rectangles), and cellular components (jungle green rectangles). Several are targets for AP-1 family of transcription factors (purple rectangles), and many of them are in Allen Brain Atlas's top 500-gene list and Chen-Plotkin's 747 genes that are related to human frontotemporal lobar degeneration (other colored rectangles) **(B)** 31 of the 65 proteins (orange hexagons) are involved in abnormal neuron morphology and abnormal synaptic morphology, as well as neurodegenerative diseases including AD and PD (sky blue and violet rectangles). Eight proteins are involved both in abnormal neuron or synaptic vesicle morphology and AD. Three are involved both in abnormal neuron or synaptic vesicle morphology and Parkinson's disease. Seven proteins are shared between AD and PD (including DNAJC6, MEF2C and PICALM).

associated protein 2 (MAP2) is proposed to be important for microtubule stabilization and may be involved in dendritic morphology (Dehmelt and Halpain, 2005). Actin binding LIM protein 3 (ABLIM3) is involved in cell-cell contacts and modulates learning and memory (Guo et al., 2018). Synaptopodin (SYNPO) may be involved in actin organization, cell motility and autophagy (Asanuma et al., 2006; Ji et al., 2019). Tankyrase-1-binding protein (TNKS1BP) regulates actin cytoskeleton rearrangement and is dysregulated both in blood and brain in AD (Ohishi et al., 2017; Pang et al., 2017). RAB11-interacting protein 5 (RABFIP5) is important for protein trafficking via binding to RAB11 GTPase (Grant and Donaldson, 2009). Reticulon 3 (RTN3) may be involved in regulation of ER autophagy, BACE1 activity and amyloid β production (Murayama et al., 2006; Grumati et al., 2017). β-synuclein (SNCB) is important for synaptic function and a negative regulator of α-synuclein aggregation (Clayton and George, 1999; Hashimoto et al., 2001). Band 4.1-like protein 1 (EPB41L1) may be involved in cortical actin cytoskeleton organization and its missense mutations were found in nonsyndromic intellectual disability patients (Hamdan et al., 2011). Dematin actin binding protein (DMTN) also plays a role in actin dynamics (Khan et al., 2008). Ankyrin 2 (ANK2) interacts with PINK2/Parkin-target proteins and ANK2 variants are associated with the risk of PD (Auburger et al., 2019).

O-GlcNAcylated proteins involved in membrane trafficking include: 1) DNAJC6, which is important for clathrin uncoating (Edvardson et al., 2012); 2) phosphatidylinositol binding clathrin assembly protein (PICALM), which can interact with LC3 and regulate amyloid precursor protein cleaved C-terminal fragment (APP-CTF) degradation (Tian et al., 2013); 3) cytoplasmic dynein one light intermediate chain 1 (DYNC1LI1) whose knockdown led to dendritic atrophy (Liu et al., 2016); 4) Perilipin 3 (PLIN3, tail-interacting protein 47 kDa, TIP47, Mannose-6-Phosphate Receptor-Binding Protein 1, M6PRBP1), which is required for endosome-to-Golgi transport and delivery of lysosomal hydrolases from the Golgi to endosomes (Diaz and Pfeffer, 1998; Carroll et al., 2001); 5) clathrin interactor 1 (CLINT1), which may be involved in clathrin coated vesicles and *trans*-Golgi network-endosome trafficking (Pimm et al., 2005); 6) ArfGAP with FG repeats 1 (AGFG1), which may be involved in both nucleocytoplasmic transport and clathrin-mediated endocytosis, 7) ankyrin2 ANK2, 8) casein kinase I

TABLE 1 | O-GlcNAcylation sites of DNAJC6 (AUXI), PICALM, alpha-synuclein (SYUA), and MAP2 identified in the present study in comparison with the two cortical O-GlcNAcome studies.

Protein	Gene name	Study	Subject	Accession number	O-GlcNAcylation sites/regions
AUXI	DNAJC6	Present study	Mouse	Q80TZ3	S591
—	—	Alfaro et al	Mouse	Q80TZ3	V600-R640
—	—	Wang et al	Human	Not detected	Not detected
PICAL	PICALM	Present study	Mouse	Q7M6Y3	S453
—	—	Alfaro et al	Mouse	Q7M6Y3	K349-K388, S443-R461
—	—	Wang et al	Human	Q13492	S565, T291
SYUA	SCNA	Present study	Mouse	O55042	T53, T72, T81
—	—	Alfaro et al	Mouse	O55042	T53, T64, T72
—	—	Wang et al	Human	P37840	T54, T64, T72, T81
MTAP2	MAP2	Present study	Mouse	P20357	S361, S472, S788
—	—	Alfaro et al	Mouse	P20357	T466-K483, V776-K795
—	—	Wang et al	Human	P11137	S199, T241, T335, S502, T522, T851, S1082

delta (CSNK1D), and 9) cortactin (CTTN), which are all thought to be involved in membrane trafficking.

Proteins Relevant to Neurodegenerative Diseases Are Dynamically Regulated by O-GlcNAcylation

Many of the 65 proteins with O-GlcNAcylation levels changed after TG administration are involved in abnormal neuron morphology and synaptic vesicle morphology, AD and PD pathogenesis (**Figure 2B**, **Supplementary Table S3**). Relevant to PD, α-synuclein in this study is O-GlcNAcylated at T72, T53 and T81 (**Table 1**), sites previously identified (Alfaro et al., 2012; Levine et al., 2019). As discussed above, nine proteins involved in membrane trafficking exhibit dynamic O-GlcNAcylation with O-GlcNAcylation levels sensitive to systemic TG administration. The most highly regulated O-GlcNAcylated protein by TG is DNAJC6 (with 12-fold difference between TG and saline injected mouse cortex). Mutations of DNAJC6 have been found to be responsible for juvenile and early onset PD (Edvardson et al., 2012; Köroğlu et al., 2013; Elsayed et al., 2016; Olgiati et al., 2016). Relevant to AD, one of the 65 peptides is phosphatidylinositol-binding clathrin assembly protein (PICALM) (showing a 2-fold difference between TG and saline injected mouse cortex), which is associated with late-onset AD in genome wide association studies (Harold et al., 2009). Both DNAJC6 and PICALM are involved in protein trafficking. Furthermore, MAP2 is associated with both AD and PD and is increased by 2-fold by TG at S472, but not S361 nor S788. The O-GlcNAcylation sites for DNAJC6 and PICALM that have changed O-GlcNAcylation levels by TG were previously known, while the MAP2 O-GlcNAcylation site S472 which is changed by TG was a previously un-identified site. While DNAJC6 and PICALM O-GlcNAcylation have not been investigated thoroughly, O-GlcNAcylation of MAP2 related protein tau has been extensively studied with various TG administration strategies (i.v., i.c.v., i.p., and drinking water), on multiple S/T residues, especially in transgenic mice overexpressing human tau or tau mutations (Mueller et al., 2021).

Related to synaptic pathway function, acute TG injection in rats impairs learning and memory (Taylor et al., 2014), in rat hippocampal slices induces long-term depression (Taylor et al., 2014), in rodent hippocampal slices attenuated epileptiform activity at CA3-CA1 synapses as well as spontaneous CA3 pyramidal cell activity (Stewart et al., 2017), and in rodent hippocampal slices decreased $GABA_AR$ currents and intrinsic excitability (Stewart et al., 2020). O-GlcNAcylation has been shown to regulate cellular clock oscillation, the level and stability of clock regulators (Li et al., 2013), as perturbation of the sleep/wake cycle occurs in neurodegenerative disease patients, O-GlcNAcylation of circadian regulators may be important for disease pathogenesis and/or therapeutic considerations (Austad et al., 2021).

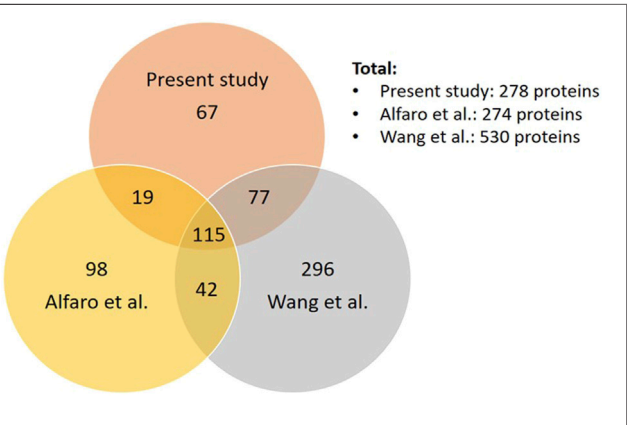

FIGURE 3 | Comparison of our dataset to the two most extensive datasets of cortical O-GlcNAcome. Venn diagram showing the number of proteins that are unique or common among our current datasets, Alfaro et al. (Alfaro et al., 2012) and Wang et al. (Wang et al., 2017).

Unique and Common Proteins Identified Compared to Two Extensive Cortical O-GlcNAcome Studies

Comparing our dataset with the two most extensive studies on O-GlcNAcome in the mouse (274 proteins) and human (530 proteins) cortex (Alfaro et al., 2012; Wang et al., 2017), we found

that there are a total of 714 proteins identified combining our current and the two prior studies, with 115 in common and 67 unique proteins in our study (**Figure 3**, **Supplementary Figure S1**). The 115 proteins shared among the three datasets include cell junction proteins BSN and PCLO, which is expected since they are abundant and possess numerous O-GlcNAcylation sites **(Supplementary Figure S1A)**. There are also proteins involved in cellular component organization and morphogenesis processes, such as cytoskeleton proteins ABLM2, ANK2, and MAP1B; synaptic proteins SYN1, SYN2, SYNPO, SYUA, and SYUB. Among synaptic signaling proteins, ULK2 and STAT3 are regulators of autophagy. The 67 proteins unique to the present study are also involved in cellular component organization and morphogenesis processes. These include synapse organization proteins such as DAB2IP, NPTN, and CTTNBP2 and localized in dendrite and microtubule components **(Supplementary Figure S1B)**. They also include HDAC6 a histone deacetylase; signaling proteins INP4A and INPP; and LRP5 which is involved in endocytosis, mTOR signaling and AD. DNAJC6/AUXI has been shown to be O-GlcNAcylated in both mouse studies but not in human postmortem mid frontal gyrus O-GlcNAcome. Potential autophagy regulators including MAP1A, MEF2C, NUP62, TFE3 and TPPP are present in both the human O-GlcNAcome and our current study but not in the prior mouse cerebrocortical tissue study. Although the Alfaro study also used mouse cortex, they used 1 year old female mice (1 each WT and 3xTg AD model). In contrast, our study used male C57BL/6J strain 2–3 each WT with Saline or Thiamet G injection at 2 months of age. Additionally, the TMT labeling might change the ionization efficiency of some peptides. These differences likely contribute to the differences in the O-GlcNAc proteome observed between our studies.

We also compared the 65 proteins with O-GlcNAcylation levels significantly increased by TG with the 81 proteins with O-GlcNAcylation levels significantly changed in human AD brains versus control brains (Wang et al., 2017). We found 15 proteins in common, which are ANK2, EPN4, EMSY, EP400, P66B, HCFC1, JHD2C, GCR, PRC2C, QRIC1, RBP2, MINT, SYNPO, ZFR, and ZYX (**Supplementary Table S4**). All of these proteins showed higher O-GlcNAcylation levels in AD brains compared to matched controls except for SYNPO, which showed lower levels in AD brains, and has been suggested to be involved in actin organization, cell motility and autophagy (Grant and Donaldson, 2009; Grumati et al., 2017). In addition, six of these 15 proteins are enriched in chromatin organization process, which include EP400, HCFC1, JMJD1C, EMSY, GATAD2B, and NR3C1. HCFC1 loss of function resulted in brain development deficits (Jolly et al., 2015). EP400 is required for oligodendrocyte survival (Elsesser et al., 2019). NR3C1 is a nuclear receptor of the steroid/thyroid/retinoic acid superfamily and whose methylation has been found to be involved in stress response (Oberlander et al., 2008). JMJD1C is involved in mitochondrial dysfunction in response to MPP+ in SH-SY5Y cells (Wang et al., 2016). GATAD2B is associated with neurodevelopmental disorders and intellectual disabilities (Willemsen et al., 2013; Kaur et al., 2019; Trubnykova et al., 2019; Ueda et al., 2019; Shieh et al., 2020a; Shieh et al., 2020b; Vera et al., 2020). There are 50 proteins that are changed with their O-GlcNAcylation by TG in our study, but the change of their O-GlcNAcylation in AD compared to control has not reached significance. This include MAP2, the abundance of which is increased in AD (with log2~05 and q~0.01) but not O-GlcNAcylation, and PICALM (Wang et al., 2017). Whether the O-GlcNAcylation of these proteins is linked to AD phenotypes and whether modulating their O-GlcNAcylation after AD pathogenesis can impact disease progression is an important question for future studies.

SUMMARY

In this study we first assessed the impact on total levels of protein O-GlcNAcylation in response to TG using the lower resolution western blotting techniques and found a robust 3-fold increase in protein O-GlcNAcylation (**Figure 1**). Using the isobaric tandem mass tag labelling combined with chemoenzymatic photocleavage (CEPC) method we examined the O-GlcNAc proteome with and without the i. p administration of the inhibitor of OGA TG (Wang et al., 2017). This approach allowed us to characterize the O-GlcNAc proteome and those sensitive to acute inhibition of OGA. We identified 506 O-GlcNAcylated peptides, and of these, 85 peptides corresponding to 65 proteins were at least 50% more O-GlcNAcylated from the TG treated mouse cortex compared to saline control. Of those sensitive to OGA inhibition the O-GlcNAcylated proteins were highly enriched for synaptic signaling, neuron projection, membrane trafficking, transcription, and cytoskeletal protein binding. The most regulated protein DNAJC6 is associated with the risk for PD, raising the question whether its O-GlcNAcylation may contribute to pathogenesis. MAP2, which is associated with both AD and PD, and PICALM which is associated with AD are also regulated by TG by 2-fold.

One important feature of this study is that the observed increase in the levels of O-GlcNAcylation of 85 peptides corresponding to 65 protein (5 peptides corresponding to four proteins exhibited decreased levels of O-GlcNAcylation) occurred within only 3 h of TG administration. These peptides/proteins are likely the most sensitive to changes of OGA/OGT activities and/or substrates in response to intracellular and extracellular stimuli. It is also of interest that pharmacological inhibition of OGA affects a sub-O-GlcNAc proteome suggesting the potential for selectivity in its mechanisms of action. Despite that these highly dynamic changes are likely to be the "first responders." they have been often ignored in studies of pathologies associated with altered levels of O-GlcNAcylation. The numbers of O-GlcNAc modification sites on a given protein range from 0 to over 50 on different proteins. The implications for the impact of the O-GlcNAcylation pathway on the network of pathway in the brain is still poorly understood. Determining how different O-GlcNAcylated proteins in different regions of the brain impact neuronal function and survival will be important for understanding cognition and neurodegeneration. Future

studies will define the role of O-GlcNAcylation in neuronal function as well as in aging and neurodegenerative disease pathogenesis.

DATA AVAILABILITY STATEMENT

The raw datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Massive.ucsd.edu with accession: MSV000088053. The data will also be available through ProteomeXchange with accession: PXD028204.

ETHICS STATEMENT

The animal study was reviewed and approved by UAB IACUC.

AUTHOR CONTRIBUTIONS

SW, XO, WW contributed to data generation. VH, MJ, BC, AJ, W-JQ, JC, VDU and JZ contributed to data analyses. VH, VDU and JZ wrote the manuscript. All authors edited and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fragi.2021.757801/full#supplementary-material

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Adipocyte-Specific Ablation of PU.1 Promotes Energy Expenditure and Ameliorates Metabolic Syndrome in Aging Mice

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Objective: Although PU.1/Spi1 is known as a master regulator for macrophage development and function, we have reported previously that it is also expressed in adipocytes and is transcriptionally induced in obesity. Here, we investigated the role of adipocyte PU.1 in the development of the age-associated metabolic syndrome.

Methods: We generated mice with adipocyte-specific PU.1 knockout, assessed metabolic changes in young and older adult PU.1$^{fl/fl}$ (control) and AdipoqCre PU.1$^{fl/fl}$ (aPU.1KO) mice, including body weight, body composition, energy expenditure, and glucose homeostasis. We also performed transcriptional analyses using RNA-Sequencing of adipocytes from these mice.

Results: aPU.1KO mice have elevated energy expenditure at a young age and decreased adiposity and increased insulin sensitivity in later life. Corroborating these observations, transcriptional network analysis indicated the existence of validated, adipocyte PU.1-modulated regulatory hubs that direct inflammatory and thermogenic gene expression programs.

Conclusion: Our data provide evidence for a previously uncharacterized role of PU.1 in the development of age-associated obesity and insulin resistance.

Keywords: Spi1/PU.1, thermogenesis, energy expenditure, inflammation, insulin resistance, obesity, aging, adipocyte

Abbreviations: Adipoq, adiponectin; GTT, glucose tolerance test; HCT, high confidence transcriptional targets; ITT, insulin tolerance test; LPS, lipopolysaccharide; MPO, Mammalian Phenotype Ontology; PGC-1, PPARG coactivator 1; RPA, Reactome Pathway Analysis; SPP, Signaling Pathways Project.

INTRODUCTION

Systemic insulin resistance is a major global public health concern. Although it impacts numerous metabolic organs including skeletal muscle, liver and adipose tissue, evidence indicates that adipose tissue is one of the primary origins of systemic insulin resistance. It is well documented, for example, that obesity induces chronic low-grade inflammation in adipose tissue, a key event leading to systemic insulin resistance and metabolic syndrome (Hotamisligil, 2017). During obesity, adipose tissue secretes elevated levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and MCP-1 (Gregor and Hotamisligil, 2011). Obesity-associated adipose inflammation, for instance, is characterized by increased infiltration of macrophages (Weisberg et al., 2003; Xu et al., 2003) and other immune cells (Liu et al., 2009; Nishimura et al., 2009; Winer et al., 2009; Ohmura et al., 2010), and macrophage infiltration has been shown to be stimulated by obese adipose tissue expression of Ccl2/MCP-1 (Kamei et al., 2006; Weisberg et al., 2006). Although the role of macrophages is well-established in the inflammatory processes accompanying insulin resistance in adipose tissue, accumulating evidence implicates adipocytes as active participants in these processes.

PU.1 (encoded by *Spi1*) is a member of the ETS family of transcription factors (Klemsz et al., 1990) with historically well-characterized roles in the development of myeloid and lymphoid lineages, in particular macrophages and granulocytes (Scott et al., 1994; McKercher et al., 1996; Iwasaki et al., 2005). Functions for PU1.1 have also been established in lineage establishment of microglia (Kierdorf et al., 2013), dendritic cells (Carotta et al., 2010), and osteoclasts (Tondravi et al., 1997). Additionally, numerous lines of evidence cast macrophage PU.1 in a central role in the coordination of inflammatory transcriptional programs in macrophages. For example, macrophages lacking PU.1 are deficient in lipopolysaccharide (LPS) induction of *Tlr4*, *Ptgs2* (encoding COX-2), *Tnf*, *Il1b*, *Il6*, and *Ccl2/MCP-1* (Karpurapu et al., 2011). In contrast to the volume of studies on PU.1 function in macrophages however, relatively little is known about its role in adipocytes. We previously reported that PU.1 was expressed in adipocytes, and that adipose tissue PU.1 expression was greatly increased in mouse models of obesity (Wang and Tong, 2008). Consistent with its pro-inflammatory role in macrophages, we found that depletion of PU.1 in cultured adipocytes led to decreased reactive oxygen species (ROS) production, increased insulin sensitivity, and reduced expression of signature obese adipose tissue cytokines, including TNF-α, IL-1β, and IL-6 [20]. In addition, Lackey and colleagues have recently reported that mice with adipocyte-specific PU.1 knockout showed improved insulin sensitivity on a high fat diet, without any difference in body weight (Lackey et al., 2019).

The age-associated metabolic syndrome may have characteristics different from that caused by diet-induced obesity (Bapat et al., 2015). Here, we investigated the adipocyte-specific functions of PU.1 in mice, particularly during aging. We found that male mice with adipocyte-specific ablation of PU.1 had elevated energy expenditure, and were protected against age-associated obesity, with increased insulin sensitivity and increased glucose tolerance. Mechanistically, we performed validated informatics analyses that connect PU.1-modulated transcriptional hubs to the observed physiological changes.

EXPERIMENTAL PROCEDURES

Animals Experiments

$PU.1^{fl/+}$ mice, containing loxP sites flanking exon 5 of the *Spi1* gene, were obtained from Dr. Stephen Nutts (Polli et al., 2005). These mice are on C57BL/6 genetic background and were used successfully for tissue specific knockout. Mice with adipocyte-specific knockout of PU.1 (aPU.1KO) were generated by crossing Adiponectin-Cre mice (Adipoq-Cre, The Jackson Laboratory) (Eguchi et al., 2011) with PU.1 floxed mice. Mice were housed with littermates in cages and were fed on a standard chow diet (Lab Diet 5053; Purina Mills). All procedures used in animal experiments were approved by the Institution of Animal Care and Use Committee at Baylor College of Medicine. Mice were maintained under conditions of controlled temperature (~75°F) and illumination (12-hour light/12-hour dark cycle, 6 am to 6 pm) with free access to water.

Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

Mice were fasted for overnight (GTT) or 4 h (ITT) and received an intraperitoneal injection of D-glucose (2 g/kg) or insulin (1 IU/kg). Blood glucose was measured by glucose meter (TrueTest Glucose Meter and Strips) from the tail vein before and at 15, 30, 60, and 120 min after the bolus glucose or insulin injection.

Body Composition Measurement and Calorimetry Experiment

Mice body composition was measured using the EchoMRI-100™ quantitative NMR instrument (Echo Medical Systems).

Indirect Calorimetry

Indirect calorimetry was measured using a computer-controlled, open-circuit system (Oxymax System) as part of an integrated Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, United States). Mice were singly housed in individual cages in adaptation for 3 days, followed by measurement for 4 days. On the last day, food was removed, and mice were fasted for 6 h. Oxygen consumption (VO2) and carbon dioxide production (VCO2) were measured for each chamber and calculated by Oxymax software (v. 5.9). Energy expenditure was calculated as $EE = 3.815 \times VO2 + 1.232 \times VCO2$. The basal energy expenditure was calculated based on the three lowest EE time points during the fasting period.

Adipose Tissue Fractionation

Epididymal fat pads from mice were minced in Krebs-Ringer phosphate buffer and digested with 1 mg/ml collagenase type I (Worthington Biochemical) at 37°C for 1 h as described in the literature (Wang and Tong, 2008). Digested tissue was filtered through a nylon mesh and centrifuged at 500 rpm for 10 min. The top layer (adipocyte fraction) was collected. Proteins were extracted from adipocyte fraction for Western blot analysis. RNA was also prepared from adipocyte fraction for RNA-sequencing and Real-time PCR analyses.

RNA-Sequencing

Epididymal adipocytes RNA were extracted from isolated adipocyte fraction of three control and three aPU.1KO mice using the RNeasy Lipid Tissue Mini Kit (QIAGEN). The cells were homogenized in 1 ml QIAzol lysis reagent and centrifuged at 12,000 g for 10 min at 4°C. The lysates under the lipid layer were transferred to a fresh tube and extracted with 200 µl chloroform, centrifuged at 12,000 g for 15 min at 4°C. The upper aqueous phase was transferred out and mixed with 1 volume of 70% ethanol. The samples were then applied to RNeasy Mini spin column and centrifuged at room temperature for 15 s at 8,000 g. The columns were washed once with 700 µL Buffer RW1, and twice with 500 µL Buffer RPE. The RNA samples were eluded with 30–50 µL RNase-free water. RNA-sequencing was performed by Novogene Corporation Inc. (Sacramento, CA).

RNA-Seq Analysis

Sequencing was performed on adipocyte samples from $PU.1^{fl/fl}$ (control) and $PU.1^{fl/fl}$-AdipoqCre (PU.1 knockout) mice, with three replicates in each group. Sequencing reads were quantified using Salmon with the option-validateMappings for a more sensitive mapping scheme (Patro et al., 2017). Transcript-level counts were summed to the gene-level for differential expression analysis using DESeq2 (Love et al., 2014).

Real-Time PCR

Adipocytes were isolated from mice gonadal adipose tissue. Total RNA of adipocytes was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). qRT-PCR reactions were performed using iTaq Universal SYBR Green in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The ΔCt method (2–ΔCt) was used to calculate the relative mRNA expression level of each gene. Specific gene expression was normalized to 18S ribosomal RNA. Sequences of primers used for real-time PCR were as follows: MCP-1-F 5′-GAAGGAATGGGTCCAGACAT-3′ and MCP-1-R 5′-ACGGGTCAACTTCACATTCA-3′; TNFα-F 5′-ACGGGTCAACTTCACATTCA-3′ and TNFα-R 5′-CTGATG AGAGGGAGGCCATT-3′; UCP-1-R 5′-AGCCACCACAGA AAGCTTGTCAAC-3′ and UCP-1-R 5′-ACAGCTTGGTAC GCTTGGGTACTG-3′; PGC1α-F 5′- GTCAACAGCAAAAGC CACAA-3′ and PCG1α-R 5′-TCTGGGGTCAGAGGAAGAGA-3′; 18S ribosomal RNA-F 5′-AACGAGACTCTGGCATGCTAA CTAG-3′ and 18S ribosomal RNA-R 5′-CGCCACTTGTCCCTC TAAGAA-3′. The expression levels of genes of interest were normalized by the levels of 18S RNA.

Western Blot Analysis

Cells were lysed in lysis buffer (50 mM Tris, 50 mM KCl, 20 mM NaF, 1 mM Na_3VO_4, 10 mM EDTA, 1% NP-40, 1 mM PMSF, 5 µg/ml leupeptin, pH 8.0). Protein concentration was determined with BCA protein assay kit (Pierce, Rockford, IL). Twenty microgram proteins of each sample were separated by SDS-PAGE and electro-transferred to nitrocellulose membrane for immunoblot analysis. The following antibodies were used: anti-PU.1 (Santa Cruz Biotechnology, Santa Cruz, CA; sc-352, 1: 500), anti-a-tubulin (Sigma, St. Louis, MO; T5168, 1:100,000), HRP-conjugated anti-mouse (Bio-Rad, Richmond, CA; 170–6,516, 1:30,000), anti-rabbit (Bio-Rad, 170–6,515, 1: 30,000. The SuperSignal West Pico Chemiluminescent kit (Pierce, Rockford, IL) was used as substrates.

3T3-L1 Adipogenesis Consensome

Full details of the methods and principles underlying consensome analysis can be found in the original publication (Ochsner et al., 2019). Briefly, five transcriptomic datasets (GSE2192, GSE60745, GSE14004, GSE12929, GSE20696) generated from 3T3-L1 cells treated with a standard adipogenic cocktail were organized into appropriate contrasts comparing gene expression levels at different time points to day 0 expression levels. These contrasts were then processed by the consensome pipeline implemented in R as previously described. For each transcript, the algorithm counts the number of experiments where the significance for differential expression is < 0.05, then generates the binomial probability, referred to as the consensome *p*-value (CPV), of observing that many or more nominally significant experiments out of the number of experiments in which the transcript was assayed, given a true probability of 0.05. Genes were ranked firstly by CPV, then by geometric mean fold change (GMFC). The 3T3-L1 adipogenesis consensome was validated against the GSEA adipogenesis Hallmark gene set using a hypergeometric test implemented in R as described in the results. The consensome analysis code has been deposited in the SPP GitHub account at https://github.com/signaling-pathways-project/ominer/.

High Confidence Transcriptional Target Intersection Analysis

Node and node family consensomes are gene lists ranked according to measures of the strength of their regulatory relationship with upstream signaling pathway nodes derived from independent publicly archived transcriptomic or ChIP-Seq datasets. In the case of ChIP-Seq datasets, the strength of the regulatory relationship is inferred from the mean ChIP-Atlas (Oki et al., 2018) MACS2 peak strength across available archived ChIP-Seq datasets in which a given pathway node is the IP antigen. In the case of transcriptomic datasets, the strength of the regulatory relationship is inferred from the frequency of significant differential expression of a given gene across independent experiments involving perturbation of a member

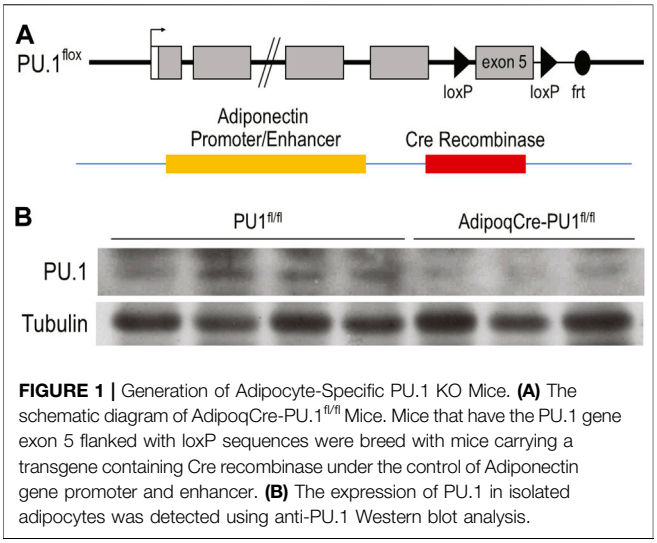


FIGURE 1 | Generation of Adipocyte-Specific PU.1 KO Mice. **(A)** The schematic diagram of AdipoqCre-PU.1$^{fl/fl}$ Mice. Mice that have the PU.1 gene exon 5 flanked with loxP sequences were breed with mice carrying a transgene containing Cre recombinase under the control of Adiponectin gene promoter and enhancer. **(B)** The expression of PU.1 in isolated adipocytes was detected using anti-PU.1 Western blot analysis.

of a given node family (Ochsner et al., 2019). Genes in the 95th percentile of a given node consensome were designated high confidence transcriptional targets (HCTs) for that node and used as the input for the HCT intersection analysis using the Bioconductor GeneOverlap analysis package implemented in R as previously described (Ochsner et al., 2020). For both consensome and HCT intersection analysis, p values were adjusted for multiple testing by using the method of Benjamini and Hochberg to control the false discovery rate as implemented with the p. adjust function in R, to generate Q values. Evidence for a transcriptional regulatory relationship between a node and a gene set was inferred from a larger intersection between the gene set and HCTs for a given node or node family than would be expected by chance after FDR correction ($Q < 0.05$). The HCT intersection analysis code has been deposited in the SPP GitHub account at https://github.com/signaling-pathways-project/ominer/.

Mammalian Phenotype Ontology Analysis

Genes mapping to MPO terms phenotypes were retrieved from MGI (Law et al., 2018). A hypergeometric test implemented in GraphPad Prism 7.0 was used to estimate the over-representation, relative to their distribution in all 691 nodes, of nodes encoded by MPO term-mapped genes among nodes with significant HCT intersections with aPU.1KO-induced or -repressed genes.

Statistics

The data are represented as the mean ± standard deviation. For GTT and ITT assays, statistical significance was determined using repeated measures two-way ANOVA with Sidak correction for multiple comparison (GraphPad Prism v 9.3). $p < 0.05$ was considered to be statistically significant. Pearson's correlation (PRISM software package v 7.0 (RRID: SCR_005375) was used to evaluate the correlation between differential expression values in the aPU.1KO v WT and those in the adipogenesis consensome.

RESULTS

Generation of Adipocyte-Specific PU.1 Knockout Mice

To investigate the systemic functions of aPU.1 *in vivo*, we generated mice with adipose-specific knockout of PU.1. Mice in which *Spi1* gene exon 5 was flanked by loxP sequences were bred with mice carrying a transgene containing Cre recombinase under the control of adiponectin (*Adipoq*) gene promoter and enhancer (**Figure 1A**). Mice with adipose-specific knockout of PU.1 (adiponectinCre-PU.1$^{fl/fl}$) and littermate control mice (PU.1$^{fl/fl}$) were used for the study. To confirm ablation of aPU.1 expression, we isolated the adipocyte fraction from gonadal adipose tissue. As shown in **Figure 1B**, PU.1 expression in the adipocytes of aPU.1KO mice was significantly down-regulated. We noticed that the PU.1KO adipocytes maintained a lower level of PU.1 expression. The Cre-LoxP mediated recombination might not be 100% efficient. Additionally, non-adipocytes with PU.1 expression, such as the macrophages, might contaminate the adipocyte fraction, likely in the form of lipid-laden foam cells or by sticking to adipocytes due to incomplete separation of cells to single cell suspension.

Phenotype of Young Adult aPU.1KO Mice

Young (4–5 months) aPU.1KO male (**Figure 2A**) or female (**Supplementary Figure S1A**) mice exhibited no difference in body weight, lean body mass or fat mass from floxed littermate controls. Moreover, glucose tolerance tests found no difference in glucose homeostasis (**Figure 2B** and **Supplementary Figure S1B**) between 4–5 months aPU.1 KO male or female mice and their floxed littermate controls. Using indirect calorimetry to measure energy expenditure however, we found that compared to wild-type littermates, male aPU.1KO mice had significantly higher average energy expenditure and basal energy expenditure under fasted and resting state (**Figure 2C**). This increase in energy expenditure was not contributed by increased physical activity, as there was no difference in ambulatory activity in these mice (**Figure 2D**). In female aPU.1KO mice, no difference of energy expenditure was observed (**Supplementary Figure S1C**).

Deficiency of Adipocyte PU.1 Protects Against Age-Associated Obesity and Glucose Intolerance

At 10–11 months of age, control male mice gained significantly more body weight than aPU.1KO mice (**Figure 3A**). Moreover, whereas lean body mass of aPU.1KO mice was indistinguishable from that of wild-type mice (**Figure 3A**), fat mass was significantly lower (**Figure 3A**), indicating that the difference of body weight was attributable primarily to a loss of adiposity. Fasted 10 months old aPU.1KO male mice exhibited significantly improved glucose tolerance compared with WT controls (**Figure 3B**) with significantly different area under the curve (21,657 ± 3,273 vs. 15,609 ± 2,697, $p = 0.006$). Older aPU.1KO mice also displayed higher insulin sensitivity (**Figure 3C**) with

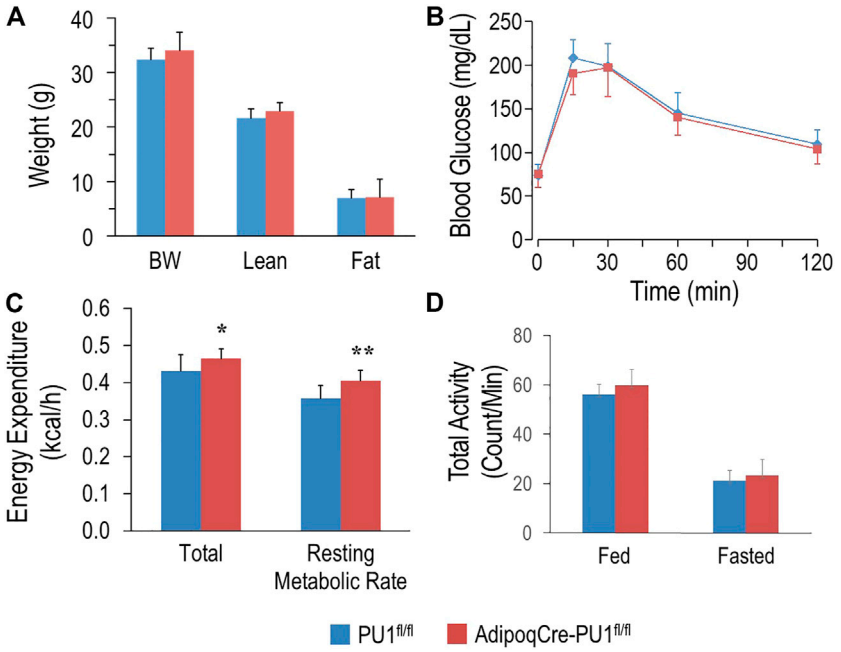


FIGURE 2 | Adipocyte PU.1 Deficiency Has No Effect on Body Weight, Body Composition and Glucose Tolerance in Young Adult Male Mice. **(A)** Body weight (BW) and body composition of young adult (4–5 months of age) male AdipoqCre-PU.1$^{fl/fl}$ mice (N = 8) and the control littermate PU.1$^{fl/fl}$ mice (N = 8). **(B)** For glucose tolerance test, mice (N = 8 vs. 8) were fasted overnight and injected with glucose. Blood glucose was then measured. **(C)** Energy expenditure was determined using indirect calorimetry in the integrated Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments) in 4–5 months old male AdipoqCre-PU.1$^{fl/fl}$ mice (N = 8) and the control littermate PU.1$^{fl/fl}$ mice (N = 8). **(D)** Total locomotor activity under fed or fasting condition. *p < 0.05, **p < 0.01.

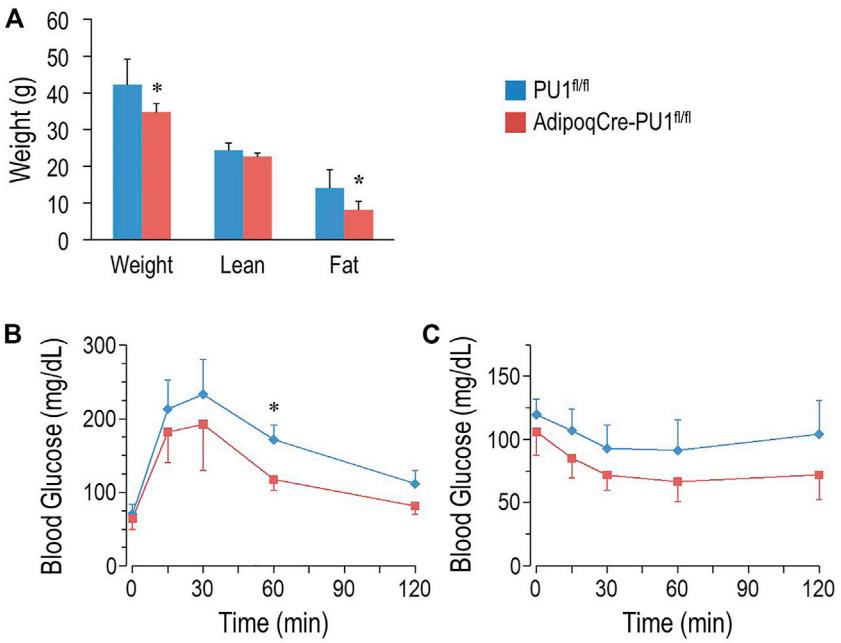
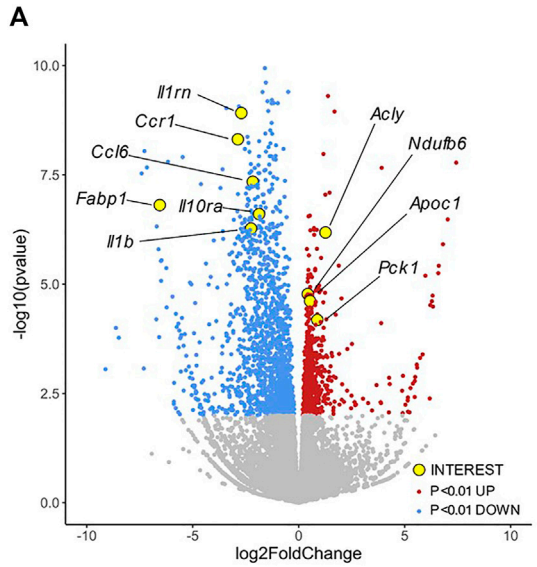


FIGURE 3 | Adipocyte PU.1 Deficiency Protects Mice Against Age-Associated Obesity and Insulin Resistance. **(A)** Body weight (BW) and body composition of 10 months old male AdipoqCre-PU.1$^{fl/fl}$ mice (N = 6) and the control littermate PU.1$^{fl/fl}$ mice (N = 6). **(B)** For glucose tolerance test, 10 months old male mice (N = 7 vs. 5) were fasted overnight and injected with glucose (2 g/kg body weight). Blood glucose was then measured. **(C)** For insulin tolerance test, 1 year old male mice (N = 8 vs. 7) were fasted for 4-hr and injected with insulin (1.0 IU/kg body weight). Blood glucose was measured afterwards. *p < 0.05.



B

Pathway Name	pValue	FDR
Up-Regulated Genes		
NR1H2 and NR1H3-mediated signaling	1.44E-05	4.91E-03
ChREBP activates metabolic gene expression	1.73E-05	4.91E-03
Glutathione conjugation	1.41E-04	2.59E-02
Activation of gene expression by SREBF (SREBP)	1.84E-04	2.59E-02
Transcriptional regulation of white adipocyte differentiation	4.79E-04	4.83E-02
PPARA activates gene expression	7.03E-04	4.83E-02
PKA-mediated phosphorylation of key metabolic factors	6.11E-03	2.87E-01
VEGF ligand-receptor interactions	7.90E-03	2.92E-01
Respiratory electron transport, ATP synthesis by chemiosmotic coupling, and heat production by uncoupling proteins	1.28E-02	3.07E-01
Down-Regulated Genes		
Extracellular matrix organization	3.36E-11	4.40E-08
Immune System	6.02E-10	3.94E-07
Neutrophil degranulation	1.31E-09	5.72E-07
Integrin cell surface interactions	2.75E-09	8.99E-07
Innate Immune System	6.69E-09	1.75E-06
Interleukin-10 signaling	7.93E-07	1.73E-04
Assembly of collagen fibrils and other multimeric structures	1.67E-06	3.12E-04
Platelet activation, signaling and aggregation	2.61E-06	4.26E-04
Interleukin-3, Interleukin-5 and GM-CSF signaling	2.11E-05	2.40E-03
Cytokine Signaling in Immune system	3.05E-05	2.58E-03
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	6.73E-05	4.38E-03

FIGURE 4 | RNA-sequencing Analysis of Gene Expression Changes in the aPU.1KO Mice. **(A)** Volcano plot showing gene expression changes in adipocytes isolated from the gonadal adipose tissue, with key genes of interest highlighted in yellow. **(B)** Gene ontology analysis of differentially expressed genes ($p < 0.01$) showing highly enriched pathways. The false discovery rate (FDR) is used for multiple hypothesis testing, with a standard cutoff of FDR<0.05 for significant pathway enrichment.

significantly different area under the curve (12,700 ± 2,463 vs. 9,677 ± 2,023, $p = 0.03$). Older female aPU.1KO mice were similar to WT controls with respect to body weight and fat mass (**Supplementary Figure S2A**). They did not present any improvement in glucose tolerance (**Supplementary Figure S2B**) or insulin sensitivity (**Supplementary Figure S2C**).

Transcriptomic Analysis Identifies Regulation of Diverse Metabolic Transcriptional Programs by PU.1

To investigate the metabolic phenotypes arising from loss of adipocyte PU.1, we performed RNA-sequencing of adipocytes isolated from epididymal adipose tissue of aPU.1KO and control male mice of more than 1 year of age. Significantly ($p < 0.01$) induced and repressed genes were visualized on a volcano plot (**Figure 4A**) and analyzed by Reactome Pathway Analysis (RPA; **Figure 4B**). RPA analysis of down-regulated genes displayed an enrichment of pathways involved in extracellular matrix and immune signaling, including interleukin-10 signaling (**Figure 4B**). Consistent with this, genes with well documented roles in these processes (*Il1rn*, *Il10ra*, *Il1b*, *Ccr1*, *Ccl6*) were highly repressed in aPU.1KO adipocytes (**Figure 4A**). In contrast, up-regulated genes were enriched for pathways involved in adipogenesis and lipogenesis, including *Cebpa*, *Srebf1*/SREBP, *Nr1h3*/LXRα, *Mlxipl*/ChREBP, *Acly*, *Apoc1* and *Pck1* (**Figures 4A,B**). These results were consistent with our previous findings of the roles of PU.1 in driving expression of inflammatory genes and transcriptional suppression of adipogenesis in cultured adipocytes (Wang and Tong, 2008; Lin et al., 2012).

Transcriptional Regulatory Network Analysis Illuminates Crosstalk of PU.1 With Adipogenic and Inflammatory Signaling Node Networks

We next set out to identify direct PU.1 transcriptional targets within the RNA-Seq dataset, and to identify evidence for PU.1-interacting proteins that coregulate expression of these transcriptional targets with PU.1. Signaling Pathways Project (SPP) consensomes are ranked consensus transcriptional signatures for signaling pathway nodes—receptors, enzymes, transcription factors and other nodes—computed from publicly archived omics datasets (Ochsner et al., 2019). As such, consensomes have value in identifying potential high confidence transcriptional targets (HCTs) for specific nodes or node families in a given biological system (Ochsner et al., 2020). To gain insight into members of the PU.1 transcriptional regulatory network, we next applied HCT intersection analysis to compute intersections between aPU.1KO-induced and aPU.1KO-repressed gene sets (FC > ±1.5, $p < 0.05$) and a library of over 700 mouse HCT gene sets derived from archived transcriptomic or ChIP-Seq consensomes as previously described (Ochsner et al., 2019; Ochsner et al., 2020). We interpreted the size and significance of these intersections as evidence for loss or gain of function of a given signaling node or node family in aPU.1KO adipocytes and, by inference, a functional relationship with aPU.1.

Figure 5A shows a heatmap displaying selected intersections between the aPU.1KO up and down gene sets and SPP mouse node family transcriptomic (upper table panel) and node ChIP-Seq (lower table panel) consensome HCTs. To assist in identifying candidate aPU.1-interacting nodes in WAT,

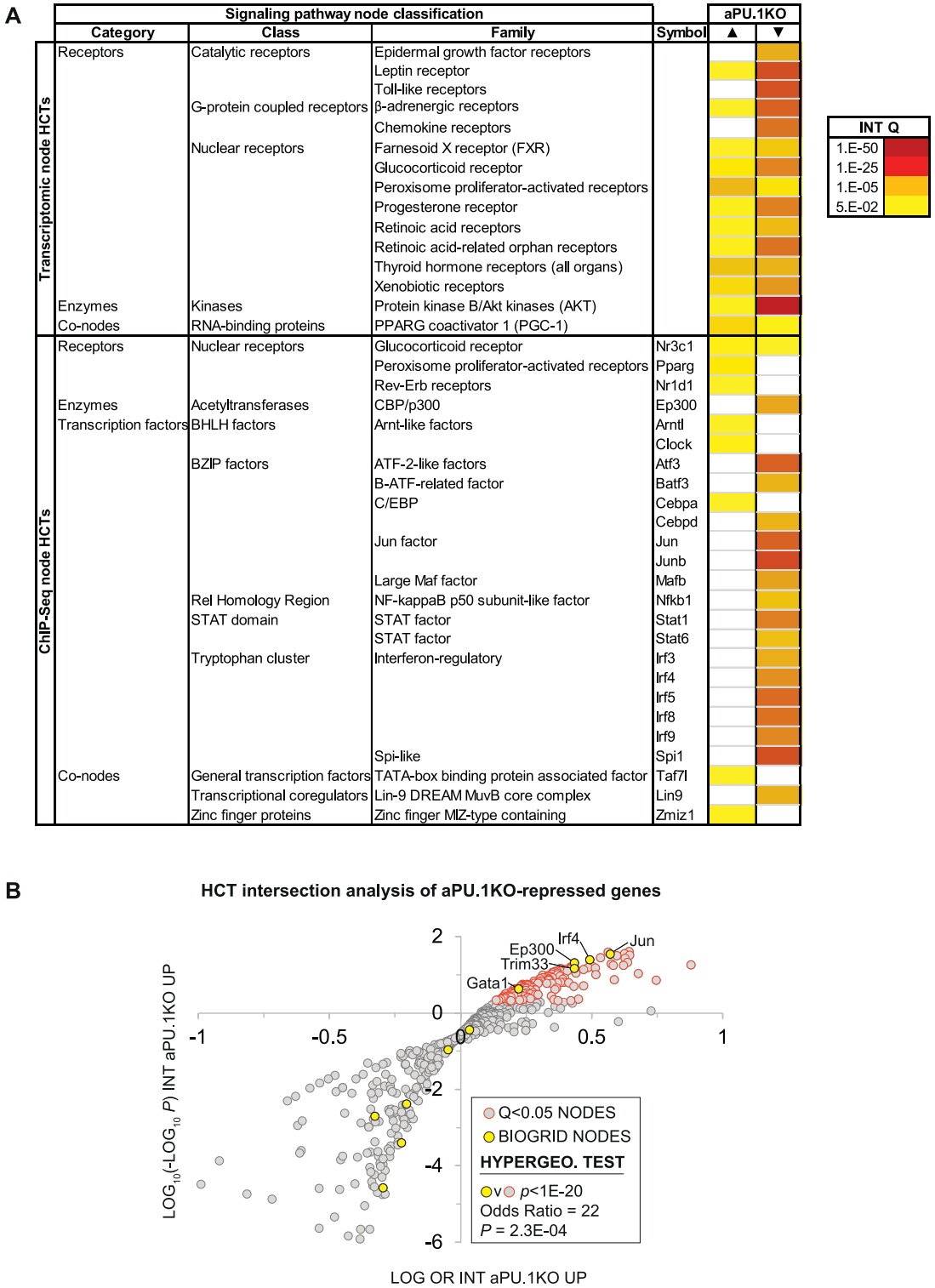


FIGURE 5 | High Confidence Transcriptional Target (HCT) Intersection Analysis Identifies PU.1-Modulated Metabolic and Inflammatory Transcriptional Regulatory Hubs. **(A)** HCT intersection Q-values (INT Q) for selected signaling pathway nodes or node families are indicated in the form of a heatmap. HCT intersection analysis was carried out as described in the Methods section. White cells represent Q > 5E-2 intersections. The intensity of the color scheme is proportional to the confidence of the intersection between high confidence transcriptional targets (HCTs) for a particular node and either **(i)** aPU.1KO-induced (▲) or **(ii)** aPU.1KO-repressed (▼) gene sets. Lower confidence (smaller Q) intersections are towards the yellow end of the spectrum and higher confidence (larger Q) intersections are towards the red end of the spectrum. Full numerical data are in **Supplementary Table S2**. **(B)** Scatterplot showing enrichment of known BioGRID-curated PU.1 interacting nodes among nodes that have the most significant intersections with aPU.1KO-repressed genes. Refer to the text for details.

Column P in **Supplementary Table S1** represents percentiles of the mean WAT expression levels of each node derived from our RNA-Seq dataset. As an objective validation of our analysis, we benchmarked it against a list of 16 proteins identified by BioGRID (Oughtred et al., 2021) as mouse PU.1 interacting proteins (**Supplementary Table S2**, column Q). **Figure 5B** shows a regulatory footprint plot, in which signaling nodes that have significant HCT footprints with aPU.1KO-repressed genes are indicated in orange outline. In this plot, nodes that have the most highly enriched and significant regulatory footprints are located towards the top right of the plot. Reflecting the reliability of our predictions, the 16 BioGRID-sourced PU.1-interacting nodes (yellow data points in **Figure 5B**) were enriched (OR = 22, p = 2.6E-04) among nodes with significant (Q < 1E-20) intersections with aPU.1-KO repressed genes (**Figure 5B**). In addition to the expected prominent footprint for PU.1 itself in the down-regulated gene set (Q = 1.9E-36), intersections with numerous canonical functional partners of PU.1 further validated our analysis. For example, robust intersections of aPU.1KO-repressed gene sets with transcriptomic HCTs for members of the toll-like (Wang et al., 2016), leptin (Dreyer et al., 2003), and chemokine (Ellis et al., 2010a) receptor and Protein kinase B/Akt (Rieske and Pongubala, 2001) families are consistent with previous studies implicating signaling through these nodes in PU.1 function (**Figure 5A**). Similarly, the increased energy expenditure of the aPU.1KO mice is reflected in the strong footprint within the aPU.1KO-induced gene set of transcriptomic HCTs for members of the PPARG coactivator 1 (PGC-1) family, which are well known mediators of metabolic control (Lin et al., 2005). Moreover, reflecting adrenergic stimulation of thermogenesis (Collins and Surwit, 2001) as well as repression of inflammatory cytokines (Ağaç et al., 2018), we observed β-adrenergic footprints in both aPU.1KO-induced (Q = 1E-4) and -repressed (Q = 7E-33) gene sets (**Figure 5A**).

Similarly, in the ChIP-Seq HCTs, robust intersections of aPU.1KO-regulated genes with HCTs for members of the interferon regulatory factor (IRF) (Marecki and Fenton, 2000), STAT (Nguyen and Benveniste, 2000), AP-1 (Steidl et al., 2006), Atf (Labzin et al., 2015), C/EBP and GATA (Wang and Tong, 2008) transcription factor families are consistent with canonical PU.1 biology. Given our previous report that PU.1 functions synergistically with GATA transcription factors to inhibit adipogenesis (Wang and Tong, 2008), we were also interested to note intersections of aPU.1KO-regulated genes with HCTs for GATA family members (**Figure 5B**). Congruent with our metabolic studies of aPU.1KO mice (**Figures 2**, **3**), aPU.1KO-induced genes contained footprints for several nodes with familiar roles in the context of whole body energy metabolism, including Pparg (3E-7), Nr3c1/GR (1.2E-05) and Cebpa (1.2E-5). Strikingly, four nodes with known roles in circadian rhythms (Clock (Debruyne, 2008), 1.2E-5; Nr3c1/GR (So et al., 2009), 1.2E-05; Nr1d1/REV-ERB (Ramakrishnan and Muscat, 2006), 1.1E-04 and Arntl/BMAL1 (Menet et al., 2014), 2.1E-04) also had appreciable intersections with the aPU.1KO-induced genes (**Figure 5A** and **Supplementary Table S2**).

In addition to corroborating canonical PU.1 biology, our analysis suggested the possibility of crosstalk of PU.1 with signaling nodes with which it has no previously established functional relationships. For aPU.1KO-induced genes these included transcriptomic HCT intersections with the PGC-1 family and ChIP-Seq HCT intersections for Zbtb11, Taf3 and Zfp57. Similarly, for aPU.1KO-repressed genes, we noted intersections with HCTs for members of the E2A-related factor family and the MuvB complex members Lin9 and E2f4 (**Supplementary Table S2**).

Some of the intersections may be attributable to transcriptional induction or repression of their encoding genes in the absence of PU.1. For example, the footprint for Cebpa in the PU.1-induced genes may be explained in part by the fact that its gene was upregulated in the knockout cells (*Cebpa* aPU.1KO v WT log2 FC = 0.35; **Supplementary Table S1**). Similarly, the footprints for Irf5, Irf8, Mafb and Mef2c in the aPU.1-repressed genes may reflect downregulation of the genes encoding these nodes in the knockout cells (aPU.1KO v WT log2 FCs: *Irf5*, −2.6; *Irf8*, −0.83; *Mafb*, −1.2; *Mef2c*, −0.95; **Supplementary Table S1**). The vast majority of nodes that had significant HCT intersections with aPU.1KO-regulated genes were however not transcriptionally regulated, suggesting that a post-translational mechanism contributes to their loss of function in the absence of PU.1. Collectively these data indicate repressive, protein-level cross-talk of PU.1 with adipogenic/lipogenic signaling nodes on the one hand, and on the other, positive cross-talk with distinct classes and families of inflammatory signaling nodes.

Consensome Analysis Identifies Broad Scale Direct Regulation by PU.1 of Adipogenic Gene Expression

We previously showed that PU.1 inhibits adipogenesis (Wang and Tong, 2008). With that in mind, our RNA-Seq analysis highlighted numerous aPU.1 KO-regulated genes that represented potentially novel, previously uncharacterized modulators of adipogenesis. We next wished to adopt a reduced-bias approach to explore this possibility in more detail. Using our previously-described consensome algorithm (Ochsner et al., 2019), we used five archived datasets (GSE2192, GSE60745, GSE14004, GSE12929, GSE20696) to generate a 3T3-L1 adipogenesis consensome, which ranks ~12,500 genes according to the frequency with which they are upregulated or downregulated across independent transcriptomic 3T3-L1 adipogenic datasets (**Supplementary Table S3** contains 3T3-L1 adipogenesis consensome genes with p < 0.05, n = 9,152). Within the 3T3-adipogenesis consensome we designated Q < 0.05 genes with a mean FC > 2 (log FC > 1) as 3T3-L1 adipogenesis induced confidence transcripts (3T3-ADIPICTs, n = 508; **Supplementary Table S3**, column I) and Q < 0.05 genes with a mean FC < 0.5 (log FC < −1) as adipogenesis repressed confidence transcripts (3T3-ADIPRCTs, n = 100; **Supplementary Table S3**, column J). We first benchmarked the 3T3-L1 adipogenesis consensome against a set of 200 genes designated as hallmark adipogenesis-induced transcripts by the GSEA (Subramanian et al., 2005) resource (GSEA

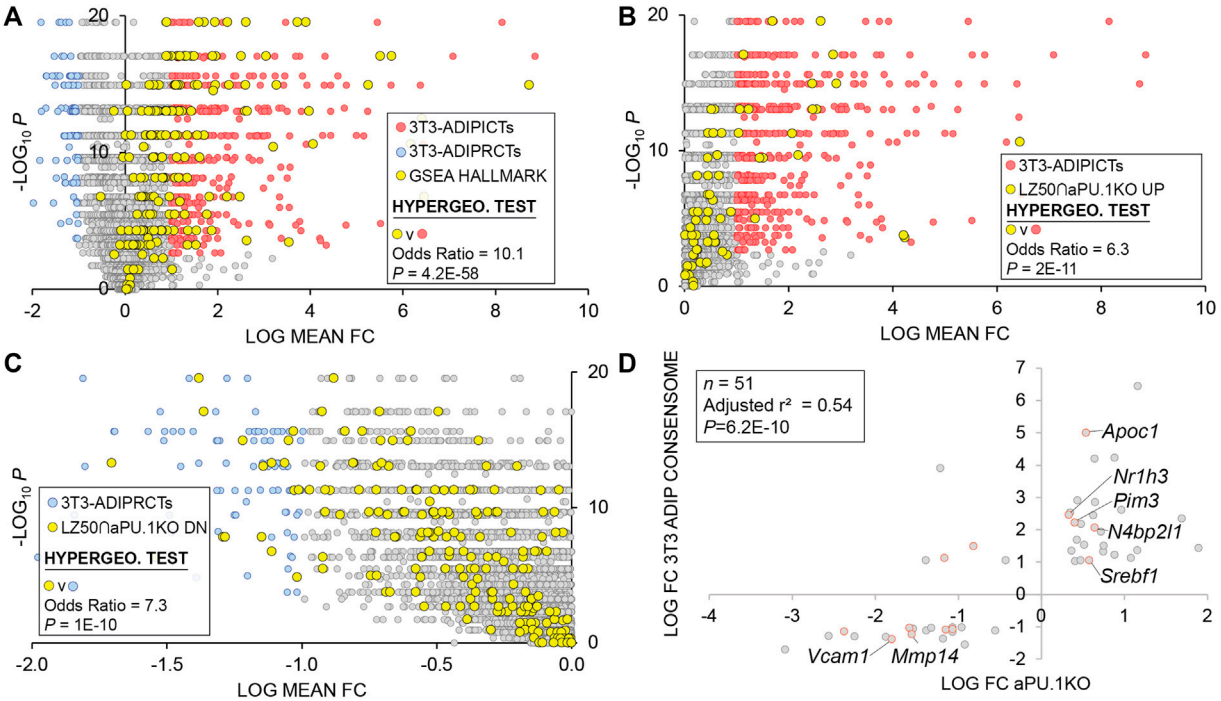


FIGURE 6 | 3T3-L1 Adipogenic Differentiation Consensome. The mouse 3T3-L1 adipogenesis transcriptomic consensome ranks mouse genes based on their discovery rates across five independent, publicly archived 3T3-L1 adipogenesis transcriptomic datasets. Hypergeometric test odds ratio and associated *p*-value are indicated in each panel. **(A)** Validation of the 3T3-L1 adipogenesis consensome against the GSEA adipogenesis Hallmark gene set. **(B)** Over-representation of aPU.1KO-induced LZ50 genes among 3T3-ADIPICTs. **(C)** Over-representation of aPU.1KO-repressed LZ50 genes among 3T3-ADIPRCTs. **(D)** Correlation between aPU.1KO log FC and adipogenesis log mean FC.

TABLE 1 | Selected PU.1 regulated genes with elevated rankings in the 3T3-L1 adipogenesis transcriptomic consensome.

	Target			aPU.1KO	3T3-ADIP CONSENSOME		
Category	**Class**	**Family**	**Symbol**	**LFC**	**LMFC**	**%ile**	***p***
aPU1.KO-induced							
Enzymes	Dehydrogenases	3 beta hydroxysteroid dehydrogenases	*Hsd3b7*	0.37	0.57	94	9.64E-14
	Reductases	Glyoxylate and hydroxypyruvate reductases	*Grhpr*	0.40	1.04	92	5.53E-12
	Regulatory factors	NEDD4 binding proteins like	*N4bp2l1*	0.64	2.07	90	5.87E-12
Co-nodes	Developmental proteins	Testis development related protein	*Tdrp*	0.45	0.89	87	2.32E-10
	Glycoproteins	Glycoprotein integral membrane	*Ginm1*	0.45	1.27	99	8.73E-18
	Membrane proteins	Tetraspanin	*Tspan12*	0.48	2.18	87	2.32E-10
	Stress response factors	DnaJ heat shock protein (Hsp40) member	*Dnaja3*	0.40	0.67	99	8.73E-18
aPU1.KO-repressed							
Receptors	G protein coupled receptors	G protein-coupled receptor	*Gpr176*	−3.09	−1.71	95	5.59E-14
	Ligands	FAT atypical cadherin	*Fat4*	−0.92	−1.54	74	4.95E-07
Enzymes	ADP ribosyltransferases	Poly [ADP-ribose] polymerases (PARP)	*Parp14*	−0.56	−1.11	95	5.59E-14
	Dehydratases	3-hydroxyacyl-CoA dehydratases	*Hacd4*	−1.07	−1.15	80	1.61E-08
	GTPases	RAB, member RAS oncogene	*Rab7b*	−2.56	−1.28	80	1.61E-08
Co-nodes	Apoptosis and apoptosis regulators	Niban apoptosis regulator	*Niban1*	−1.13	−1.01	90	5.87E-12
	Cytoskeleton components and regulators	FERM domain containing	*Frmd4a*	−1.07	−1.06	95	5.59E-14
	Membrane proteins	CKLF like MARVEL transmembrane domain containing	*Cmtm3*	−1.55	−1.05	97	1.17E-15
	Pleckstrin domain	Pleckstrin homology domain containing	*Plekho2*	−1.59	−1.03	98	2.44E-16
	Other co-nodes	MAM domain containing	*Mamdc2*	−2.25	−1.29	80	1.61E-08
		Vesicle amine transport	*Vat1*	−0.86	−1.11	80	1.61E-08

HALLMARK; **Supplementary Table S3**, column Q). Validating the 3T3-L1 adipogenesis consensome, the GSEA HALLMARK gene set was robustly over-represented among 3T3-ADIPICTs (OR = 10.1, p = 4.2E-58; **Figure 6A**). Many of the aPU.1KO-regulated 3T3-ADIPICTs are immediately familiar in the context of adipogenesis, including *Cidec* (Keller et al., 2008), *Adipoq* (Hu et al., 1996) and *Acsl1* (Ellis et al., 2010b). Interestingly, numerous aPU.1KO-induced (**Supplementary Table S3**, column R) and aPU.1KO-repressed (**Supplementary Table S3**, column S) genes have 3T3-L1 adipogenesis consensome rankings that are comparable to or exceed those of classic adipogenic markers, but have potential roles in adipogenesis that to date are unexplored in the research literature (**Table 1**).

To focus on direct aPU.1 targets among genes with elevated rankings in the 3T3-adipogenesis consensome, we next percentilized peak call heights (n = 679) from a previously published PU.1 3T3-L1 ChIP-Seq analysis by Lazar and colleagues (DiSpirito et al., 2013) and mapped these to genes in the 3T3-L1 adipogenesis consensome (**Supplementary Table S3**, column T). For the purposes of subsequent statistical analyses, the 50th percentile of this gene set (n = 444) is referred to here as LZ50. Consistent with broad, direct antagonism by PU.1 of the 3T3-L1 adipogenic transcriptional program, we observed robust enrichment of aPU.1KO-induced LZ50 genes (n = 78) in 3T3-ADIPICTs (**Figure 6B**; OR = 6.3, p = 2.2E-11) and of aPU.1KO-repressed LZ50 genes (n = 293) among 3T3-ADIPRCTs (**Figure 6C**; OR = 7.3, P = 1E-10). Further reflecting the role of aPU.1 as an important direct regulator of adipogenic differentiation, we observed a clear positive correlation (adjusted r^2 = 0.54, P = 6E-10, Pearson's correlation) between aPU.1KO log FCs and mean 3T3-adipogenesis consensome log FCs for 3T3-ADIPRCTs or 3T3-ADIPICTs in the 50th percentile of PU.1 3T3-L1 peaks (n = 51. **Figure 6D**). Studying these 51 genes further, we identified two prominently pro-adipogenic members of the 3T3-ADIPICTs subset, *Srebf1* (encoding SREBP1) and *Nr1h3* (encoding LXRα), that have not been previously appreciated as direct PU.1 transcriptional targets. Similarly, uncharacterized candidate direct PU.1 targets within the 3T3-ADIPRCT gene subset included *Vcam1*, previously shown to mediate adhesion of inflammatory macrophages to adipocytes as a potential mechanism driving insulin resistance (Chung et al., 2017), and *Mmp14*, whose role in adipogenic collagen turnover has been linked to obesity (Chun et al., 2010). Collectively, our *in vivo* analysis confirms and adds value to previous *in vitro* studies implicating PU.1 as an anti-adipogenic, pro-inflammatory driver of gene expression in adipocytes.

SPP Web Resource Facilitates the Generation of Novel Hypotheses Around PU.1 Regulation of Adipogenic Gene Expression

To make full use of the adipogenesis consensome for hypothesis generation around aPU.1 regulation of adipogenic gene expression, it is important that the data points be placed in the context of data points from other mouse adipose transcriptomic experiments. For each gene in **Supplementary Table S3** therefore, column U links to an SPP website interface showing the specific experimental data points for that gene from the 3T3-L1 adipogenesis expression profiling datasets. In addition, the interface includes data points from transcriptomic experiments in mouse adipose tissue or cell lines involving genetic or small molecule perturbation of various receptor and enzyme signaling nodes, as well as data points from metabolic challenges such as cold exposure. For insight into nodes directly regulating expression of genes in the adipogenesis consensome, **Supplementary Table S3** column V links to an interface showing data points from ChIP-Seq experiments carried out in mouse adipose tissue or cell lines. Data points in both the transcriptomic and ChIP-Seq interfaces link to contextual pop-up windows, which in turn point to the full source datasets on the SPP website.

Confirmation of PU.1 Regulation of Pro-inflammatory Cytokines and Thermogenesis Genes in Adipose Tissues

Given the global transcriptional repression of cytokine production in aPU.1KO adipocytes indicated by the RNA-Seq analysis (**Figure 4**), we next used quantitative real-time RT-PCR (Q-PCR) to validate this observation. We confirmed the reduction of the expression of *Ccl2*/MCP-1 and *Tnf* in adipocytes isolated from epididymal WAT of 1 yo male aPU.1KO mice (**Figure 7A**). Since aPU.1KO mice exhibited elevated energy expenditure, we speculated that we might also observe induction of thermogenic expression programs in brown adipose tissue (BAT) of aPU.1KO mice. Consistent with this hypothesis, Q-PCR analysis identified transcriptional induction in aPU.1KO BAT of two key thermogenic genes, *Ppargc1a*/PGC-1α and *Ucp1* (**Figure 7B**). These results suggest a molecular mechanism underlying PU.1 regulation of adipocyte inflammation and thermogenesis and offer an explanation of the phenotype of the aPU.1KO mice.

Integrated Analysis of aPU.1KO Transcriptional Regulatory Networks and Mouse Phenotypes

The Mammalian Phenotype Ontology (MPO) (Smith and Eppig, 2009), uses evidence from the research literature to assign specific metabolic and physiological functions to the products of mouse genes. As such, MPO annotations represent a potentially powerful approach to inferring the metabolic impact of transcriptional regulatory networks in the aPU.1KO mouse. Given the increased energy expenditure of aPU.1KO mice (**Figure 2**), we first wished to gather evidence for nodes that are transcriptional mediators of increased whole body energy expenditure in these mice. To do this we examined the intersection between nodes that had significant intersections with aPU.1KO-UP genes, and those encoded by genes whose

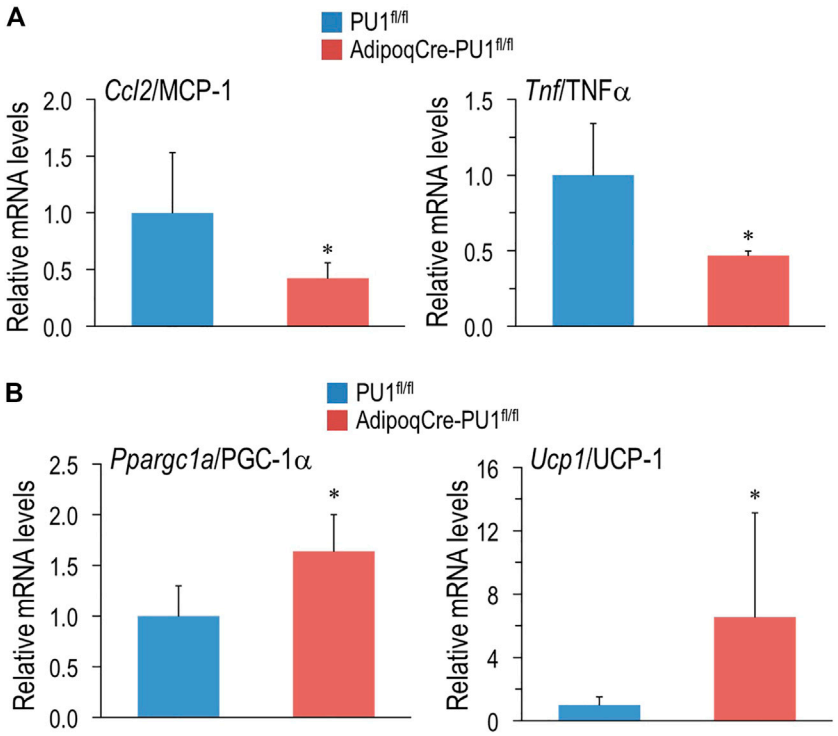


FIGURE 7 | Loss of PU.1 in Adipocytes Reduces Inflammatory Gene Expression and Promotes Thermogenic Gene Expression. **(A)** Adipocytes were isolated from the gonadal adipose tissue and mRNA expression of *ccl2*/MCP-1 and TNFα were measured using real-time RT-PCR. **(B)** Brown adipose tissue mRNA expression of PGC-1α and UCP1 and TNFα were measured using real-time RT-PCR. *N*= 5 vs. 5, **p* < 0.05.

disruption in mice mapped to the MPO term "abnormal energy expenditure" (AEE; **Supplementary Table S2**, column R). Consistent with the increased energy expenditure in the aPU.1KO mice, nodes encoded by AEE-mapped genes were strongly enriched among the top ranked nodes that had significant intersections with aPU.1KO-induced genes (OR = 14, P = 9E-5, **Figure 8A**) but not the aPU.1KO-repressed genes. These nodes included the nuclear receptors Pparg (Kubota et al., 1999) and Ppara (Finck et al., 2005), the circadian clock regulators Clock (Turek et al., 2005) and Arntl (Shimba et al., 2011), and the C/EBP family member Cebpa (Wang et al., 1995) which, as previously noted, was strongly transcriptionally induced in adipocytes in the aPU.1KO mice (**Figure 2**).

Next, to identify potential transcriptional mediators of thermogenic gene expression in the aPU.1KO mice (**Figure 4**), we compared nodes that had significant intersections in aPU.1KO-UP genes with those encoded by a set of genes that mapped to at least one of the MPO terms "decreased brown adipose tissue amount", "decreased core body temperature" or "impaired adaptive thermogenesis" (collectively referred to as THERMO genes; **Supplementary Table S2**, column S). Consistent with the induction of BAT thermogenic genes in the aPU.1KO animals, nodes encoded by THERMO genes were strongly enriched among the top ranked nodes that had significant intersections with aPU.1KO-induced genes (OR = 20, *P* = 2E-05; **Figure 8B**) but not aPU.1KO-repressed genes. These included the nuclear receptor Pparg (Imai et al., 2004), the C/EBP family transcription factor Cebpa (Wang et al., 1995), the general transcription factor Taf7l (Zhou et al., 2014) and the cohesin complex member Stag1 (Remeseiro et al., 2012). Collectively these data indicate that a non-redundant role for PU.1 in repressing a transcriptional regulatory network driving thermogenic gene expression *in vivo*.

Node HCT intersection analysis had previously indicated that numerous inflammatory transcription factors with roles in cytokine production were functionally impacted by the loss of PU.1 (**Figure 5A**). To gain insight into the PU.1 adipocyte transcriptional hub supporting inflammatory cytokine production *in vivo*, we compared nodes that had significant intersections with aPU.1KO-DOWN genes and those encoded by genes whose disruption in mice mapped to the MPO term "abnormal cytokine levels" (ACL; **Supplementary Table S2**, column T). Reflecting transcriptional repression of numerous inflammatory cytokine genes in the aPU.1KO mice (**Figures 4**, **7A**, and **Supplementary Table S1**), nodes encoded by ACL genes were strongly enriched among the top ranked nodes that had significant intersections with aPU.1KO-repressed genes (OR = 11, *P* = 3E-05; **Figure 8C**) but not the aPU.1KO-induced genes. These included members of the IRF (Irf3, Irf5), STAT (Stat1, Stat6) and AP-1 (Jun, Junb) transcription factor families, as well as Fosl1, Cebpd and Nfkb1. Collectively these data indicate that PU.1 has a non-redundant role in anchoring a transcriptional

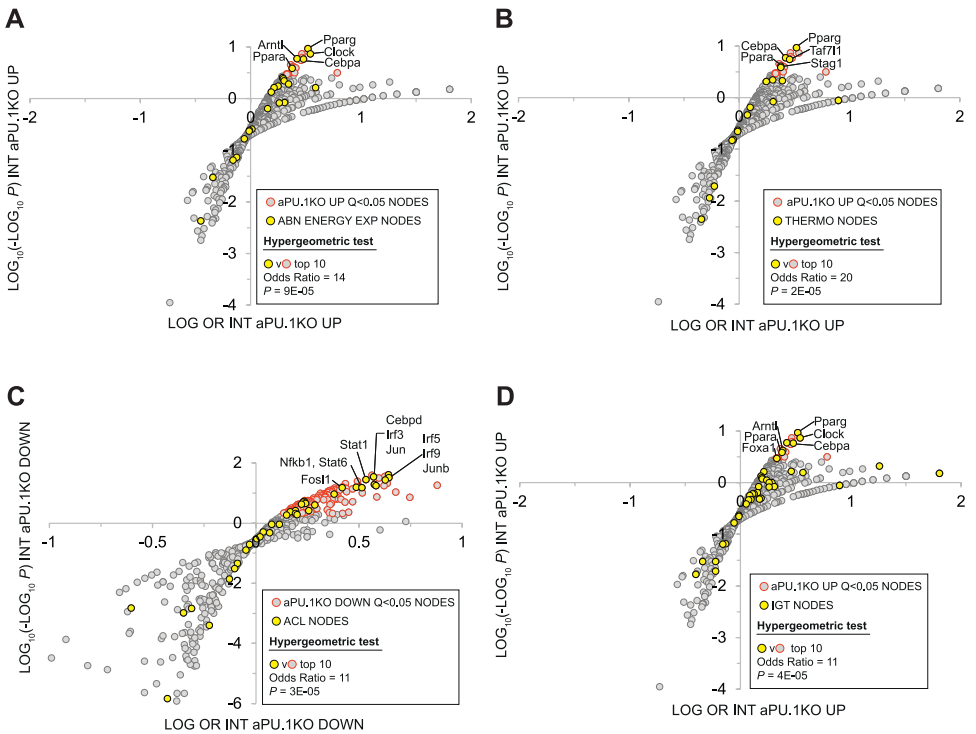


FIGURE 8 | Mammalian Phenotype Ontology Analysis Of Nodes With Significant HCT Intersections With aPU.1KO-Induced and Repressed Gene Sets. **(A)** Enrichment of nodes encoded by genes that map to the MPO term "abnormal energy expenditure" (AEE) among nodes that have significant HCT intersections with aPU.1KO-induced genes. **(B)** Enrichment of nodes encoded by genes that map to the MPO terms "decreased brown adipose tissue amount", "decreased core body temperature" or "impaired adaptive thermogenesis" (collectively referred to as THERMO genes) among nodes that have significant HCT intersections with aPU.1KO-induced genes. **(C)** Enrichment of nodes encoded by genes that map to the MPO term "abnormal cytokine levels" (ACL) among nodes that have significant HCT intersections with aPU.1KO-repressed genes. **(D)** Enrichment of nodes encoded by genes that map to the MPO term "impaired glucose tolerance" (IGT) among nodes that have significant HCT intersections with aPU.1KO-induced genes. Please refer to the Methods for details of the analysis.

regulatory network supporting adipocyte cytokine gene expression *in vivo*.

We showed that aPU.1KO mice exhibited improved glucose homeostasis (**Figure 3**). To gather evidence for the specific signaling nodes contributing to this phenotype, we compared nodes that had significant intersections in aPU.1KO-UP genes with those encoded by genes mapped to the MPO term "impaired glucose tolerance" (IGT; **Supplementary Table S2**, column U). Consistent with improved glucose homeostasis in the aPU.1KO mice, nodes encoded by IGT genes were strongly over-represented among nodes with Q < 0.01 intersections with aPU.1KO-induced genes (OR = 6, p = 5.7E-05, **Figure 8D**). Confirming the link between improved glucose tolerance and energy expenditure, these included the five previously identified AEE nodes (Pparg, Ppara, Clock, Arntl and Cebpa), in addition to Foxa1.

DISCUSSION

In this study, we set out to investigate the role of the transcription factor PU.1 in adipocytes *in vivo,* particularly during aging. We observed that although young (4–5 months) aPU.1KO mice had no overt phenotypes with respect to body weight, body composition, or glucose homeostasis, they did exhibit elevated energy expenditure. Moreover, at around 1 year of age, aPU.1KO mice were protected against age-associated obesity, adipose tissue inflammation, and insulin resistance. Mechanistically, and consistent with their elevated energy expenditure, we found that loss of adipocyte PU.1 suppressed inflammatory transcriptional programs in WAT and promoted thermogenic gene expression in BAT. Using a combination of conventional, literature-based pathway analysis and a novel 'omics dataset-centric analytic platform, we identified numerous PU.1-modulated signaling systems and downstream functional pathways that shed mechanistic light on these phenotypes.

Consistent with the enhanced energy expenditure of aPU.1KO mice, we identified a robust transcriptional footprint within aPU.1KO-induced genes for members of the PGC-1 family (**Figure 5A**), which are well known transcriptional drivers of thermogenesis and energy metabolism (Puigserver et al., 1998). This represented strong evidence, validated by subsequent Q-PCR analysis (**Figure 7A**), that PU.1 suppresses thermogenic transcriptional programs in mice. To afford insight into the functional pathways involved, **Supplementary Table S2** column L indicates aPU.1KO-induced genes that are HCTs for the PGC-1 family. Many of these are familiar players in

cellular energy metabolism that have emerged from studies in the research literature. The induction of *Ndufb6*, *Ndufb10* and *Ndufs6*, for example, reflects our finding from RPA analysis that respiratory electron transport chain pathway genes were enriched among aPU.1KO-induced genes (**Figure 4A**). Moreover, upregulation of the transferrin (*Trf*) gene may reflect potential endocrine or paracrine signaling from the white adipocytes to activate brown or beige adipocyte thermogenesis in aPU.1KO mice (Qiu et al., 2020). The power of consensome analysis, however, is that it illuminates genes for which a specific function is not described in the research literature, but for which, based upon close regulatory relationship with a node family computed from 'omics datasets, that function can be inferred with a high level of confidence. This is the case with genes such as *Blcap*, *Gkap1*, *Proca1* and others, for which, as aPU.1KO-induced PGC-1 HCTs, a functional contribution to enhanced bioenergetics of the aPU.1KO animals can be reasonably surmised. Supporting this assertion, and confirming the clinical relevance of our study, the human ortholog of *Fam13a* (aPU.1KO logFC = 1.3; PGC-1 family consensome 95th percentile) has been recently shown to regulate fat distribution and metabolic traits through its action on adipose tissue (Fathzadeh et al., 2020). In summary, we conclude that activation of PGC-1 family signaling in aPU.1KO mice contributes to the increased energy expenditure, reduced adiposity, improved glucose homeostasis and insulin sensitivity of aPU.1KO mice, and ultimately protects against age-related metabolic abnormalities in these animals.

Transcriptomic analysis has cast PU.1 in the global maintenance of pro-inflammatory transcriptional programs in response to sepsis or lipopolysaccharide stimulation (Karpurapu et al., 2011). Although transcriptional induction by PU.1 of pro-inflammatory factors, including *Tnf*, *Il1b* and *Il6*, as well as *Ccl3* and *Ccl2* has been extensively documented in macrophages and other immune cell lineages (Karpurapu et al., 2011), there is increasing interest in its role as a pro-inflammatory transcription factor in adipose tissue. Building on our previous study showing that PU.1 activates inflammatory cytokine expression in cultured adipocytes (Lin et al., 2012), our current study shows that depletion of adipocyte PU.1 results in broad, transcriptome-scale suppression of inflammatory programs in adipocytes. Since inflammation is a key mediator for insulin resistance and metabolic syndrome (Hotamisligil, 2017), suppression of this inflammatory transcriptional program likely contributes to the phenotypes of the aPU.1KO mice. Going to the underlying mechanism, we found that the expression levels of many proinflammatory cytokines driven by the PU.1 transcription factor, such as *il1b*, *tnf* and *ccl2*/MCP-1, are down regulated in the adipocytes of aPU.1KO mice (**Figures 4**, **7**). IL-10 signaling, which has been shown to inhibit thermogenesis and energy expenditure in adipocytes (Rajbhandari et al., 2018), was also downregulated in PU.1-deficient adipocytes (**Figure 4B**). Members of the NOD-like family of receptors have prominent roles in the transcriptional regulation of inflammasome pathways. Our transcriptomic analysis identified significant down-regulation of genes encoding two members of the NOD-like family, *Ciita* and *Nrpl3,* in aPU.1KO adipocytes (**Supplementary Table S1**). Similarly, consistent with the strong TLR regulatory footprint in the aPU.1KO-repressed genes (**Figure 5A**), genes encoding four members of the TLR family (*Tlr1*, *Tlr6*, *Tlr7* and *Tlr8*) are repressed in the aPU.1KO. Given that TLRs (Kim et al., 2012), *Ciita* (Deng et al., 2013) and *Nrpl3* (Stienstra et al., 2010) are known to be induced in obese adipose tissue or to support adipocyte inflammation, it can be justifiably speculated that their transcriptional induction in adipocytes makes an important contribution to PU.1's action in promoting inflammation and insulin resistance.

On a broader scale, HCT intersection analysis (**Figure 5A**), validated by integration with literature-based mouse phenotype annotations (**Figure 8C**), reflects the profound impact of loss of PU.1 on the function of numerous inflammatory node families. For example, aPU.1KO-downregulated genes contain a sizeable regulatory footprint for members of the IRF transcription factor family (**Figure 5A** and **Supplementary Table S2**). Given that the roles of members of the IRF family in the regulation of adipogenesis, inflammation and thermogenesis in adipocytes are well-documented (Eguchi et al., 2008; Kumari et al., 2016), we interpret the presence of this footprint as evidence for strong, network-level interactions between PU.1 and IRFs in adipocytes. On the other hand, transcription factors that suppress inflammatory gene expression, such as PPARγ (Lefterova et al., 2010) and LXRs (Heinz et al., 2010), have DNA binding sites adjacent to PU.1 binding sites, potentially reflecting mutual functional antagonism. Given that we observed evidence for activation of both PPAR and LXR in response to aPU.1 loss of function (**Figure 4B**), we speculate that aPU.1 may also drive inflammation through the suppression of PPAR and LXR transcription factors.

Although 'omics datasets have intrinsic value for metabolic research, they realize their full value when integrated with existing data resources to facilitate the generation of hypotheses around metabolic signaling pathways not explored in the research literature. A unique aspect of our RNA-Seq dataset is that rather than limiting it to a standalone analysis of aPU.1KO-regulated genes, we have placed it in the context of millions of regulatory data points curated from archived 'omics datasets by the SPP cell signaling knowledgebase (Love et al., 2014). Annotation of aPU.1KO-regulated gene list (**Supplementary Table S1**) according to the SPP classification, for example, provides for an immediate appreciation of the diversity of cellular functions impacted by PU.1 depletion. Similarly, HCT intersection analysis (**Figure 5** and **Supplementary Table S2**) affords a unique perspective on the various receptors, enzymes, transcription factors and co-nodes that are functionally impacted by PU.1 depletion and which, by extension, are candidate PU.1-interacting proteins. Finally, the adipose-centric SPP transcriptomic and ChIP-Seq Regulation Reports to which the 3T3-L1 adipogenic consensome (**Supplementary Table S3**) links provide the user with a rich, contextual perspective to generate hypotheses around transcriptional regulation of novel effectors of adipose tissue biology. By integrating these three data resources in a single study, we provide for a unique perspective on PU.1-dependent transcriptional

regulatory networks in adipocytes, and an appreciation of how diverse signaling nodes impact expression of a specific PU.1 target gene (Ochsner et al., 2019).

The collective value of our supplementary material to researchers in generating novel metabolic hypotheses can be illustrated with reference to *Gpr176*, identified in **Supplementary Table S1** as a gene encoding a member of the G protein-coupled receptor family that is strongly transcriptionally dependent upon PU.1. With the exception of a role in the regulation of circadian clock in the suprachiasmatic nucleus (Doi et al., 2016), the function of this receptor is largely uncharacterized. The 3T3-L1 adipogenesis consensome ranks *Gpr176* 445th of 12525 genes (mean log FC −1.71, CQV 1E-11, 95th %ile), indicating that *Gpr176* is robustly and consistently downregulated during adipogenic differentiation. The SPP transcriptomic Regulation Report for *Gpr176* (**Supplementary Table S3** column U) contains data points documenting its regulation by prominent regulators of lipid metabolism, including FGF21, PPARG and members of the PGC-1 family. Similarly, the ChIP-Seq Regulation Report (**Supplementary Table S3** column V) provides evidence for direct regulation of *Gpr176* by PU.1 and numerous other nodes, including Polycomb group proteins and members of the C/EBP, STAT and BRD families. Finally, the enrichment among aPU.1KO-induced genes of HCTs for numerous characterized transcriptional regulators of circadian rhythms (Arntl/BML1, Nr1d1/Rev-Erbα, Clock, and Nr3c1/GR; **Figure 5A** and **Supplementary Table S2**) suggests that PU.1 regulation of circadian transcriptional programs in adipocytes may well extend beyond *Gpr176*. Set in the context of existing evidence documenting circadian connections between adipose tissue biology, lipid metabolism and the immune system (Krueger and Feldman, 2013; Lekkas and Paschos, 2019; Lananna and Musiek, 2020), the SPP data points suggest a hypothesis implicating PU.1 as a transcriptional co-ordinator of circadian programs in adipocytes and immune cells. Indeed, such a notion is supported by a previous report of global enhancement of PU.1 transactivation in Arntl/BML1-depleted macrophages (Oishi et al., 2017).

The recent characterization of PU.1 as a transcriptional driver of fibrosis (Wohlfahrt et al., 2019) is interesting given the known role of fibrosis in supporting the inflammatory state (Crewe et al., 2017) and obesity (Chiang et al., 2011). Interestingly, RPA identified a strong repression in the aPU.1KO adipocytes of pathways related to the extracellular matrix (ECM), a critical player in the development of fibrosis (Herrera et al., 2018) (**Figure 4B**). Inspecting the aPU.1KO-repressed genes more closely, we identified three members of the fibrinogen family (OR = 318, *P* = 1E-08, hypergeometric test), six members of the integrin family (OR = 7.5; P = 1E-04, hypergeometric test), 12 members of the cluster of differentiation group (OR = 13, *P* = 1E-10, hypergeometric test) and nine members of the collagen family (OR = 6.9, P = 5E-06, hypergeometric test), many of which have been implicated in fibrosis and obesity (Ditschuneit et al., 1995; Féral et al., 2008; Wynn, 2008; Khan et al., 2009; Pasarica et al., 2009; Yamauchi et al., 2011; Craciun et al., 2014; Dankel et al., 2014; Schnittert et al., 2018). Most intriguingly of all perhaps, aPU.1KO-repressed genes contain eight members of the major urinary protein (MUP) family (OR = 417, *P* = 4E-21), several of which are among the most strongly repressed genes. MUP proteins are related to members of the lipocalin family, which have documented connections to a variety of fibrotic conditions (Eichler et al., 1999; Ikezoe et al., 2014; Chen et al., 2020), as well as obesity and insulin resistance (Wang et al., 2007; Yan et al., 2007). Collectively, our analysis data point to a pivotal role for PU.1 in driving fibrotic transcriptional programs that support inflammatory pathways in adipocytes. Taken together, our transcriptome and bioinformatics analyzes provide valuable insights into the action of PU.1 in adipocytes. However, we need to validate these leads with a larger set of samples in follow-up studies.

Our observation that PU.1 plays a role in the development of age-associated metabolic syndrome shed light on not only a novel PU.1 action in adipocytes, but also the nature of age-related metabolic defects. Metabolic changes developed during the aging process share similarities with that caused by obesity, but also possess some unique characteristics (Bapat et al., 2015). The underlying mechanism is not well characterized. A recent study identified a sub-population of adipocytes present only in the subcutaneous adipose tissue of older mice or humans (Nguyen et al., 2021). These cells have elevated PU.1 expression, which causes defective adipogenesis and proinflammatory cytokines secretion to inhibit adipogenesis of neighboring cells. This finding is in agreement with our results, supporting an important role of PU.1 in aging adipose tissue, in the development of age-associated adipocyte dysfunction, with a likely consequence of whole-body metabolic defects.

As an adipocyte-specific knockout, our model underscores the contribution of adipocyte-autonomous functions of PU.1 to disorders of systemic metabolism. However, PU.1 in tissues other than adipose may also contribute to metabolic syndrome. For example, expression of hepatic PU.1 is also elevated in diet-induced obese and diabetic mice, and is positively correlated with insulin resistance and liver inflammation in humans (Liu et al., 2020). Depletion of PU.1 in non-parenchymal liver cells, likely in liver macrophages, inhibited liver inflammation, hepatic steatosis and whole body insulin resistance (Liu et al., 2020). Taken together, PU.1 regulates metabolic functions in both adipocytes and in liver macrophages. Therefore, PU.1 is an important driver for metabolic disorders when animals get older and may serve as a therapeutic target for the treatment of metabolic syndrome.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE188497.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institution of Animal Care and Use Committee at Baylor College of Medicine.

AUTHOR CONTRIBUTIONS

Conceptualization: QT Investigation: KC, AD, XG, and WP. Validation: EL and DS. Methodology/Software: SO and NM. Formal analysis: AM, SO, and NM. Writing and editing: QT, AM, YS, and NM. Funding acquisition: QT and NM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fragi.2021.803482/full#supplementary-material

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REVIEW
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Role of BAG5 in Protein Quality Control: Double-Edged Sword?

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Cardiovascular disorder is the major health burden and cause of death among individuals worldwide. As the cardiomyocytes lack the ability for self-renewal, it is utmost necessary to surveil the protein quality in the cells. The Bcl-2 associated anthanogene protein (BAG) family and molecular chaperones (HSP70, HSP90) actively participate in maintaining cellular protein quality control (PQC) to limit cellular dysfunction in the cells. The BAG family contains a unique BAG domain which facilitates their interaction with the ATPase domain of the heat shock protein 70 (HSP70) to assist in protein folding. Among the BAG family members (BAG1-6), BAG5 protein is unique since it has five domains in tandem, and the binding of BD5 induces certain conformational changes in the nucleotide-binding domain (NBD) of HSP70 such that it loses its affinity for binding to ADP and results in enhanced protein refolding activity of HSP70. In this review, we shall describe the role of BAG5 in modulating mitophagy, endoplasmic stress, and cellular viability. Also, we have highlighted the interaction of BAG5 with other proteins, including PINK, DJ-1, CHIP, and their role in cellular PQC. Apart from this, we have described the role of BAG5 in cellular metabolism and aging.

Keywords: BAG5, mitophagy, cardiovascular, Hsp70, autophagy, chaperone, aging, neuroprotection

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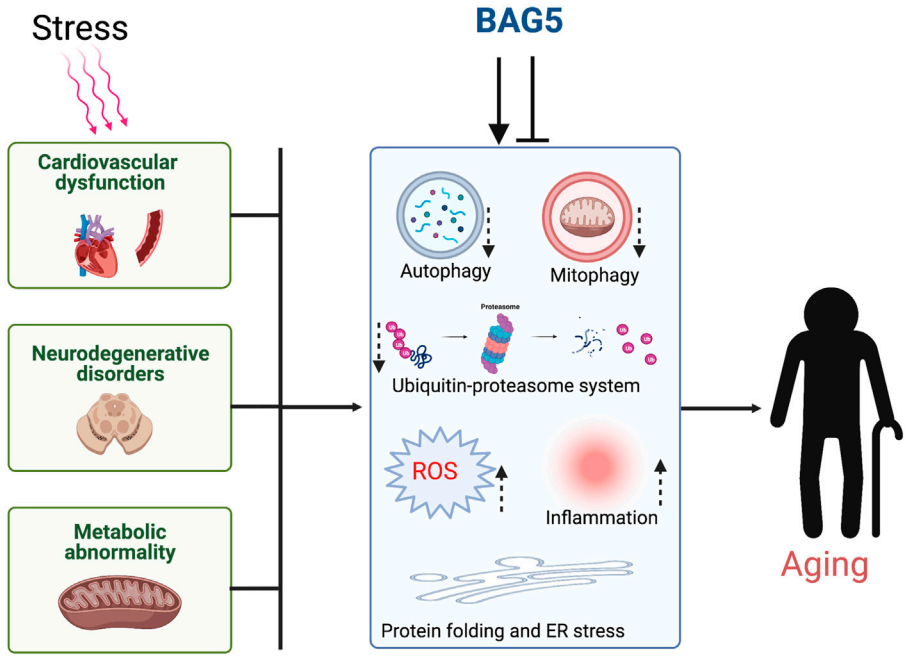
INTRODUCTION

Cardiovascular diseases (CVDs), including ischemic heart disease, heart failure, or other vascular conditions, constitute the leading cause of mortality among individuals worldwide (Pinto et al., 2021; Virani et al., 2021). According to the World Health Organization estimation, in the year 2019, 18.6 million people died due to CVDs globally (Randhawa and Gupta, 2020; Virani et al., 2021). Although we have made considerable progress in the cardiovascular field, there is a dire need to develop treatment strategies to treat CVDs in a fundamental way rather than in a symptomatic way. Cellular function and viability depend on the quality and quantity of cellular protein, and cellular protein quality control (PQC) plays an active role in maintaining cellular proteostasis (Henning and Brundel, 2017; Hohfeld et al., 2021). Cellular chaperones and co-chaperones actively maintain the cellular PQC by helping nascent protein fold properly and translocating the folded protein to correct destinations (Santra et al., 2019; Hartl et al., 2011). Additionally, chaperones and co-chaperones help in the removal and recycling of misfolded protein by cellular protein degradation systems. Most of the cellular protein gets degraded through the ubiquitin-proteasome system (UPS) (Sun-Wang et al., 2020; Li et al., 2021). The cell also uses a vesicle-mediated protein degradation system called autophagy to degrade cellular proteins and subcellular organelles (Delbridge et al., 2017). In addition, to these processes, misfolded proteins in the endoplasmic reticulum (ER) also get degraded through ER-associated protein degradation (ERAD) system. However, genetic mutation, hemodynamic stress, cellular toxicity, and cellular infection change cellular proteostasis and dysregulate a



GRAPHICAL ABSTRACT | Cellular stress dysregulates the major organ function through inhibition of cellular protein quality control and induces aging.

quintessential environment required for normal cellular metabolism. Impairment of cellular PQC and accumulation of protein aggregates with faulty subcellular organelles leads to the generation of oxidative stress, inflammation, and cell death (Li et al., 2021). These modifications are known to be associated with the impairment of several organ functions and the development of several life-threatening diseases including, CVD, neurological disorders, and premature aging (Gong et al., 2016; Kaur et al., 2020). This review will discuss the evolving role of co-chaperone Bcl-2 associated anthanogene protein (BAG5) in UPS, autophagy, mitophagy, oxidative stress, metabolism, and its association with CVDs, Alzheimer's disease, and Parkinson's disease.

ROLE OF PROTEIN QUALITY CONTROL IN HEART DISEASE

The adult mammalian cardiomyocytes are post-mitotic cells that lack self-renewal ability (Lázár et al., 2017). Therefore, it is critical to maintain protein homeostasis and monitor PQC to limit cellular dysfunction in the cardiomyocytes (Wang and Robbins, 2006; Maejima, 2020). The maintenance of protein homeostasis includes a range of events, including gene transcription, translation, post-translational modification, complex formation, and protein degradation (Webster et al., 2020). Cellular PQC is maintained *via* two major mechanisms, the UPS and or autophagy, to eliminate misfolded proteins and damaged organelles (Maejima, 2020). Also, the ERAD system tags the unfolded proteins of ER with ubiquitin and facilitates the degradation of proteins through the UPS pathway. Studies have indicated that the accumulation of misfolded proteins may impart mechanical stress and also induce oxidative stress in the cardiomyocytes such that it may lead to the development of heart diseases, including myocardial infarction and even heart failure (Wang and Robbins, 2006; Maejima, 2020). Therefore, the maintenance of protein integrity and quality control in the cell is crucial for the normal functioning of the myocardium.

MOLECULAR CHAPERONES IN MAINTAINING CELLULAR PROTEOSTASIS

Molecular chaperones are the class of proteins that create equilibrium between protein synthesis and degradation in the cell (Arakawa et al., 2010; Friesen et al., 2020). The molecular chaperones and co-chaperones participate in enhancing protein refolding and protecting cells against the buildup of misfolded proteins (Arakawa et al., 2010; Friesen et al., 2020). In the cardiomyocytes, molecular chaperones encompass the major heat shock proteins (HSPs), such as HSP70, HSP90 and co-chaperones, including carboxy terminus of HSP70-interacting protein (CHIP), and BAG family proteins (BAG 1-6) (**Figure 1**) (Hartl et al., 2011; Balchin et al., 2020). The HSP70 family chaperones are monomeric proteins that are expressed in the heart and play a pivotal role in protecting the cardiomyocytes under stressful conditions (Willis and Patterson, 2010; Ranek et al., 2018). The stress-inducible forms include HSP70-1a, HSP70-1b, HSP70-6, and constitutive forms include HSP70-2, HSP70-5, HSc70, and HSP70-9 (Trcka et al., 2019). Among the inducible forms, HSP70-1a, HSP70-1b is exclusively found in the cytosol, nucleus, and lysosomes, whereas HSP70-6 is usually localized in the cytosol and the nucleus (Trcka et al., 2019).

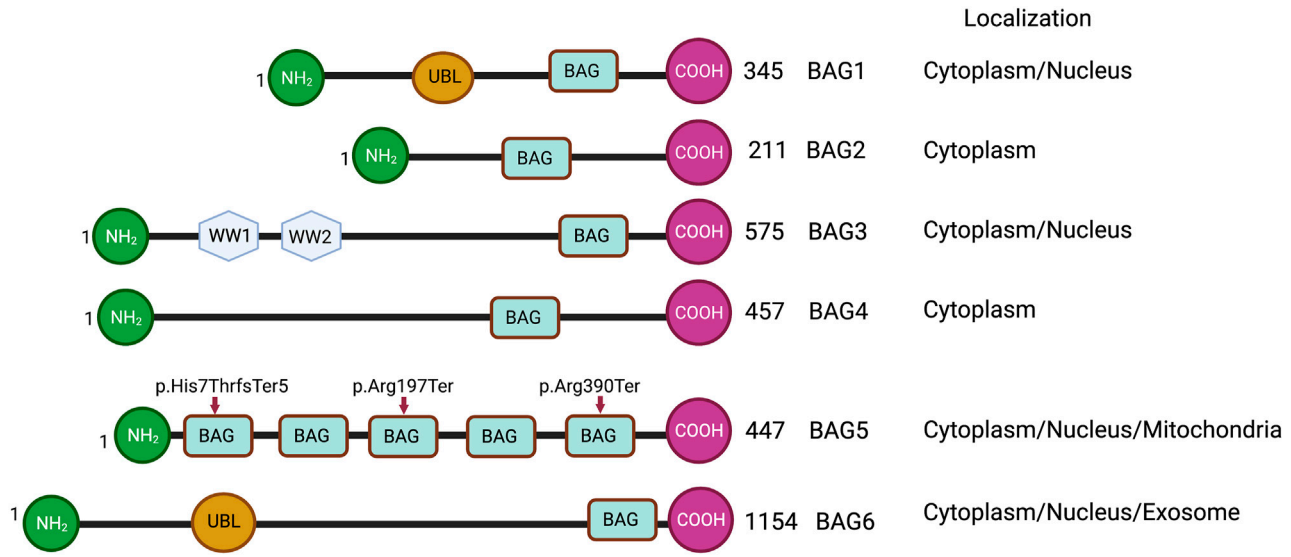


FIGURE 1 | Schematic representations of BAG family proteins, showing conserved domains and cellular localization. NH2 represents the N-terminal and COOH represents the C-terminal of protein. UBL represents a ubiquitin-like domain. BAG represents the BAG domain. WW represents WW domain. Arrowheads show the truncating variants of the BAG5 gene.

These proteins possess two domains, i.e. ATPase domain and substrate-binding domain (SBD), responsible for regulating their activity. Oxidative stress can induce the expression of HSP70, whereas its protein homolog HSC70 (heat shock cognate) is constitutively expressed in the myocardium (Ranek et al., 2018; Trcka et al., 2019). HSP70 proteins can fold the misfolded proteins to a native protein conformation *via* a ATP-dependent cyclic process and protect cells from proteotoxic stress (**Figure 2**). However, under physiological conditions, HSP70s facilitate the folding of newly synthesized polypeptides into a typical protein and help in the transport of proteins to the intracellular environment (**Figure 2**) (Ranek et al., 2018).

HSP 90 proteins are molecular chaperones that stabilize the proteins against heat stress and facilitate their degradation, and are more selective compared to other chaperones in identifying misfolded proteins (Zhao and Houry, 2005; Morán Luengo et al., 2019). These proteins comprise three domains i.e. ATP binding domain, protein-binding domain, dimerizing domain, and they interact with misfolded protein regions of the substrate protein to provide stability to the substrate and reduce the protein aggregation (**Figure 2**). The members of the HSP90 family include HSP90N, glucose-related protein (GRP) 94 (GRP94), and TNF receptor-associated protein 1 (TRAP1) (Morán Luengo et al., 2019). These chaperones are abundantly expressed in the cytosol and exist in two isoforms i.e. HSP90α (inducible) and HSP 90β (constitutive) (Chen et al., 2005; Morán Luengo et al., 2019). In addition, it has been reported that ischemic conditions cause reactive oxygen species (ROS) build-up, induction of HSP90α, followed by augmentation of HSF1 expression (Ranek et al., 2018).

PROTEIN HOMEOSTASIS IN THE ER

Apparently, ER-resident chaperones play a significant role in the cellular protein folding and degradation through ERAD pathways. HSP70 member protein, GRP78 also known as BiP, plays a diverse role in maintaining ER-mediated proteostasis. Like Hsp70, GRP78 has SBD and during ER stress, its expression is significantly upregulated to assist the protein folding. Additionally, BiP plays a significant role in maintaining the translocation of proteins in the ER by retrograde transport of misfolded proteins for degradation through the ERAD system (Pobre et al., 2019). Intriguingly, ER is equipped with enzymes and chaperones that assist in the folding and assembly of proteins. The folding of proteins in a proper way requires the formation of an intramolecular or intermolecular disulfide bond in the native proteins to achieve the proper structure. ER-resident protein disulfide isomerase (PDI) plays a critical role in crosslinking of native proteins via disulfide bond formation. PDI expression is found to be upregulated during ER stress, and overexpression of PDI protects cells from ER stress induced cell death (Feige and Hendershot, 2011; Shin et al., 2021). Another member of heat shock protein HSP90, known as a GRP94 found to be in the ER. The chaperonic activity of GRP94 is restricted to selective proteins. It mainly assists the folding of membrane-associated proteins or secretory proteins (Feige and Hendershot, 2011; Shin et al., 2021). Most of the proteins that form in the ER undergo post-translational modification by forming an N-glycosidic bond with the glycans N- (carbohydrate-based polymers). This PTM help in protein folding, translocation, solubilization, and degradation. Apart from this, ER proteins calnexin and calreticulin also act as chaperones for the glycoprotein and helps in protein folding and

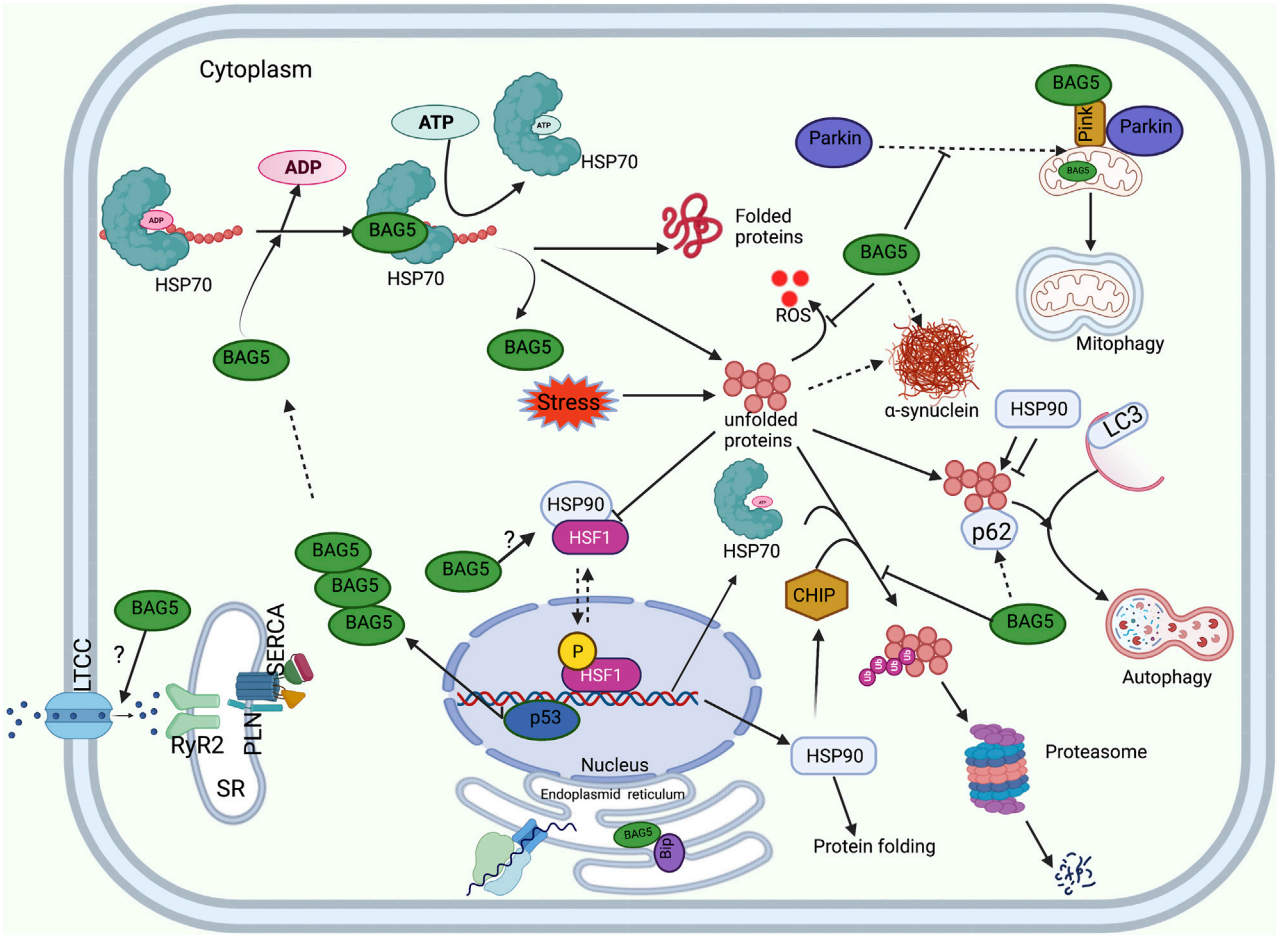


FIGURE 2 | Functions of BAG5 in cellular protein quality control. A diagram shows the involvement of BAG5 in the major cellular functions: regulation of HSP70 mediated protein folding, degradation of protein by autophagy and UPS system, regulation of PINK and Parkin mediated cellular mitophagy, regulation of cellular ER stress, generation of heat shock response, inhibition of ROS production, and handaling of Ca2+ ions. Sarcoplasmic reticulum (SR), L-type calcium channel (LTCC), Ryanodine receptor (RyR), Sarcoplasmic Ca2+ -ATPase (SERCA), Phospholamban (PLN).

terminal release of proteins (Ihara et al., 2020; Kozlov and Gehring, 2020).

HSF1 MEDIATED REGULATION OF CELLULAR PROTEOSTASIS

Interestingly, the expression of chaperones is regulated by transcription factors HSF1 and HSF2 (Willis and Patterson, 2010). HSF1 transcription factor is present in the cytosol as a monomer and binds to HSP90 to impede its chaperone activity (Willis and Patterson, 2010). However, under stressful conditions, denatured proteins bind HSP90 and release HSF1, which then translocate to the nucleus to bind to the heat shock response elements of the stress-induced genes (promoter region) (Willis and Patterson, 2010). In the nucleus, augmented levels of HSF1 up-regulate the expression of HSP70 chaperone. After that, HSP70 binds HSF-1 to reduce its activity via the feedback mechanism. Apart from this, HSF-1 dependent stress response causes up-regulation of ubiquitin such that it enhances the ability of the cell to degrade proteins under stressful conditions (Willis and Patterson, 2010). Interestingly, a recent study indicated that HSF-1 regulates the expression of BAG family proteins as well (Prince et al., 2020). In fact, it was shown that the expression of HSF1 protein and its nuclear localization shows a positive correlation with BAG3 expression in the human cardiac tissue (Martin et al., 2021a). Previous studies have indicated that the expression of HSP90 is induced during cardiac arrhythmia, cardiac remodeling associated with cardiac hypertrophy, and heart failure (Ranek et al., 2018). This indicates that HSP90 proteins play a crucial role in the maturation of proteins and the progression of cardiac disorders (**Figure 2**). Interestingly, it was demonstrated that BAG5 protein strongly interacts with HSF1. However, the functional significance of BAG5 and HSF1 interaction needs to be established (Taipale et al., 2014).

BAG PROTEIN FAMILY AND ITS MOLECULAR INTERACTION WITH HSP70 AND OTHER CELLULAR PROTEINS

The BAG family is a set of multifunctional proteins that play a pivotal role in maintaining cellular homeostasis (Arakawa et al., 2010; De Snoo et al., 2019). The human BAG family comprises of six members viz. BAG1, BAG2, BAG3, BAG4, BAG5 and BAG6 (Arakawa et al., 2010; Friesen et al., 2020) (**Figure 1**) and found to be localized in nucleus, cytoplasm, mitochondria as well as in exosome (**Figure 1**). The members of this family contain a unique BAG domain which facilitates their interaction with the ATPase domain of molecular chaperone heat shock protein (HSP70) (Arakawa et al., 2010). Interestingly, BAG3 protein has some additional domains like WW domains in addition to the BAG domain, which helps in interacting with other proteins (Merabova et al., 2015; Sherman and Gabai, 2022). BAG family proteins play a significant role in regulating several physiological functions, like autophagy, UPS mediated protein degradation, and apoptosis (Kögel et al., 2020). Among the family members, BAG5 is considered exceptional as it comprises five domains in tandem. It has been reported that the fifth domain (BD5) is quintessential for exhibiting the interaction between HSP70 and BAG5 (Arakawa et al., 2010). Furthermore, it is manifested that the binding of BD5 induces certain conformational changes in the nucleotide-binding domain (NBD) of HSP70 such that it loses its affinity for binding ADP. In an *in-vitro* assay, it was found that BD5 or full-length BAG5 acts as a nucleotide exchange factor of HSP70 and accelerates the protein refolding activity of HSP70 (**Figure 2**) (De Snoo et al., 2019).

Interestingly, among all the members of the family, BAG5 has drawn considerable attention as it is expressed in the cardiomyocytes (Gupta et al., 2016) and plays a pivotal role in monitoring cell homeostasis under stressful conditions (Gupta et al., 2016; Duggan et al., 2021). In fact, studies have indicated that BAG5 protein overexpression can limit oxidative stress and mitochondrial dysfunction in the cells (Wang et al., 2014; Gupta et al., 2016).

BAG FAMILY AND ITS ASSOCIATION WITH CARDIAC DISEASE

BAG-family proteins participate in various cellular processes, including cell survival, migration, and proliferation (Rosati et al., 2011). Interestingly, over-expression of BAG-family proteins has been found in various cancers and is known to promote cell survival and induce cell proliferation. However, these proteins also possess anti-apoptotic activity, which depends on their interactions with either Hsc70/Hsp70 protein or binding to Bcl-2 protein (Doong et al., 2002; Rosati et al., 2011). Anticancer drug JG-98 is found to be effective in reducing the cancer cells growth by inhibition of BAG3-HSP70 interaction (Martin et al., 2022). However, this drug induces cardiac toxicity through reduction of BAG3 proteins half-life and reduction of cellular autophagy (Martin et al., 2022).

BAG family proteins are expressed in various tissues, including the heart (Gupta et al., 2016; Qin et al., 2017), lungs, brown adipose tissue (Qin et al., 2016), and are associated with various cellular components, including endoplasmic reticulum (Saxena et al., 2012), mitochondria (De Snoo et al., 2019; Li et al., 2010), microtubules (de Paula et al., 2016; He et al., 2020). In addition, BAG family proteins play a pivotal role in maintaining the structural and functional integrity of the heart (Knezevic et al., 2015). In fact, mutations in these proteins may result in structural as well as functional defects in the heart such that it can make an individual more vulnerable to CVDs, including heart failure (Knezevic et al., 2015; Diofano et al., 2020). A recent study shows that cardiac patients having truncation mutations (p.Arg197Ter and p. His7ThrfsTer5) of the BAG5 gene develop tachycardia-induced cardiomyopathy (**Figure 1**). Furthermore, this study shows that mouse models with the BAG5 gene mutation (Arg197Ter) have ventricular dilatation and dysregulation of calcium handling with arrhythmogenicity, suggesting that BAG5 is important for cardiac function (Hakui et al., 2022).

Apparently, studies have indicated that the BAG family proteins play a crucial role in mediating the adaptive cell survival response against sustained ischemia and thereafter reperfusion via induction of autophagy (Gurusamy et al., 2009). It has been reported that BAG-1 protein works in consensus with LC3-II (autophagosomal protein) and HSC70 protein to induce autophagy (Gurusamy et al., 2009). Also, it has been shown that BAG-3 protein participates in inducing autophagy in association with HSPB8 protein. A recent study shows that reduction of BAG3 mediated protein turnover may cause cardiomyocytes' contractile dysfunction during heart failure (Martin et al., 2021b). Apparently, this indicates that BAG family proteins promote cell survival under stressful conditions via induction of autophagy (Kögel et al., 2020).

ROLE OF BAG5 IN MODULATING MITOPHAGY

Cardiac muscle cells are constantly beating to maintain heart function, and these cells have high energy demand and are therefore, rich in mitochondria content compared to other types of cells. Mitochondria provide most of the cellular energy through oxidative phosphorylation, and maintaining a healthy mitochondrial pull is important for cardiac health (Murphy and Hartley, 2018). Under mild stress conditions, mitochondrial components can deteriorate, and mitochondria can revive by the process of mitochondrial fusion or fission event and maintain the cellular energy demands (Wai et al., 2015; Murphy and Hartley, 2018). However, under severe stress conditions, mitochondria cannot revive and need to be removed from the cell to maintain a healthy cellular condition. Mitochondrial protein quality control (MQC) is the most vital process for maintaining mitochondrial homeostasis, removing dysfunctional or superfluous mitochondria such that the cells are shielded against oxidative stress (Picca et al., 2018; Bakula and Scheibye-Knudsen, 2020). Damaged mitochondria can be removed from the cell by a vesicular process, where

mitochondria with the help of some chaperones are engulfed into a double membrane structure and then fused with the lysosome for degradation and recycling of degraded materials. Several studies suggest that dysfunctional MQC accumulates damaged mitochondria and generates ROS production, leading to the progression of several diseases, including CVD, Alzheimer's disease, and Parkinson's disease (Kornfeld et al., 2015; Kolodkin et al., 2020; Wenzhang Wang et al., 2020). PTEN-induced kinase 1 (PINK1) and Parkin play an important role in maintaining healthy mitochondrial pull and regulation of MQC (Gong et al., 2015).

Accumulating evidence from different studies suggests that PINK1 plays a dual role in the regulation of MQC (Ge et al., 2020). PINK1 is a mitochondrial serine/threonine-protein kinase that undergoes post-translational cleavage in the inner mitochondrial matrix and generates a c-terminal PINK1 (c-PINK1). Previous studies suggest that c-PINK1 in association with cellular chaperone can stay in the cytosol or be degraded through the UPS system. It was demonstrated that c-PINK is important for the biogenesis of mitochondria as well as to protect the healthy mitochondrial pull in the cytosol. c-PINK inhibits the Parkin protein translocation to the mitochondria as well as degradation of Parkin by the UPS system. Additionally, c-PINK can protect the mitochondria from mitophagy by promoting the phosphorylation of LC3 and upregulation of the mTORC2 pathway (Nan Wang et al., 2020). However, during severe stress and irreversible mitochondrial damage, PINK1 promotes mitophagy to remove damaged mitochondria from the cell. During stress, PINK1 is not processed by the mitochondria, and unprocessed PINK1 accumulates on the outer mitochondrial membrane (OMM) of the damaged mitochondria. Membrane-associated PINK1 can undergo autophosphorylation as well as phosphorylate the ubiquitin molecule tagged with the OMM proteins. These changes promote the translocation of the Parkin protein to the damaged mitochondria and ubiquitinate the mitochondrial surface proteins. Ubiquitinated proteins can be recognized by the mitophagy adaptor proteins like P62, LC3 and undergo the mitophagy process (Nan Wang et al., 2020).

Earlier studies have reported that the physical interaction between PINK1 and BAG5 plays a pivotal role in modulating PINK1 protein degradation (Wang et al., 2014). It was shown that BAG5 overexpression led to a marked increase in the level of PINK1 protein accompanied by a reduction in PINK1 degradation. Also, BAG5 knockdown significantly reduced PINK1 levels, which was associated with increased PINK1 ubiquitination. This indicated that BAG5 monitors the PINK1 level and abrogates its degradation via stabilizing PINK1 protein and inhibiting Parkin E3 ligase activity (Wang et al., 2014). Apart from this, it was also demonstrated that the decline in the PINK1 level or suppression of PINK1 expression could lead to a significant increase in the BAG5 level and increased localization of BAG5 protein in the mitochondria. Probably increased expression of BAG5 in PINK1-suppressed cells is a compensatory feedback regulatory mechanism of cells (Wang et al., 2014). Besides, the authors reported that administration of MPP^+ (neurotoxin) induced mitochondrial dysfunction (loss of mitochondrial membrane potential, cytochrome c release, augmentation in ROS level) was overcome by increasing the expression of BAG5 protein and subsequent upregulation of endogenous PINK1 protein (Wang et al., 2014). Furthermore, another group of researchers reported that MPP^+ (1-methyl-4-phenylpyridinium, neurotoxin)-administration induced apoptosis in PC12 cells (cells derived from rat adrenal medulla). However, BAG5 overexpression protected the PC12 cells against MPP^+-induced apoptosis by significantly upregulating various proteins, including Bcl-2 (anti-apoptotic protein) and Bcl-xl, decreasing cleaved caspase-3 level, reducing cytochrome c release, and inactivating downstream signaling cascade of apoptosis (Ma et al., 2012).

Another study reported that under mild stress, Parkin-dependent mitophagy is minimized as BAG5 impedes recruitment of Parkin into the depolarized mitochondria in the human osteosarcoma U2OS cell line. Meanwhile, simulation of chronic stress *via* dissipation of mitochondrial membrane potential with carbonyl cyanide m-chlorophenyl hydrazine (CCCP), BAG5 promoted apoptosis by enhancing Parkin-dependent Mcl-1 (pro-survival factor) polyubiquitination and degradation in SH-SY5Y neuroblastic cells. However, knockdown of BAG5 partially reduced Parkin-dependent Mcl-1 degradation in CCCP treated cells. Also, BAG5 overexpression-dependent enhanced cell death was overcome by knocking out Parkin using CRISPR/Cas. This indicates that Parkin interacts with BAG5 and other chaperone proteins during stressful conditions, which can promote Parkin's switching from pro-survival to the pro-apoptotic pathway (De Snoo et al., 2019).

A recent study with rat cardiomyocytes by Tan et al. showed that Hexokinase-II (HK-II) could play an important role in MQC. HK-II can sense metabolic disturbance of cells and modulates its intracellular localization to modulate mitophagy and cell survival. It has been reported that administration of mitochondrial HK-II (mitoHK-II) dissociating peptide reduces mitochondrial HK-II levels but increases Parkin translocation to the mitochondria and the ubiquitination of mitochondrial proteins in the cardiomyocytes. Interestingly, BAG5 owes the ability to localize to mitochondria and form a complex with HK-II. The exposure of the cardiomyocytes to an ischemic condition induced modest dissociation of mitoHK-II. In fact, the enhancement of this process protected the cardiomyocytes against ischemic damage via inducing mitophagy. The authors reported that BAG5 overexpression inhibits HK-II translocation from mitochondria and protects the mitochondria from Parkin-mediated mitophagy. This study suggests that BAG5 is an important co-chaperone of the HK-II and helps in maintaining an optimum level of mitophagy in basal conditions (Tan et al., 2019).

ROLE OF BAG5 IN REGULATING ENDOPLASMIC RETICULUM STRESS AND CELL VIABILITY

Our previous study indicated that during endoplasmic reticulum (ER) stress, unfolded proteins accumulate, which activates unfolded protein response (UPR). To counter the UPR during ER stress, the cells might upregulate the expression of ER stress-related chaperone and co-chaperone proteins to facilitate the protein folding capacity of ER and subsequently reduce ER stress (Ren et al., 2021). We reported that tunicamycin (antibiotic to induce endoplasmic reticulum stress) administration in the

cardiomyocytes was associated with an enhancement in the expression of ER-associated proteins, i.e., GRP78, and pro-apoptotic C/EBP homologous protein (CHOP). Additionally, it was found that during tunicamycin-mediated ER stress, BAG5 expression increased in a time-dependent manner in the cardiomyocytes, and the interaction between BAG5 and GRP78 was considerably increased. It is manifested that GRP78 elicits protective effects against ER stress either by acting as a molecular chaperone or reducing the load of UPR. It was seen that during ER stress, the interaction of BAG5 with GRP78 was significantly increased such that it tended to stabilize GRP78. Intriguingly, it was found that BAG5 protein overexpression considerably reduced cell death and improved the viability of cardiomyocytes via reducing the expression of CHOP and caspase-3 (Gupta et al., 2016). This indicates that BAG5 plays a protective role against stress via improving the survival of cardiomyocytes (Gupta et al., 2016). However, knockdown of BAG5 remarkably increased cell death and reduced cell viability in the cardiomyocytes. This indicates that BAG5 exhibits protective effects against endoplasmic stress during stressful conditions by modulating the expression of stress-induced CHOP and GRP78 proteins (**Figure 2**) (Gupta et al., 2016).

Similarly, another recent study reported that BAG5 acts as an ER stress regulator and its expression contributes to maintaining endothelial cell viability and protects cells from endothelial dysfunction (Zhu et al., 2021). This study found that catecholamine-dependent endothelial dysfunction immensely causes an increase in oxidative stress via augmenting ROS production and decreasing the levels of antioxidant factors viz. superoxide dismutase, glutathione, and glutathione peroxidase. The study unraveled that BAG5 overexpression could overcome catecholamine-dependent endothelial dysfunction and improve cell survival *via* decreasing oxidative stress and improving endothelial cell survival. Mechanistically, it was found that catecholamine treatment significantly increases protein kinase R-like endoplasmic reticulum kinase (PERK) and activates transcription factor 6 (ATF6) levels in the endothelial cells. Apparently, PERK and ATF6 are endoplasmic stress transducers and induce either apoptosis or autophagy. It was found that BAG5 overexpression considerably reduced the level of PERK and ATF6 in the endothelial cells indicating that the endoplasmic stress was negatively correlated to BAG5 expression. Also, it was found that catecholamine treatment inhibits ERK activity in the cells, accompanied by a reduction in BAG5 transcription. Intriguingly, MAPK-ERK activation using a pharmacological agonist reversed the decline in BAG expression in the dysfunctional cells. This indicated that catecholamine-dependent BAG5 downregulation was possible because of MAPK-ERK inactivation and reactivation of MAPK-ERK abrogated the endoplasmic reticulum stress, oxidative stress, and improved the cell survival via modulating MAPK/ERK signaling and BAG5 transcription (Zhu et al., 2021). The role of BAG5 as a stress regulator is further substantiated by a very recent study which revealed that exposure of primary neuronal cultures to H_2O_2 for 6 h induced oxidative stress. Also, this was accompanied by a reduction in the expression of BAG5 protein (Duggan et al., 2021). This indicates that stressful conditions downregulate BAG5 expression and induce cell death.

ROLE OF microRNAs IN REGULATING BAG5 EXPRESSION

A previous study showed that microRNA miR-155 is involved in various pathological processes including CVDs (Gangwar et al., 2018). Xi et al. reported that exposure of H9C2 cells to hypoxia and thereafter, reperfusion led to a significant increase in cell death, accompanied by an increase in the expression of miR-155. Interestingly, the increase in the expression of miR-155 was accompanied with downregulation in the expression of BAG5 protein. Further, the authors found that transfection of miR-155 mimic into the H9C2 cells also reduced BAG5 protein expression and cell viability. This indicated that miR-155 negatively regulated BAG5 protein expression and cell viability. However, the authors also found that inhibition of miR-155 reduced myocardial apoptosis, improved cell injury, and this protective effect was further promoted by BAG5 protein overexpression. Also, the inhibition of miR-155 was followed by reduced JNK/MAPK signaling. This indicated that miR-155 modulated myocardial hypoxia-reperfusion injury via regulating the JNK/MAPK signaling and the downregulation of BAG5 expression (Xi et al., 2020).

This is further corroborated by a recent study where miR-155 (siRNA) transfection into UE6E7T-2 cells (human mesenchymal stem cell) disrupted mitophagy and interfered with mitochondrial PQC in the cells. The authors found that miR-155 directly reduced BAG5 expression and considerably augmented the ubiquitination and degradation of PINK1 protein for clearing the damaged mitochondria. This indicated that BAG5 expression regulates ubiquitination status and a decline in its expression results in exaggerated ubiquitination of the mitochondrial proteins (Tsujimoto et al., 2020). Thus, microRNAs modulate BAG5 expression, and its interaction with PINK1 plays a critical role in maintaining mitochondrial homeostasis under stressful conditions. Additionally, it was demonstrated that miR-4454 and miR-127 suppress the cancer cells through regulation of BAG5 expression and over expression of BAG5 can neutralize the suppressive effect of miR-127 and miR-4454 (Bi et al., 2016; Dasari et al., 2020).

ROLE OF BAG5 IN THE REGULATION OF THE MTOR PATHWAY

A recent study published by Wang et al. showed that expression of BAG5 significantly decreased in the cisplatin-resistant ovarian cancer tissue, and down regulation of BAG5 made the cancer cells chemoresistant (Wang et al., 2021). Further, the authors found that knockdown of the BAG5 causes metabolic reprogramming and maintenance of cancer stem cells like feathers of the ovarian cancer cells. Mechanistically, it was demonstrated that Bcl6 protein binds to the promoter of the BAG5 gene and causes transcriptional suppression of BAG5 gene expression. Additionally, it was demonstrated that suppression of BAG5 may cause the upregulation of the rictor-mTORC2 pathway which modulates cellular metabolism and cancer stem cell-like feathers of cisplatin-resistant ovarian cancer cells. Furthermore, the authors concluded that BAG5 could be useful as a novel target to control cancer cell growth. However, the role of BAG5 in the

regulation of autophagy through mTOR pathway needs to be investigated.

ROLE OF BAG5 IN PARKINSON'S DISEASE

Astonishingly, some studies have reported that BAG5 interaction with other chaperones might interfere with their E3 ubiquitin ligase activity such that it might cause accumulation of toxic oligomers of α-synuclein (α-syn) (Chen et al., 2020). Parkinson's disease is a neurodegenerative disorder characterized by abnormal protein homeostasis causing accumulation of the protein α-syn in the form of Lewy bodies (Orme et al., 2018; Goldman et al., 2020). Previous studies have indicated that the carboxyl terminus of HSP70-interacting protein (CHIP, a co-chaperone) exhibits E3 ubiquitin ligase activity such that it may hamper the accumulation of toxic α-syn oligomers (Kalia et al., 2011). The authors unveiled that in the brain, CHIP, α-syn, and BAG5 interact *via* HSP70 protein.

CHIP comprises of tetratricopeptide repeat (amino-terminal), which facilitates its interaction with HSP70/90, whereas the U-box domain (carboxy-terminal) promotes the interaction with BAG5. The interaction of BAG5 with CHIP protein (Carboxy terminus of HSP-70 Interacting protein) impedes its E3 ubiquitin ligase activity in human H4 neuroglioma cells. This results in reduced α-synuclein ubiquitinylation and increases the propensity for α-synuclein to oligomerize and form intracellular protein aggregates (Lewy bodies), which are toxic to the cells. This is further supported by the fact that knockdown of CHIP promoted α-synuclein oligomerization, indicating that CHIP-dependent ubiquitinylation reduces the formation of α-synuclein oligomers (Kalia et al., 2011). Additionally, another study demonstrated that oxidative stress inducers like H2O2 and etoposide could induce the expression of transcription factor p53. Oxidative stress-induced P53 binds to the promoter region of the BAG5 gene and causes upregulation of BAG5 protein expression. Also, it was shown that colocalization of BAG5 and α-synuclein significantly increases in the cells which might result in α-synuclein aggregation. This suggests that BAG5 protein may play an important role in the progression of Parkinson's disease (Chen et al., 2020).

Apart from the Parkin gene, DJ-1 is another pathological gene involved in the onset of Parkinson's disease. DJ-1 is a multifunctional protein with an antioxidant property and acts as a molecular chaperone to maintain cellular homeostasis. Under normal physiological conditions, DJ-1 localizes in the mitochondria and monitors its activity *via* binding to subunits of mitochondrial complex I. However, during oxidative stress, DJ-1 dimerizes and with the help of chaperones, translocates to the mitochondria to prevent oxidative stress-induced cell death. In fact, DJ-1 interacts with proteins including Parkin, PINK1, and HSP70 to protect the cells against oxidative stress-induced disorders. Interestingly, it has been reported that the interaction of BAG5 and DJ-1 protein may enhance apoptotic cell death in neurodegenerative disorders, including Parkinson's disease. Qin et al. showed that exposure of HEK293 cells to rotenone (inhibitor of complex I of the mitochondrial respiratory chain) co-expressing BAG5 and DJ-1 significantly increased the ROS production in comparison to the cells expressing DJ-1 alone. This indicated that the protective effects of DJ-1 against rotenone-induced cell apoptosis are abolished in the presence of BAG5 protein. Further, the authors reported that overexpression of BAG5 in HEK293 cells decreases DJ-1 levels indicating that BAG5 inhibits its dimerization and mitochondrial translocation. However, this effect could be reversed by overexpressing HSP-70 protein. This suggests that HSP-70 and BAG5 protein act in concert to regulate the stability of DJ-1 protein and subsequently regulate cell viability (Qin et al., 2017).

ROLE OF BAG5 IN ALZHEIMER'S DISEASE

Various studies have reported that the expression of BAG5 protein in the brain monitors the development of neurodegenerative diseases (Kalia et al., 2011; Guo et al., 2015). Using human neuroblastoma cells (SH-SY5Y), Guo and co-workers reported that BAG5 expression is modulated in Alzheimer's disease. Furthermore, the authors revealed that BAG5 expression at both transcriptional and translational levels is upregulated in the transgenic mice suffering from Alzheimer's disease. In fact, *in-vitro* experiments revealed that administration of Aβ1-42 (10 μM) increased the expression of BAG5 in a dose-dependent manner. However, inhibition of BAG5 expression using siRNA exaggerated ROS generation, augmented malondialdehyde levels, and promoted Aβ1-42-mediated neurotoxicity. This was also accompanied by caspase-3 cleavage and elevation in the number of apoptotic cells (Guo et al., 2015). However, further research is needed to understand the role of BAG5 in neurodegenerative diseases.

CONCLUSION

It is now believed that aging is the most significant risk factor associated with CVDs, neurological disorders, inflammation, metabolic abnormality, and drug toxicity which causes premature death worldwide. The past 2 decades of research have generated several novel findings and determined the risk factors associated with aging. It was noticed that dysregulation of cellular proteostasis could cause formation of cellular protein aggregates, which can lead to the development of CVDs and neurological disorders. Autophagy and UPS are two important cellular PQC systems, which remove the unfolded or aggregated proteins from the cell and maintain a clean cellular environment. Mitochondria plays a vital role in the supply of high energy demands in the muscles and brain. Environmental stress can affect mitochondrial function and lead to ROS production through stressed mitochondria. Although, mitochondrial biology is known, we are still not clear about the role of chaperones and co-chaperones in maintaining healthy mitochondria in the cells. BAG5 protein plays a critical role in maintaining cellular PQC under normal and stressful conditions. Additionally, BAG5 can regulate the turnover of mitochondria during normal and stressful conditions. Earlier studies suggest

that BAG5 can promote cell death, but recent studies from own laboratory as well as from other laboratories show that BAG5 can protect cells from oxidative stress and modulate autophagy in the cell to improve cell survival. Also, micro RNAs can regulate the expression of BAG5 protein to limit cell apoptosis and subsequently death. Since BAG5 protein expresses in the heart and brain, it has become a novel target that can be exploited for the development of treatment strategies for curing cardiovascular and cerebrovascular disorders.

AUTHOR CONTRIBUTIONS

Writing and editing the manuscript MG, PR, and MM.

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REVIEW
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STIM and Orai Mediated Regulation of Calcium Signaling in Age-Related Diseases

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Tight spatiotemporal regulation of intracellular Ca^{2+} plays a critical role in regulating diverse cellular functions including cell survival, metabolism, and transcription. As a result, eukaryotic cells have developed a wide variety of mechanisms for controlling Ca^{2+} influx and efflux across the plasma membrane as well as Ca^{2+} release and uptake from intracellular stores. The STIM and Orai protein families comprising of STIM1, STIM2, Orai1, Orai2, and Orai3, are evolutionarily highly conserved proteins that are core components of all mammalian Ca^{2+} signaling systems. STIM1 and Orai1 are considered key players in the regulation of Store Operated Calcium Entry (SOCE), where release of Ca^{2+} from intracellular stores such as the Endoplasmic/Sarcoplasmic reticulum (ER/SR) triggers Ca^{2+} influx across the plasma membrane. SOCE, which has been widely characterized in non-excitable cells, plays a central role in Ca^{2+}-dependent transcriptional regulation. In addition to their role in Ca^{2+} signaling, STIM1 and Orai1 have been shown to contribute to the regulation of metabolism and mitochondrial function. STIM and Orai proteins are also subject to redox modifications, which influence their activities. Considering their ubiquitous expression, there has been increasing interest in the roles of STIM and Orai proteins in excitable cells such as neurons and myocytes. While controversy remains as to the importance of SOCE in excitable cells, STIM1 and Orai1 are essential for cellular homeostasis and their disruption is linked to various diseases associated with aging such as cardiovascular disease and neurodegeneration. The recent identification of splice variants for most STIM and Orai isoforms while complicating our understanding of their function, may also provide insight into some of the current contradictions on their roles. Therefore, the goal of this review is to describe our current understanding of the molecular regulation of STIM and Orai proteins and their roles in normal physiology and diseases of aging, with a particular focus on heart disease and neurodegeneration.

Keywords: calcium, neurodegeneration, cardiovascular disease, STIM, orai, cell survival, SOCE channels

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1 INTRODUCTION

It is universally recognized that tight spatiotemporal regulation of cytoplasmic Ca^{2+} is essential for cellular homeostasis and that dysregulation of Ca^{2+} signaling is associated with the development of pathophysiology. Homologs of human plasma membrane Ca^{2+} channels have been found in organisms as distant as the protozoan *Naegleria gruberi* demonstrating that regulation of

extracellular influx as a Ca^{2+} signaling mechanism has existed for over 1 billion years (Collins and Meyer, 2011). Eukaryotic cells have developed evolutionary highly conserved mechanisms for controlling Ca^{2+} influx and efflux across the plasma membrane, and Ca^{2+} release and uptake from intracellular stores, such as the endoplasmic reticulum (ER).

In the late 1970s, Putney reported a potential link between the transient release of Ca^{2+} from intracellular stores to subsequent influx of extracellular Ca^{2+} (Putney, 1977). The biophysics underlying this phenomenon, which subsequently became known as store-operated Ca^{2+} entry (SOCE), became increasingly well characterized over the following two decades (Parekh and Putney, 2005). It was found that physiologically, Ca^{2+} release from ER/SR was triggered in an agonist-dependent manner, typically, although not exclusively, via inositol 1,4,5-trisphosphate (IP_3)-mediated activation of the IP_3 receptor (IP_3R). This was followed by the activation of a highly selective non-voltage gated, Ca^{2+} channel in the plasma membrane. In contrast to IP_3-induced release of Ca^{2+} from intracellular stores, which results in transient increases in Ca^{2+} of the order of seconds or less, SOCE can remain active for minutes or longer (Soboloff et al., 2012). The longer duration of SOCE is an important factor in its role in Ca^{2+}-dependent regulation of gene transcription, such as the canonical Ca^{2+}/calmodulin-dependent activation of the phosphatase calcineurin, followed by dephosphorylation and nuclear translocation of transcription factors such as nuclear factor of activated T cells (NFAT) and nuclear factor kappa B (NF-κB) (Parekh and Putney, 2005). However, the identity of the molecular mediators of SOCE remained elusive until a remarkable series of papers published in 2005 and 2006 identified Stromal Interaction Molecule-1 (STIM1) and the Calcium Release-Activated Calcium Modulator 1 (CRACM1, now known as Orai1) as the ER/SR Ca^{2+} sensor and the plasma membrane Ca^{2+} channel respectively, that together regulated SOCE (Roos et al., 2005; Zhang et al., 2005; Vig et al., 2006b; Feske et al., 2006; Mercer et al., 2006; Peinelt et al., 2006; Prakriya et al., 2006; Soboloff et al., 2006; Taylor, 2006; Yeromin et al., 2006; Zhang et al., 2006).

Since their identification, STIM1 and Orai1 have been widely accepted as being essential components of SOCE. As discussed below, the detailed molecular interactions between the two proteins required to facilitate SOCE have been elucidated; however, the role of their homologs STIM2, Orai2, and Orai3 remain poorly understood. To complicate matters further, several variants of STIM1, STIM2, Orai1, and Orai2 have also been identified (Gross et al., 2007; Darbellay et al., 2011; Fukushima et al., 2012; Miederer et al., 2015; Rana et al., 2015; Knapp et al., 2020; Ramesh et al., 2021). While the molecular mechanisms underlying the regulation of SOCE have been almost exclusively studied in non-excitable cells, the expression of STIM1 and Orai1 is ubiquitous, and consequently they are also found in excitable cells including myocytes and neurons. However, ongoing controversies regarding the presence of SOCE in excitable cells has suggested possible non-canonical functions of STIM1, Orai1, and their homologs in such cells. Therefore, the goal of this review is to provide a thorough understanding of the molecular regulation of STIM and Orai proteins, their roles in normal physiology. We also discuss their roles in regulating mitochondrial function and metabolism, redox regulation, and cell survival mechanisms—all of which are components of normal healthy aging. Much of the work on the roles of STIM and Orai has been focused on non-excitable cells, particularly that related to the immune system; however, there is growing evidence that they are also involved in regulating the function of excitable cells such as neurons and cardiomyocytes. Therefore, we have also discussed the contributions of defects in STIM and Orai function in key age-related diseases such as cardiovascular disease and neurodegeneration. We have also summarized the few studies that have examined the potential roles of STIM and Orai dysfunction in the normal aging process.

2 STIMS—GENE AND PROTEIN STRUCTURES

2.1 STIM1

In 2005 two independent studies, both using siRNA arrays, identified for the first time, that STIM1 played a central role in mediating SOCE (Liou et al., 2005; Roos et al., 2005). In 1996 there were two reports describing a protein of unknown function, one identified a gene called *GOK* that was predicted to encode a protein that contained a transmembrane helix (Parker et al., 1996), the other identified a stromal interacting molecule (SIM) (Oritani and Kincade, 1996). SIM and GOK were subsequently named STIM1. Some lines of evidence suggested it might be a tumor suppressor gene (Parker et al., 1996; Sabbioni et al., 1997), but its function remained elusive. Early studies correctly characterized STIM1 as a type 1 transmembrane protein that was widely expressed and highly conserved. It was also shown to be phosphorylated in the C-terminal region, a possible target for mitogen-activated protein kinases (MAPK), and initially identified as cell surface protein (Manji et al., 2000). In addition, it was recognized that the N-terminal region contained consensus sequences for EF-hand calcium binding motifs (Williams et al., 2001). In 2005, in addition to demonstrating that STIM1 was essential for SOCE, Liou *et al.* reported that STIM1 was located primarily in the ER (Liou et al., 2005). Moreover, they also showed that ER Ca^{2+} depletion resulted in the redistribution of STIM1 into puncta that were close to the plasma membrane, and that this redistribution of STIM1 occurred because its EF-hand motifs sensed decreases in ER Ca^{2+} (Liou et al., 2005). These fundamental observations regarding STIM1 function, were confirmed later the in same year by Zhang *et al.* (Zhang et al., 2005). Although predominantly located in the ER, depending on cell type and cell cycle, 5–20% of STIM1 is also found at the plasma membrane (Mignen et al., 2007; Hewavitharana et al., 2008; Ercan et al., 2012).

2.1.1 STIM1 structure

The domain structure of mammalian STIM1 (**Figure 1**) is characterized by an ER signal peptide, followed by a canonical EF-hand (cEF) Ca^{2+} binding domain in the N-terminal region of the protein. The cEF-hand domain localized to the lumen of the ER (Gudlur et al., 2020), is critical to the Ca^{2+} sensing function of

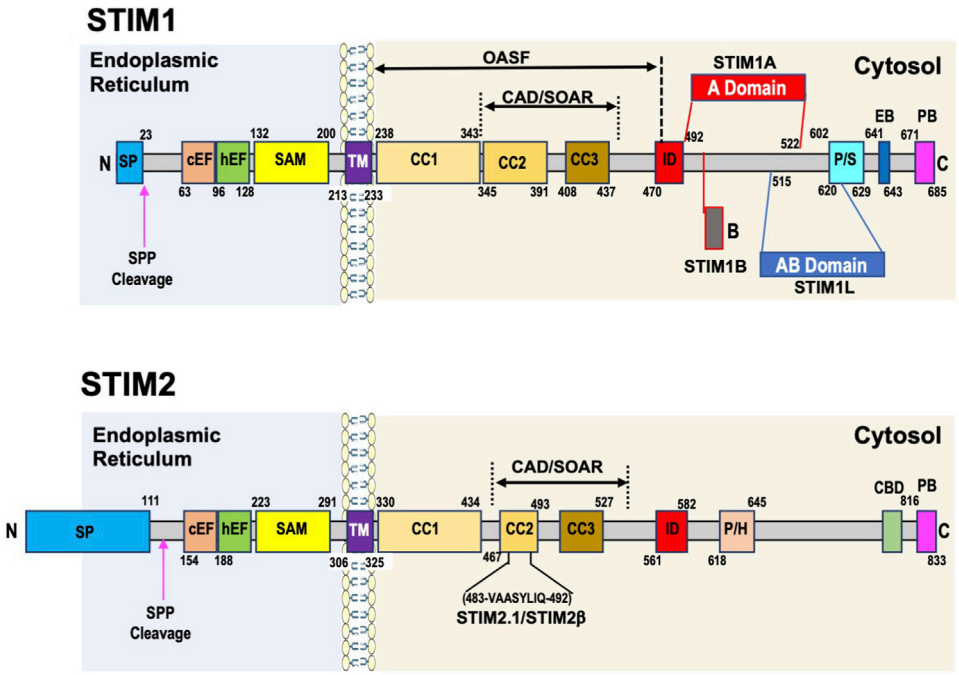

FIGURE 1 | Domain structure of human STIM1 and STIM2 proteins. STIM1 has a short signaling peptide (SP) at the N-terminus which is cleaved by a signaling peptide peptidase (SPP) when the protein is localized to ER membrane. In the endoplasmic reticulum the key domains are canonical and hidden EF hand domains (cEF, hEF) and the sterile alpha motif (SAM domain. These regions play a critical role in regulating SOCE, with the cEF domain as the sensor of ER Ca^{2+}, and the hEF and SAM domains contributing to the initial conformation changes that are transmitted via the transmembrane domain (TM) to coiled coil (CC)1 domain, which releases the CAD/SOAR region (CRAC-activating domain/STIM-Orai-activating region), which includes CC2 and CC3 domain, facilitating both the extension of the C-terminal domain towards the plasma membrane and STIM1 oligomerization, both of which are required for SOCE activation. Other important cytosolic domains include the inhibitory domain (ID), which is involved in Ca^{2+} dependent inhibition of SOCE; the proline/serine domain (P/S), which contains many phosphorylation sites that can regulate STIM1 function; the End binding protein domain (EB) and the polybasic domain (PB) at the C-terminus. STIM1L is a splice variant found predominantly in striated muscle includes the insertion of an actin binding domain (ABD) between aa515 and aa620. STIM1A is another splice variant with insertion of an A domain just after the ID domain between aa492 and 522. STIM2 architecture overall is very similar to STIM1 with a few key differences. Like STIM1 it has cEF, hEF, and SAM domains in the endoplasmic reticulum, a TM domain, followed by CC1, CC2, and CC3 domains in the cytosol with a PB region at the C-terminus. As described in the text subtle differences in these regions lead to changes in function compared to STIM1. Differences with STIM1 include an unusually long signaling peptide region of ~100aa, a proline/histidine region (P/H), and a calmodulin binding domain (CBD) close to the PB region. It is unclear whether STIM2 contains an ID region. There are two known STIM2 splice variants, one STIM2.1 also known as STIM2β involves the insertion of an 8 amino acid sequence (VAASYLIQ) within CC2 domain.

STIM1. Mutations in the cEF region of STIM1 decrease its sensitivity to ER Ca^{2+} concentrations and result in a constitutively active STIM1 (Liou et al., 2005; Zhang et al., 2005; Spassova et al., 2006). The cEF domain is followed by a hidden or non-canonical EF hand (hEF or ncEF), which does not bind Ca^{2+} and a sterile alpha-motif (SAM) domain (Stathopulos et al., 2008). The combined EF-SAM domains are key to regulating SOCE, with the cEF domain as the sensor of EF Ca^{2+} and the hEF domain essential for regulating the stability of the EF-SAM region. The transmembrane domain (TM) connects the ER and cytosolic regions of STIM1. In the Ca^{2+} bound state, the EF-SAM domains on STIM1 are kept apart to prevent spontaneous activation.

While the ER luminal region of the protein is critical for initiating the response of STIM1 to changes in ER Ca^{2+} levels, Huang *et al.*, demonstrated that the cytoplasmic carboxy-terminal domain of STIM1 was sufficient to activate Ca^{2+} entry in the absence of store depletion (Huang et al., 2006). Immediately following the TM domain are three coiled coil domains CC1, CC2, and CC3. An Orai1 activating STIM1 fragment (OASF) (Muik et al., 2009) as well as a minimal region required for gaiting Orai1 channels, the STIM1-Orai activating region (SOAR) also known as the Ca^{2+}-release-activated Ca^{2+} (CRAC) activation domain (CAD) were identified in the CC domains (Derler et al., 2016a; Lewis, 2020). The OASF spans all three CC domains while the CAD/SOAR region encompasses CC2 and CC3 domains. The CAD/SOAR region is divided into four helices, the first, Sα1, corresponds to CC2 and the last Sα4 to CC3, with Sα2 and Sα3 located between CC2 and CC3 domains (Yang et al., 2012). A mutation of a single amino acid Phe394 to histidine, in the Sα2 helix of the SOAR domain, completely prevented STIM1 activation of Orai1 (Wang et al., 2014). The Sα3 helical segment while not involved with STIM1 colocalization with Orai1 is essential for activating channel opening (Butorac et al., 2019). The CC2 component of the SOAR domain has been shown to contain a cholesterol binding region, which following store depletion binds cholesterol, acting as a negative regulator of SOCE (Pacheco et al., 2016).

When the SR Ca^{2+} stores are full (i.e., 1 to 5×10^{-4} M (Bagur and Hajnoczky, 2017)) the CC1 domain interacts with the SOAR/CAD region keeping it in an inactivated state by clamping it close to the ER membrane (Lewis, 2020). The CC1 domains contain 3 CC regions $CC1\alpha_1$, $CC1\alpha_2$, and $CC1\alpha_3$ and evidence suggests that the $CC1\alpha_1$ and $CC1\alpha_3$ regions, in combination with the CC2 and CC3 domains, play a key role in keeping the SOAR/CAD domain in the inactivated state (Fahrner et al., 2014). In response to store depletion, reorganization of the EF-SAM region coupled with reorientation of both the transmembrane helices leads to the homomerization of $CC1\alpha_1$ region and release of SOAR/CAD domain from its inactive state (Fahrner et al., 2020). The importance of the TM domain in contributing to this initial STIM1 conformational change was demonstrated by gain of function mutations in this region leading to constitutive STIM1 puncta formation and Ca^{2+} influx (Ma et al., 2015). The subsequent reorientation of the $CC1\alpha_1$, $CC1\alpha_2$, and $CC1\alpha_3$ regions in the CC1 domain not only helps to extend the SOAR/CAD domain towards the plasma membrane but also contributes to the oligomerization of STIM1 necessary for activation of SOCE (Fahrner et al., 2020). Of note however it is only the $CC1\alpha_1$ region that is essential for activating SOCE (Fahrner et al., 2014). The CC3 domains also contributes to STIM1 oligomerization leading to larger STIM1 clusters (Fahrner et al., 2014); the region aa420-450 of the CC3 domain has been described as a STIM1 homomerization domain (SHD) (Muik et al., 2009). The resulting extension of the STIM1 cytoplasmic section, enables the short polybasic (PB) region at the C-terminus to interact with plasma membrane phospholipids thereby partly facilitating the localization of STIM1 to ER-PM junctions. This is supported by the observation that deletion of this region decreases the size of STIM1 plasma membrane clusters that form following ER Ca^{2+} depletion (Maleth et al., 2014; Sauc et al., 2015). While the PB region is not essential for SOCE it appears to improve the efficiency with which STIM1 interacts with Orai1 (Lewis, 2020). Another important C-terminal regulatory domain is the Inhibitory domain (ID). Like other Ca^{2+}-channels, the STIM1-Orai1 channel is inhibited by Ca^{2+} in a feedback manner that occurs in a time frame of milliseconds. This process, called Ca^{2+} dependent inhibition (CDI), requires the ID domain although this domain itself is not the primary Ca^{2+} sensor for CDI of SOCE (Mullins and Lewis, 2016). Full CDI requires the interaction of the ID with key Orai1 tryptophan and tyrosine residues. Early studies suggested that calmodulin, similar to its role in regulating CDI in voltage gated Ca^{2+} channels, was the SOCE Ca^{2+} sensor for CDI (Mullins et al., 2009; Liu Y. et al., 2012); however, subsequent studies indicated that this was not the case (Mullins et al., 2016). Calmodulin has been implicated in a slower Ca^{2+}-dependent inactivation process via interaction with the SOAR/CAD domain facilitating dissociation between STIM1 and Orai1 (Li et al., 2017).

2.1.2 STIM1 regulation

Additional key regulatory domains in STIM1 are the end binding protein1 (EB1) domain and the Proline/Serine rich region (P/S). In 2008 Grigoriev *et al.*, identified STIM1 as an microtubule associated protein RP/EB family member 1 (EB1) interacting protein; however, the function of this interaction was unclear as loss of EB1 had no effect on SOCE (Grigoriev et al., 2008). More recent studies have shown that EB1 dynamically traps STIM1 thereby limiting excess STIM1 in ER-PM junctions, potentially preventing ER Ca^{2+} overload (Chang et al., 2018). EB1 is a microtubule plus-end tracking protein (+TIP) and is recognized as a master regulator of +TIP function and thus microtubule dynamics (Akhmanova and Steinmetz, 2008). STIM1 has also been identified as a +TIP and its EB domain contains a Thr-Arg-Ile-Pro sequence (TRIP), a motif common to other EB1 binding proteins (Akhmanova and Steinmetz, 2008; Grigoriev et al., 2008). Phosphorylation in regions adjacent to the EB/TRIP domain negatively regulate the interactions of +TIP with EB1 (Smyth et al., 2012). Of note, the P/S region of STIM1 is close to the STIM1 EB/TRIP domain, and phosphorylation of Ser575, Ser608, and Ser621 in that region by extracellular signal-regulated kinases 1/2 (ERK1/2) regulates the interactions between STIM1 and EB1, which is required for activation of SOCE (Pozo-Guisado and Martin-Romero, 2013). On the other hand, phosphorylation of Ser668 by cyclin dependent kinase 1 (CDK1) has been implicated in inactivation of SOCE during mitosis (Smyth et al., 2009). To date over 30 STIM1 phosphorylation sites have been mapped many of which are located in or adjacent to the P/S region (Hornbeck et al., 2004). Phosphorylation outside of the P/S domain has also been shown to modulate STIM1 function. For example, in endothelial cells ER Ca^{2+} depletion leads to phosphorylation of Tyr361 in the SOAR/CAD domain by proline rich kinase 2 (Pyk2) thereby facilitating SOCE (Yazbeck et al., 2017). AMPK phosphorylates STIM1 at Ser257, located in the CC1 domain, and phosphorylation of this site favors an inactive STIM1 conformation (Nelson et al., 2019). PKA phosphorylates Thr389 regulating a non-SOCE function of STIM1 (Thompson and Shuttleworth, 2015) and dual-specificity tyrosine phosphorylation-regulated kinase (DYRK2) phosphorylates Ser519 and Ser521, enhancing STIM1 and Orai1 interactions (Wei et al., 2021). A number of phosphorylation sites have also been identified in the N-terminal luminal domain of STIM1 (Hornbeck et al., 2004); however their function and kinases are not known.

STIM1 is also subject to oxidative modifications that affect its function. For example, Cys56 in the STIM1 luminal region is subjected to S-glutathionylation in response to oxidant stress, resulting in constitutive Ca^{2+} entry independent of Ca^{2+} store levels (Hawkins et al., 2010). Both Cys49 and Cys56 undergo nitric oxide (NO)-mediated S-nitrosylation, which resulted in stabilization of the EF-SAM region inhibiting SOCE (Gui et al., 2018). The modification of serine and threonine residues with O-linked N-acetylglucosamine (O-GlcNAc) is increasing recognized as an important nutrient mediated signaling mechanism (Chatham et al., 2021). STIM1 has been shown to be O-GlcNAcylated and pharmacologically mediated increases in O-GlcNAc attenuated STIM1 puncta formation and SOCE (Zhu-Mauldin et al., 2012). Nomura *et al.*, reported that Ser621 and Thr626 in STIM1 were O-GlcNAcylated (Nomura et al., 2020). They observed that decreased O-GlcNAcylation at Thr626 and increased O-GlcNAcylation at Ser621 both attenuated SOCE, possibly by decreasing Ser621 phosphorylation thereby changing STIM1 interactions with EB1.

2.1.3 STIM1 variants

Alternative splicing is another important mechanism for regulating protein function and STIM1L was the first STIM1 splice variant identified (Darbellay et al., 2011). Alternative splicing on exon 11 results in the insertion of 106 residues between the SOAR/CAD and P/S region in the C-terminal region of STIM1, which functions as an actin binding domain (ABD) (**Figure 1**). In contrast to the ubiquitous expression of STIM1, STIM1L appears to be restricted to striated muscle and brain in rodents (Darbellay et al., 2011) and skeletal muscle in humans (Horinouchi et al., 2012), although it is found in neonatal rat cardiomyocytes and in adult rodent hearts under stress (Luo et al., 2012; Sabourin et al., 2018). In skeletal muscle, the actin binding domain in STIM1L enables it to form permanent clusters with Orai1 thereby allowing for immediate activation of SOCE, which may be critical in excitable cells where there are large rapid changes in ER and cytosolic Ca^{2+} levels facilitating faster and more efficient refilling of ER (Darbellay et al., 2011). Database analysis predicts that several other STIM1 splice variants may occur and to date, two variants STIM1A and STIM1B have been characterized (Knapp et al., 2021; Ramesh et al., 2021). STIM1A contains an additional A domain comprising 31 residues, adjacent to the ID domain (**Figure 1**) and is highly conserved from fish to birds to mammals. STIM1A was found in heart, kidney, astrocytes, and testes, but was not present in T-cells. STIM1 and STIM1A both co-localized with Orai1 after ER Ca^{2+} depletion; surprisingly, however, STIM1A appears to function in a dominant negative manner, resulting in a decrease in SOCE possibly by interfering with the interaction between the STIM1 CAD/SOAR domain and Orai1 (Knapp et al., 2021). STIM1B has a truncated C-terminus that includes a novel B domain downstream of the ID domain (Ramesh et al., 2021). STIM1B was reported to be exclusively found in the brain and compared to STIM1 exhibits slower formation of oligomers in response to store depletion and differential interactions with all 3 Orai isoforms. The altered function of STIM1B appears to be primarily linked to the new B-domain rather that the absence of P/S, EB, and PB domains (Ramesh et al., 2021).

2.2 STIM2

In contrast to invertebrates that have a single STIM gene, mammals have two genes, STIM1 and STIM2. The STIM2 gene was cloned in 2001 and its fundamental structure characterized (Williams et al., 2001); however, its function was unknown.

2.2.1 STIM2 structure

In contrast to STIM1 where a variable fraction is located at the plasma membrane, a di-lysine ER-retention signal restricts STIM2 to the ER (Ercan et al., 2012). The overall structure of STIM2 is similar to STIM1 particularly in the N-terminal ER region (**Figure 1**), which contains SP, cEF, hEF, and SAM domains. The unusually long 101 residue STIM2 SP appears to contribute to reduced ER localization leading to a pool of uncleaved cytosolic preSTIM2 (Graham et al., 2011). It has been reported that the cytosolic preSTIM2 interacts with Orai1 at the plasma membrane potentially regulating basal Ca^{2+} levels (Graham et al., 2011). In addition, a 91aa fragment of the STIM2 signal peptide (SPF) is also released into the cytosol and may regulate NF-κB transcription (Graham et al., 2011). The rest of the luminal STIM2 N-terminus shares >80% homology with STIM1 (Stathopulos et al., 2009); however, differences in only 3 amino acids in the cEF hand sequence results in a 2-fold lower affinity for Ca^{2+} than STIM1 making it more sensitive to small changes in ER Ca^{2+} concentrations (Zheng et al., 2011). Despite a high degree of similarities between the SAM domains for STIM1 and STIM2, subtle differences in the STIM2 SAM domain result in a substantial increase in its stability (Zheng et al., 2008), which attenuates its rate of oligomerization in response to ER Ca^{2+} depletion (Zheng et al., 2011). In addition, small differences in the STIM2 TM domain compared to STIM1-TM also slows the transduction of ER Ca^{2+} depletion signal to the cytosolic portion of the protein (Zheng et al., 2018).

The C-terminal cytosolic region of STIM2 contains similar CC1, OASF, SOAR/CAD, ID, and terminal PB domains to those found in STIM1 (Grabmayr et al., 2020). There is exceptional homology between the STIM1 and STIM2 SOAR/CAD sequences; however, the switch of a single phenylalanine in STIM1 SOAR/CAD to leucine in STIM2 markedly reduces its ability to open Orai1 channels (Wang et al., 2014). Small differences in the S1α helix in the STIM2 CAD/SOAR domain compared to the STIM1 domain weakened the interactions between the CC1 and CC3 domains of STIM2. This resulted in a more open conformation of the STIM2 CAD/SOAR region resulting in increased clustering in ER-PM junctions under resting conditions (Subedi et al., 2018; Zheng et al., 2018). Moreover, the STIM2 PM domain has higher affinity for phosphatidylinositol biphosphate (PIP_2) than STIM1, which also helps facilitate STIM2 clustering with minimal changes in ER Ca^{2+} levels (Bhardwaj et al., 2013). On the other hand, these changes in the STIM2 CAD/SOAR domain reduced its activation of Orai1 compared to STIM1 (Zheng et al., 2018). STIM2 also contains a proline/histidine (P/H) rich region instead of the P/S region found in STIM1. While an EB domain in STIM2 has not been conclusively identified, in neurons STIM2 has been shown to bind EB3 via a similar TRIP motif to that seen in STIM1 (Pchitskaya et al., 2017). There is also a calmodulin binding site close to the PB domain (Bauer et al., 2008).

2.2.2 STIM2 regulation

STIM2 like STIM1 has numerous phosphorylation sites (>30), most of which are in the cytosolic C-terminal region (Hornbeck et al., 2004); however, little is known about their function or which kinases are involved. Like STIM1, STIM2 has cysteine residues in its luminal domain; two of them Cys53, and Cys60 are conserved with STIM1, and one Cys15 is unique to STIM2. All three residues can be S-nitrosylated leading to a synergistic stabilization of the EF-SAM region, reduced basal cytosolic Ca^{2+} and lower STIM2-mediated SOCE (Novello et al., 2020). In contrast to STIM1, STIM2 constitutively clusters at the ER-PM junctions in both mobile and immobile clusters with changes in both IP_3R function and ER Ca^{2+} levels being the driving factors contributing to the increases or decreases of immobile clusters of

STIM2 in ER-PM junctions (Ahmad et al., 2022). Under basal conditions the STIM2/Orai1 complex regulates basal Ca^{2+} concentrations whereas following agonist stimulation STIM1 forms clusters with STIM2 in response to a decrease in ER Ca^{2+} combined with a close association with IP_3R. Collectively these findings suggest that immobilization of STIM2 clusters is an early response to decreased ER Ca^{2+} levels, which is facilitated by IP_3R in the region of STIM2 clusters and acts as a "checkpoint" for Ca^{2+} entry (Ahmad et al., 2022).

2.2.3 STIM2 variants

In 2015, there were two reports describing a novel STIM2 splice variant, STIM2β (also referred to as STIM2.1), which antagonized STIM1-Orai1 mediated SOCE (Miederer et al., 2015; Rana et al., 2015). In different cell/tissue types there is a wide range in the expression ratio of STIM2.1 to the original STIM2 variant now known as STIM2.2 (or STIM2α). STIM2.1 also blunted the STIM2.2-mediated SOCE (Miederer et al., 2015). The antagonistic effects of STIM2.1 and wide range of cell-dependent ratios of STIM2.1/STIM2.2, might in part, account for the different conclusions of the earlier studies on STIM2 function. STIM2.2 is characterized by an 8 amino acid insertion in SOAR domain of STIM2.1; however, the mechanism by which this leads to SOCE inhibition remains unclear. It is possible that STIM2.1 forms heterodimers with STIM1 or STIM2.2, thereby preventing them from binding to Orai1, or STIM2.1 could actively inhibit SOCE via direct interaction with Orai1 (Rana et al., 2015). It is worth noting that bioinformatics analysis predicts at least an additional 4 human STIM2 splice variants, although to date, only STIM2.1 and STIM2.2 have been identified (Berna-Erro et al., 2017), suggesting that there is still much left to discover regarding STIM2 and its variants.

3 ORAIS—GENE AND PROTEIN STRUCTURES

3.1 Orai1

In 2001, Rao and colleagues identified major Ca^{2+} signaling defects in T-cells from a patient with severe combined immunodeficiency (SCID) (Feske et al., 2001). Subsequent studies with these cells demonstrated that although SOCE was almost completely abolished, STIM1 levels were normal (Feske et al., 2005), illustrating that while STIM1 was essential for SOCE it did not act alone. The fact that STIM1 was primarily localized to the ER strongly suggested that an unidentified plasma membrane Ca^{2+} channel was also involved in activating SOCE. Using genetic linkage analysis of the SCID patients and their family combined with a high throughput siRNA screen of SOCE in *Drosophila* S2 cells, a novel protein they named Orai1 and two human homologues Orai2 and Orai3 were identified (Feske et al., 2006); a single point mutation in Orai1 was responsible for the defective SOCE in cells from the SCID patients (*The name Orai originates from Greek mythology where Orai are the keepers or guardians of the gates of heaven (Feske et al., 2006)*). Two additional studies published in 2006 confirmed the essential role of Orai1 in SOCE and correctly predicted that it had 4 transmembrane domains with both C- and N-terminal regions in the cytosol (Vig et al., 2006b; Zhang et al., 2006). While Orai1 was clearly essential for SOCE, it had no homology with any other ion channel. As a result, initially it was unclear whether Orai1 was the elusive SOCE channel or instead a regulator of the channel (Shuttleworth, 2012). However, subsequent studies quickly established that interactions between STIM1 and Orai1 were required for SOCE and that Orai1 itself formed the plasma membrane channel that allows for Ca^{2+} entry to occur (Mercer et al., 2006; Prakriya et al., 2006; Soboloff et al., 2006; Yeromin et al., 2006).

3.1.1 Orai1 structure

The domain structure of Orai1 (**Figure 2**) consists of four transmembrane (TM) helices connected by two extracellular loops and one intracellular loop, with both the N- and C-terminal regions located in the cytosol. There is a proline arginine (PA) region close to the end of the N-terminal region that is involved in Orai1 reactivation (Frischauf et al., 2011), and includes an interacting site for adenylate cyclase-8 (AC8) (Willoughby et al., 2012). PIP_2 binding in the same region as AC8 has been reported to enhance Orai1-STIM1 interactions (residues 28–33) (Calloway et al., 2011). Adjacent to the plasma membrane is an α-helical extension of the TM1 domain known as the Extended Transmembrane Orai1 N-terminal (ETON) region. Almost the entire ETON region has been reported to be essential for binding with STIM1 and allowing STIM1-dependent Ca^{2+} entry (Derler et al., 2013); however, others have suggested that STIM1 interaction with the ETON region is not necessary for channel activation (Fahrner et al., 2018a). The ETON region contains calmodulin and cholesterol binding domains. The calmodulin binding domain has been reported to play a role in Ca^{2+} dependent inhibition (CDI) of SOCE (Mullins et al., 2009; Kar et al., 2014); however, it has also been suggested that this region is involved in CDI-independent of calmodulin binding (Mullins et al., 2016).

The interaction of cholesterol with Orai1 is complex with reports that it inhibits its activity and decreases SOCE (Derler et al., 2016b) on the other hand decreasing cholesterol reduced SOCE due to increased internalization of Orai1 channels (Bohorquez-Hernandez et al., 2017). Derler *et al.*, found that cholesterol depletion increased SOCE and identified a cholesterol binding motif in the region of the ETON domain that interacts with calmodulin (Derler et al., 2016b). Others have reported that cholesterol depletion reduces SOCE via increased internalization of Orai1 (Bohorquez-Hernandez et al., 2017). The role of cholesterol on SOCE is complicated by the fact that the SOAR region of STIM1 also has a cholesterol binding site (Pacheco et al., 2016). A caveolin binding domain has also been identified in the N-terminus and caveolin binding to Orai1 has been reported to increase SOCE (Yeh and Parekh, 2015; Bohorquez-Hernandez et al., 2017). However, mutation of these residues did not prevent the enhancement of SOCE that occurs in the presence of caveolin suggesting that Orai1 may contain another caveolin binding domain (Yeh and Parekh, 2015).

The first extracellular loop (loop 1) contains a Ca^{2+} accumulating region (CAR), formed by aspartate residues,

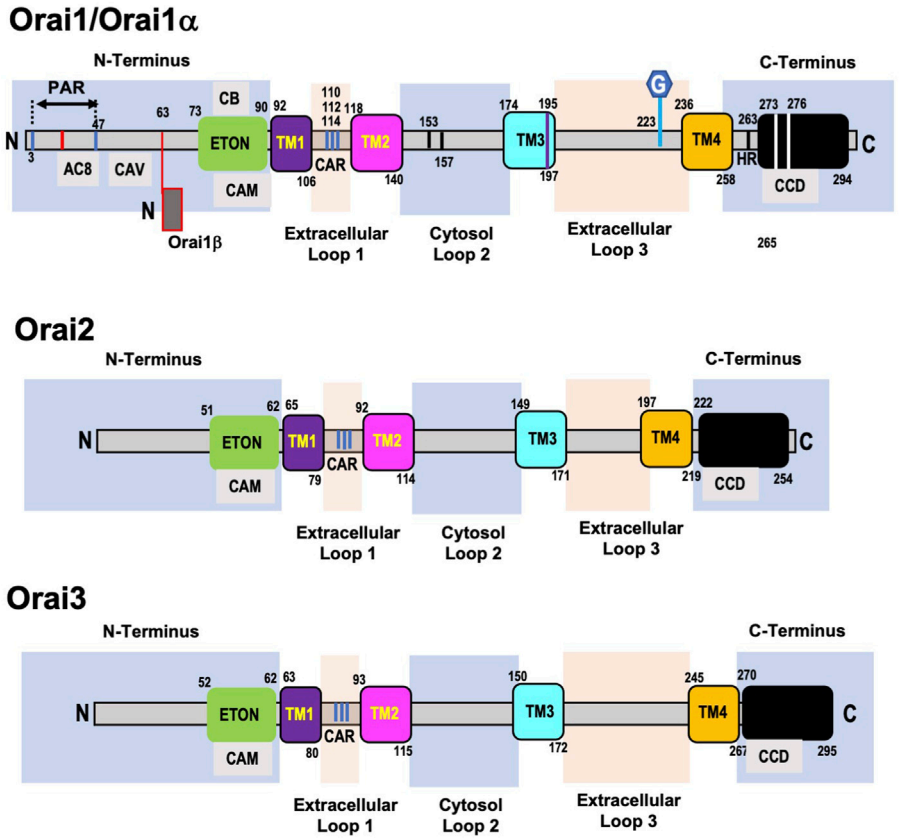

FIGURE 2 | Domain structure of human ORAI1, ORAI2, and ORAI3 proteins. Orai1 (Orai1α) has a proline arginine region (PAR) close to the end of the N-terminal domain, which includes an interacting site for adenylate cyclase-8 (AC8). This is followed by a caveolin binding region (CAV) and adjacent to the plasma membrane is the Extended Transmembrane Orai1 N-terminal (ETON) region, which contains calmodulin (CAM) and cholesterol binding (CB) domains. Following the first transmembrane domain (TM1) is the first extracellular loop, which includes a Ca^{2+} accumulating region (CAR). A region in the intracellular loop 2—153–157—is thought to regulate Ca^{2+} dependent inactivation of Orai1 and interaction between loop2 and the ETON region regulate channel activation. A cysteine residue (Cys195) at the end of the third transmembrane domain (TM3) has been implicated in the redox regulation of Orai1 function. The second extracellular loop (loop3) contains a N-glycosylation site at N223, of unknown function. The cytosolic C-terminus of Orai1 is connected to the 4th transmembrane domain (TM4) via a highly conserved hinge region (HR) and contains highly conserved coiled-coil domain (CCD). The C-terminal region essential for recruiting STIM1 and Orai1 channel activation. Alternative translation initiation results in Orai1β which lacks the first 63 amino acids of Orai1/Orai1α. The architecture of Orai2 and Orai3 are very similar to Orai1 with a few key differences. Orai2 and Orai3 have a truncated N-terminal region which lacks the PAR present in Orai1. They also lack an 18aa region in the N-terminus which regulates the slow reactivation that follows fast CDI in Orai1. Similar to Orai1 both Orai2 and Orai3 contain ETON region adjacent to the TM1, which includes a CAM domain. The TM1 domains are almost completely conserved, and the other 3 transmembrane domains exhibit a high level of homology across all Orai isoforms. However, Orai2 and 3 lack the cysteine residue in TM3 and the N-glycosylated site on extracellular loop 3 which are present in Orai1. Orai3 has a much longer loop 3 compared to both Orai1 and Orai2, but like Orai1, Orai2, and Orai3 also have coiled-coil domains their C-terminal regions.

which increase local Ca^{2+} concentrations facilitating Ca^{2+} entry even when extracellular Ca^{2+} concentrations are low (Frischauf et al., 2015). The TM2 and TM3 domains, are connected via an intracellular loop (loop2) and both have short helical extensions in the cytosol. Loop2 has been implicated in regulating fast CDI of the Orai1 channel possibly via blocking Ca^{2+} entry (Srikanth et al., 2010) and interactions between loop2 and the ETON region have been shown to regulate channel activation (Fahrner et al., 2018a). A cysteine residue was identified at the end of the TM3 domain, close to extracellular loop3, which has been implicated in the redox regulation of Orai1 function (Bogeski et al., 2010; Alansary et al., 2016). The second extracellular loop (loop3) that connects TM3-TM4 was shown to interact with loop1, potentially fine tuning Ca^{2+} accumulation in the CAR (Frischauf et al., 2015). Loop3 of Orai1 also contains a distinct N-glycosylation site at N223; the function of this modification is not known, but in some cell types, loss of this modification resulted in an increase in Ca^{2+} entry, suggesting that it may play a cell-specific role in regulating SOCE (Dorr et al., 2016). TM4 is connected to the cytosolic C-terminus of Orai1 via a highly conserved hinge region (Hou et al., 2012; Fahrner et al., 2018b). Residues in the C-terminal of Orai1 are essential for recruiting STIM1 and channel activation (Mcnally et al., 2013).

Each of the transmembrane domains contribute to the regulation of the Orai1 channel as indicated by the numerous gain and a loss of function mutations that have been identified throughout the regions (Yeung et al., 2018; Tiffner et al., 2020). Mutations of Gly98 and Val102 in the TM1 domain led to

constitutively active, non-selective currents, indicating they contribute to keeping the channel closed (Zhang et al., 2011; Mcnally et al., 2012). Multiple mutations in TM2 also result in constitutively active Ca^{2+} channels and are associated with various diseases including cancer (Endo et al., 2015; Frischauf et al., 2017). The TM3 domain contributes to Orai1 channel gating and ion selectivity as shown by the effects of mutations of Trp176 and Gly183 (Srikanth et al., 2011). The mutation of Pro245 in the TM4 domain to lysine still required STIM1 for activation of Orai1 but resulted in very slow inactivation of the channel and is associated with a myopathy in humans (Nesin et al., 2014). A key feature of the Oria1 channel is its high selectivity for Ca^{2+}, and this has been shown to be due to a set of conserved amino acids, Glu106, Glu190, Asp110, Asp112, Asp114 in TM1 and TM3 and the extracellular loop 1 (Prakriya et al., 2006; Vig et al., 2006a; Yeromin et al., 2006; Yamashita et al., 2007). As noted above mutation of Val102 in the TM1 also contributes to the Ca^{2+} selectivity of the Orai1 channel (Mcnally et al., 2012).

3.1.2 Orai1 regulation

Six phosphorylation sites have been identified in the N-terminal region and four in the C-terminal region of Orai1 (Hornbeck et al., 2004). Ser27 and Ser29 have both been shown to be phosphorylated by PKCβ (Kawasaki et al., 2010); Ser34 is a target of PKG (Wang et al., 2015) and PKA (Zhang et al., 2019). In each case phosphorylation either directly inhibits SOCE or contributes to CDI. The kinases and function of other phosphorylation sites have yet to be identified (Hornbeck et al., 2004). Alternative translation initiation results in a long variant Orai1α, which is the full length Orai1 and a short variant Orai1β adding another layer of functional regulation (Fukushima et al., 2012). Orai1β lacks the first 63 amino acids of Orai1α, a region that as noted above contains several potentially important signaling regions (Putney, 2018). Orai1α exhibited substantially slower plasma membrane mobility compared to Orai1β possibly as a result of the absence of the caveolin and PIP_2 binding regions; nevertheless, both isoforms form puncta with STIM1 and facilitated SOCE (Fukushima et al., 2012). While Orai1α and Orai1β appear to be functionally indistinguishable regarding SOCE, Orai1α exhibited stronger CDI (Desai et al., 2015). Interestingly, only Orai1α participated in the non-store-dependent arachidonic acid regulated Ca^{2+} (ARC) channels, suggesting the possibility of physiologically distinct roles for the two isoforms (Desai et al., 2015).

3.2 Orai2 and Orai3

Feske *et al.*, identified Orai2 and Orai3 during their initial studies characterizing the role of Orai1 in SOCE (Feske et al., 2006). Phylogenetic analysis showed that while Orai1 and Orai2 were present in vertebrates, Orai3 was only observed in mammals, suggesting that Orai3 evolved from Orai1 not Orai2 (Cai, 2007).

3.2.1 Orai2 and Orai3 structure

Like Orai1, Orai2, and Orai3 are ubiquitously expressed (Hoth and Niemeyer, 2013). The TM1 domains are almost completely conserved and the other 3 transmembrane domains exhibit a high level of homology across all Orai isoforms (Hoth and Niemeyer, 2013). Both Orai2 and Orai3 have a truncated N-terminal region which lacks the PA region seen in Orai1 (Shuttleworth, 2012). They also lack an 18aa region in the N-terminus with contributes to regulation of slow reactivation that follows fast CDI in Orai1 (Frischauf et al., 2011). This is consistent with the observation that Orai2 and Orai3 both exhibit fast CDI but this is followed by a slower inhibitory phase rather that the reactivation observed in Orai1 (Lis et al., 2007). There is a highly conserved region of 22 amino acids immediately before TM1, which includes the CAM binding domain (Shuttleworth, 2012). Despite 75% homology between cytosolic loop2 of Orai3 and Orai1, differences are sufficient to eliminate the role of the N-terminal domain in channel activation (Fahrner et al., 2018a). Orai2 and 3 lack the cysteine residue in TM3 that occurs in Orai1, resulting in decreased sensitivity of Orai2 and 3 to redox stress (Bogeski et al., 2010). Unlike Orai1, Orai2 and 3 are not N-glycosylated on extracellular loop 3; moreover, Orai3 has a much longer loop 3 compared to both Orai1 and Orai2, although the functional consequence of this is not known (Frischauf et al., 2011; Shuttleworth, 2012). Like Orai1, Orai2 and 3 also have coiled-coil domains in their C-terminal regions. Differences in binding affinities for STIM1 in the Orai C-terminal regions is reflected by the extent to which they trigger SOCE when overexpressed with STIM1, with Orai1 exhibiting larger SOCE compared to either Orai2 or Orai3.

3.2.2 Orai2 and Orai3 variants

There are two murine Orai2 splice variants with one Orai2S lacking 14 N terminal amino acids of Orai2L, with Orai2S potentially acting in a dominant negative fashion to block STIM1/Orai1 SOCE (Gross et al., 2007); to date this has not been observed in humans.

4 STIM AND ORAI MEDIATED Ca^{2+} SIGNALING

SOCE is characterized by a very specific Ca^{2+} current, I_{crac}, which reflects key biophysical properties including very high specificity for Ca^{2+}. STIM and Orai proteins are also involved in less selective store operated Ca^{2+} channels, resulting in a Ca^{2+} current known as I_{soc}, which can involve interactions of STIM and Orai proteins with transient receptor potential (TRP) channels (Ong and Ambudkar, 2011). While STIM and Orai proteins are essential for SOCE (I_{crac}), it has been shown that TRP channel mediated Ca^{2+} entry is not dependent on either STIM or Orai proteins (Dehaven et al., 2009). Therefore, the discussion below of STIM and Orai mediated Ca^{2+} signaling will not include consideration of TRP channels, which are reviewed in detail elsewhere (Vazquez et al., 2004; Chen X. et al., 2020). There is however, a store-independent Ca^{2+} channel, that is activated by arachidonic acid (AA) or its metabolite leukotriene and is dependent on both STIM and Orai proteins (Zhang et al., 2018). This channel commonly known as arachidonate-regulated Ca^{2+} (ARC) channel is responsible for a highly

selective Ca^{2+} current, I_{arc}, which has distinct physiological roles from SOCE (Zhang et al., 2018). Considering the essential role of STIM and Orai proteins in ARC channel activity, this is also discussed below.

4.1 Store Operated Ca^{2+} Entry

As noted above, STIM1 and Orai1 are essential for SOCE and required for I_{crac}; therefore, we will focus initially on the canonical function of STIM1 and Orai1 in the regulation of SOCE. The potential roles of STIM2, Orai2, and Orai3 will be considered later.

Under basal resting conditions, the cEF hand of STIM1 is bound to Ca^{2+} and STIM1 is distributed diffusely in the ER membrane. The cytosolic CAD/SOAR region, via interactions with CC1 domain, is locked in an inactive conformation close to the ER/SR membrane. When ER/SR Ca^{2+} levels decrease, Ca^{2+} dissociates from the cEF hand initiating a conformational change in the hEF and SAM domains, which begins the formation of STIM1 oligomers. This conformational change within the ER/SR lumen is transmitted to the cytosol via the STIM1 TM domain, resulting in a release of the SOAR/CAD region. Subsequent conformational changes of all three CC domains enhances STIM1 oligomerization, exposes the SOAD/CAD region to facilitate binding to Orai1 as well as extending the C-terminal region towards the plasma membrane (Derler et al., 2016a; Lewis, 2020). Under resting conditions, STIM1 diffuses freely in the ER membrane, whereas Orai1 diffusion is somewhat constrained possibly due to binding with other proteins (Wu et al., 2014) or the formation of supra-molecular Orai1 clusters (Peckys et al., 2021). Once activated the extended STIM1 region is trapped at ER-PM junctions via interactions of the PB domain with the plasma membrane, facilitated in part by PIP_2. Subsequently, STIM1 traps Orai1 via binding of the SOAR/CAD region to the Orai1 C-terminal region (Wu et al., 2014). The trafficking chaperone, uncoordinated 93 homolog B1 (UNC93B1), has been reported to play an important role in the early activation of STIM1, facilitating its extension. This appears to result in a more efficient interaction between STIM1 and Orai1 channels. However, UNC93B1 does not play a role in the translocation of STIM1 to the plasma membrane or in gating of the Orai1 channel (Wang and Demaurex, 2022).

Key regions of C-terminal domains of both STIM1 and Orai1 form a STIM1-Orai1 association pocket (SOAP) and mutations in this region prevent STIM1 activation of Orai1 (Derler et al., 2016a). The Ca^{2+} channel itself is composed of hexameric Orai1 subunits arranged around a pore created by TM1 domains that extend across the membrane and into the cytosol (Hou et al., 2012; Hou et al., 2020). The precise mechanism by which binding of STIM1 to the Orai1 C-terminal region leads to opening of the Ca^{2+} channel, remains uncertain. However, it has been proposed that binding of the SOAR/CAD region of STIM1 to Orai1 results in a conformational change in the hinge region of the cytosolic extension of TM4. This results in conformational changes in TM4 itself, disrupting interactions with TM3 followed by further conformational changes in TM3/TM2 leading to rotation of the TM1 helices and subsequent channel activation (Zhou et al., 2017). While the N-terminal region of Orai1 is essential for channel activation, it is unclear whether this involves interaction with STIM1. It has been suggested that the N-terminus might regulate channel activity via interactions with other domains such as TM3 or cytosolic loop2 (Fahrner et al., 2018b). It is important to note that while STIM1 and Orai1 are essential for SOCE, there are a growing number of accessory proteins that have been identified as regulating SOCE, which are reviewed in detail elsewhere (Srikanth and Gwack, 2012; Woo et al., 2018; Berlansky et al., 2021).

Most Ca^{2+} channels are regulated by feedback inhibition by Ca^{2+}, a process known as CDI; this is also true for Orai1-mediated SOCE. As noted earlier, STIM1 contains an inhibitory domain (ID_{STIM}) that is essential for CDI; surprisingly however, it is does not appear to be the primary Ca^{2+} sensor responsible for initiating CDI. Calmodulin, which binds to the N-terminal of Orai1, was thought to be the CDI sensor, but this turned out not to be the case (Lewis, 2020). Mullins *et al.*, found that two residues in the Orai1 pore, Trp76 and Tyr80 played a key role in CDI leading to conformation changes, which inactivated the channel (Mullins et al., 2016). Subsequently, they found that ID_{STIM} binding to Trp76 was required for full CDI (Mullins and Lewis, 2016). The Orai1β splice variant did not exhibit CDI indicating that the first 63 amino acids of Orai1 that are absent in Orai1β, contributed to CDI (Zhang et al., 2019). There are AC8 and caveolin binding domains in that 63 amino acid region, which have been shown to be essential for CDI (Zhang et al., 2019). Based on these findings a model was proposed where cAMP generated by Ca^{2+} dependent AC8 resulted in phosphorylation of Ser34 of Orai1 by protein kinase A (PKA), which induced CDI (Zhang et al., 2019). How Ser34 phosphorylation regulates CDI remains to be determined, although it was speculated that it may facilitate binding of ID_{STIM1} to Orai1 (Zhang et al., 2019). However, concern has been raised regarding the generalization of this mechanism due to the limited tissue distribution of AC8 (Hofer, 2019). Interestingly, compared to Orai1, Orai2, and Orai3 exhibit faster CDI, which is mediated by three conserved glutamates in their C-terminal domains (Lee et al., 2009).

A consequence of the emphasis on STIM1 and Orai1 in understanding the molecular mechanisms underlying SOCE is that our understanding of the potential roles of STIM2, Orai2, and Orai3 has been neglected. Early studies showed that overexpression of Orai2 and Orai3 with STIM1 resulted in SOCE and generation of I_{crac} albeit with some differences in their biophysical characteristics compared to Orai1 (Mercer et al., 2006; Dehaven et al., 2007; Lis et al., 2007). However, the physiological role of Orai2 and Orai3 in regulating physiological Ca^{2+} signaling remained unclear. The role of STIM2 is also not well understood. Early studies reported contradictory findings regarding STIM2 function, with some reports suggesting that it facilitated SOCE in a similar manner to STIM1, whereas others indicated that STIM2 inhibited the actions of STIM1. STIM2 was found to form pre-made clusters with Orai1 and it was believed that this played an important role in regulating basal cytosolic and ER Ca^{2+} levels (Brandman et al., 2007). It has also been suggested that STIM2 might act as an adaptor protein regulating STIM1 function (Berna-Erro et al., 2017).

It has been proposed that the difficulty in identifying clear roles for Orai2/3 and STIM2 is because the protocols used to generate maximal SOCE signals and I_{crac} currents do not represent normal physiological stimuli for Ca^{2+} signaling, thereby hiding potentially more subtle roles for these proteins (Yoast et al., 2020; Emrich et al., 2021). Studies by Trebak and colleagues suggest that under more physiological conditions, Orai2 and Orai3 form heteromultimers with Orai1, attenuating its activity, resulting in a larger bandwidth of Ca^{2+} signals (Yoast et al., 2020). Moreover, they have also proposed that physiological Ca^{2+} signaling requires STIM1 and STIM2 interactions to further finetune intracellular Ca^{2+} signaling (Emrich et al., 2021). While the concept that all five STIM/Orai isoforms work together to regulate the Ca^{2+} signaling responses to agonist stimulation clearly complicates the understanding of the function of individual proteins, it also represents a potentially elegant solution for the diverse roles of SOCE channels. Such a model would allow for Ca^{2+} signaling to be fine-tuned due to cell/tissue specific differences in expression of these five proteins. Clearly, a great deal of additional work is needed to determine how the five STIM/Orai isoforms work together under physiological conditions and whether alterations in stoichiometry could account for the diverse functions of SOCE in different tissues and cells. Understanding how different STIM and Orai variants fit in with this model also remains to be determined.

4.2 Store Independent Ca^{2+} Entry

For many years SOCE was widely considered to be the primary agonist-mediated Ca^{2+} signaling pathway, but in 1996 Shuttlesworth and Thompson identified a plasma membrane Ca^{2+} entry pathway that was independent of intracellular Ca^{2+} stores (Shuttleworth and Thompson, 1996). In a series of studies, they identified arachidonic acid as the agonist responsible and named the resulting Ca^{2+} current I_{ARC} (for arachidonate-regulated calcium current) (Shuttleworth, 1996; Shuttleworth and Thompson, 1998; Mignen and Shuttleworth, 2000). Several different agonists were subsequently shown to activate a store-independent, arachidonic acid (AA) dependent Ca^{2+} entry pathway in several cell types (Munaron et al., 1997; Broad et al., 1999; Guibert et al., 2004); however, the identity of the channel proteins remained elusive (Shuttleworth et al., 2004). While the focus on STIM1 had been its role as the ER/SR Ca^{2+} sensor regulating SOCE, it had originally been identified as a plasma membrane protein (Manji et al., 2000; Williams et al., 2001; Williams et al., 2002); consequently, Mignen *et al.*, examined whether it also played a role in ARC mediated Ca^{2+} entry (Mignen et al., 2007). They demonstrated that ARC channels were regulated by the plasma membrane pool of STIM1, with its N-terminal domain in the extracellular environment (Mignen et al., 2007). In subsequent studies they found that Orai1 and Orai3, but not Orai2 were also required for ARC channel activity (Mignen et al., 2008; 2009).

Activation of an Orai1/Orai3 Ca^{2+} channel by leukotrieneC_4 (LTC_4) that was also STIM1-dependent was reported to have very similar biophysical characteristics as the ARC channel (Gonzalez-Cobos et al., 2013; Zhang et al., 2013). However, in contrast to ARC channel activation ER/SR STIM1 rather than plasma membrane STIM1 was found to be sufficient for LTC_4 regulated Ca^{2+} (LRC) channel activation (Zhang et al., 2013). There was no formation of STIM1 puncta in response to LTC_4, but the interaction between the STIM1 CC domains and Orai3 was necessary for channel activity (Zhang et al., 2013). It was subsequently shown that the biophysical characteristics of LTC_4 and ARC channel activation were identical requiring both Orai1 and Orai3, and that metabolism of AA to LTC_4 was necessary for full activation of the channels (Zhang X. et al., 2014).

It has been suggested that the apparent differences in the pools of STIM1 required for channel activation by AA and LTC_4 was dependent on whether patch clamped cells or intact cells were studied and that ER/SR STIM1 was sufficient for ARC activation in intact cells (Zhang X. et al., 2014). However, the precise role of STIM1 in the regulation of ARC channels remains unclear because some studies have shown that while Orai1 and Orai3 are essential for ARC activation, STIM1 may not be required (Dubois et al., 2014; Goswamee et al., 2018). On the other hand, Thompson and Shuttlesworth reported that PKA-mediated phosphorylation of Thr389 of the cytosolic domain of plasma membrane STIM1 was necessary for ARC channel activation (Thompson and Shuttleworth, 2015). Thus, while there appears to be a consensus that Orai1 and Orai3 are essential components of ARC/LRC channels, the role and cellular pool of STIM1 remains an open question (Zhang et al., 2018). It is has also not been settled whether AA and LTC_4 activate the channels independently or if metabolism of AA to LTC_4 is required (Zhang et al., 2018).

5 METABOLIC AND MITOCHONDRIAL ROLES OF STIM AND ORAI

The metabolic roles of STIM and Orai have been described in immune cell populations (Vaeth et al., 2017); however, the role of these proteins in regulating metabolism and mitochondrial function has been less studied in other organs and cell types. In this section, we will discuss the contribution of STIM and Orai isoforms to the regulation of glucose and lipid metabolism and mitochondrial function in various non-immune cells of different organs, including cardiomyocytes, hepatocytes, and skeletal muscle cells.

Plenty of evidence points in the direction of STIM and Orai proteins regulating fatty acid and lipid metabolism. A recent study from Maus *et al.*, (Maus et al., 2017), showed that cells lacking either STIM1 or Orai1 had reduced SOCE, which mediated significantly high levels of lipid droplet deposition and increased lipophagy, and was shown in numerous organs including the liver, heart, and skeletal muscle. Consistent with that study, we showed that hearts from cardiomyocyte-specific STIM1-KO mice had lipid droplet accumulation, triglyceride accumulation, and altered expression of several fatty acid metabolism proteins (Collins et al., 2019). We also found reductions in insulin-mediated cardiac protein kinase b (Akt) activation, which has been shown to occur in other STIM1 cardiomyocyte knockdown models. Other studies have shown

that activation of STIM1/Orai1-mediated SOCE activated Akt, glycogen synthase kinase 3 beta (GSK3β) and mTORC2 signaling whereas pharmacological inhibition attenuated this pathway (Benard et al., 2016), providing further support for the key role of STIM1 in the regulation of metabolism.

In addition to impaired cardiac glucose metabolism in cardiomyocyte-specific STIM1-KO mice, we also showed significant changes in mitochondrial size and shape as well as evidence of increased mitochondrial fission through reductions in Mitofusin (Mfn2) and increased Dynamin related protein 1 (Drp-1) expression (Collins et al., 2014; Collins et al., 2019). Mitochondrial structural abnormalities have been identified in virtually all STIM and Orai KO and overexpression models in various cell types. For example, skeletal muscle overexpression of STIM1 has been shown to have a dystrophic-like phenotype associated with the presence of swollen mitochondria (Goonasekera et al., 2014). Henke *et al.*, (Henke et al., 2012), also showed that fibroblasts lacking either STIM1 or Orai1 were more susceptible to oxidative stress and showed that the mitochondria from STIM1-KO cells were abnormally shaped with abnormal cristae, had increased Ca^{2+} load, increased glutathione levels, and there was a significant increase in transcription of antioxidant genes, suggesting that STIM1 is an important regulator of mitochondrial function. The same group also showed that oxidative stress reduced SOCE in hippocampal neurons and that knockdown of Orai1 was protective against glutathione depletion (Henke et al., 2013). In support of these studies, several of the cardiomyocyte-specific KO and overexpression models of STIM and Orai proteins show significant mitochondrial structural abnormalities correlating with reductions in mitochondrial function and alternations in mitochondrial quality control (Collins et al., 2014; Correll et al., 2015; Collins et al., 2019; Segin et al., 2020; Gammons et al., 2021). Silva-Rojas *et al.* examined gain of function mutations in both STIM1 and Orai1 and found that this resulted in increased SOCE and promoted abnormal Ca^{2+} handling and mitochondrial activity. Specifically, the authors used mice with mutant STIM1, $STIM1^{R304W/+}$ mice, and found that the abnormal Ca^{2+} handling was the result of changes in the expression of several key proteins including sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and ryanodine receptor (RyR). The abnormal mitochondrial activity was the result of changes in the expression of several mitochondrial proteins which include mitochondrial transcription factor A (Tfam), peroxisome proliferator-activated receptor gamma coactivator (PGC1α), nuclear respiratory factor 1 (Nrf1), Sirtuin 1 (Sirt1), and mitochondrial fission 1 protein (Fis1) and an increase in apoptosis (Silva-Rojas et al., 2021). These studies suggest that STIM1 and Orai play significant roles in modulating mitochondrial function but could also regulate mitochondrial quality control and redox signaling.

It has also been shown that mitochondrial Ca^{2+} uptake is essential for regulating STIM1, Orai1, and SOCE (Naghdi et al., 2010). In addition, it has been shown that the mitochondrial protein, Mfn2 contributes to STIM1 membrane trafficking (Singaravelu et al., 2011) and that knockdown of either the mitochondrial Ca^{2+} uniporter (MCU) or uncoupling protein 2 (UCP2) resulted in slowed STIM1 oligomerization and reduced SOCE (Deak et al., 2014). This relationship appears to be reciprocal since the lack of STIM1, Orai1, and the inositol triphosphate receptor (IP_3R) in lymphocytes has been shown to not only result in reductions in mitochondrial proteins such as MCU, but also these lymphocytes had altered mitochondrial metabolism dependent on cAMP response element-binding protein (CREB) (Shanmughapriya et al., 2015). Also, the mitochondrial K^{ATP} channel opener, Diazoxide, has been shown to promote upregulation of STIM1 and Orai1 expression (Sampieri et al., 2019) through mechanisms involving increased phosphorylation of ERK1/2 and NFκB (Gavali et al., 2020), which suggests that STIM1 and Orai may contribute to the cardioprotection associated with diazoxide (Katoh et al., 2002; Hausenloy et al., 2004).

The lack of STIM1/Orai1 seems to adversely impact mitochondrial ultrastructure and function in many cell types although this does not appear to be consistent in neuronal cells. For example, in neuron-like PC12 cells it has been shown that siRNA knockdown of STIM1 increased cell viability in response to injury with 1-methyl-4-phenylpyridinium. This was associated with reductions in apoptosis, ROS production, and prevented mitochondrial dysfunction which was believed to be dependent on Homer1a (Li X. et al., 2013). On the other hand, hydroxydopamine-induced injury in PC12 cells was increased following knockdown of STIM1, resulting in increased apoptosis, decreased mitochondrial function, and mitochondrial Ca^{2+} uptake (Li et al., 2014). Interestingly, Rao *et al.*, (Rao et al., 2015), showed that shRNA knockdown of STIM2 in a traumatic brain injury model also improved neuronal survival through the targeting of mitochondrial apoptosis and preservation of mitochondrial function. Overall, these data suggest that lack of STIM and Orai proteins may be beneficial to mitochondrial-dependent cell survival in some cell types although this does not appear to hold true for cardiomyocytes.

STIM1 has been described as a "metabolic checkpoint" for tumor growth and metastasis in hepatocytes. For example, reductions in STIM1 in hepatocytes mediated a switch from glycolysis to AMPK-mediated fatty acid oxidation (Zhao et al., 2020). It has also been shown that hepatocytes from obese mice have reduced SOCE, occurring due to a reduction in STIM1 translocation, and these changes were associated with both glucose and insulin intolerance and lipid droplet accumulation. Of note, it was shown that the reduction in STIM1 translocation in this study was due to abnormal O-GlcNAcylation (Arruda et al., 2017). It has been reported that increased O-GlcNAcylation of STIM1 in neonatal cardiomyocytes was linked to a reduction in SOCE (Zhu-Mauldin et al., 2012); however, these studies did not examine the impact of these changes on metabolism and mitochondrial function which should be interrogated.

STIM1 and Orai1 have been shown to regulate whole body metabolism via their regulation of insulin secretion in β-cells. For example, STIM1 and Orai1 knockdown in β-cells leads to reduced glucose-induced insulin secretion (Usui et al., 2019). Of note, STIM1 has been shown to be reduced in expression in islets from type 2 diabetic patients, STZ diabetic mice, and INS-1 cells, resulting in impaired insulin secretion, abnormal Ca^{2+}

handling, and ER stress (Kono et al., 2018). Of note, this change may be cell specific, because Orai1 has also been shown to be reduced in lymphocytes from type 2 diabetic patients without changes in STIM1 levels (Wang et al., 2018). These studies suggest that STIM1, Orai1, and SOCE are important for insulin secretion and diabetic cell phenotypes; however, it is unclear whether STIM2, Orai2, and Orai3 contribute to these processes.

In summary, changes in STIM1 and Orai1 isoforms have been shown to alter mitochondrial function and metabolism in various cell types and organs; however, the specific signals connecting them to mitochondrial function and metabolism have yet to be fully elucidated. Furthermore, our knowledge of the potential roles of STIM2 and Orai2/3 in regulating mitochondrial function and metabolism is much less known.

6 STIM AND ORAI IN CELL SURVIVAL

Cellular Ca^{2+} homeostasis plays a pivotal role in determining cell death and survival. The relationship between Ca^{2+} and cell fate is complex due in part to the fact that Ca^{2+} can act as a stressor and also a second messenger that is involved in multiple pathways in cell death and survival (Orrenius et al., 2003). Consequently, a fine balance between Ca^{2+} depletion and Ca^{2+} overload is key for cell fate determination. A moderate rise of cytosolic Ca^{2+} level promotes cell survival by enhancing mitochondrial bioenergetics and therefore ATP synthesis, as well as activating cell survival signaling, such as Akt and NFAT dependent pathways (Yano et al., 1998; Pu et al., 2003; Rizzuto et al., 2012). Sustained increases in Ca^{2+} level, however, leads to mitochondrial Ca^{2+} overload and subsequent cell death (Naon and Scorrano, 2014). Historically, three processes of cell death were characterized: apoptosis, necrosis, and autophagy. During the past decade or so, new types of cell death such as pyroptosis and ferroptosis have been identified and their importance gradually appreciated (Yu et al., 2021). Indeed, there is growing evidence for a Ca^{2+} related mechanism in ferroptosis in cancerous and noncancerous cells (Chen P. et al., 2020; Angelova et al., 2020), suggesting a unique yet ubiquitous role of Ca^{2+} in general cell death processes. While there is some evidence for members of the less selective TRP cation channels in mediating pyroptosis and ferroptosis (Shi et al., 2021), evidence for STIM/Orai mediated SOCE involvement in these processes are lacking. Therefore, in this section, we will focus on the role of STIM and Orai proteins in the more widely studied cell death pathways, apoptosis, necrosis, and autophagy.

6.1 Apoptosis and Necrosis

Apoptosis can be initiated through intrinsic and extrinsic pathways. The intrinsic pathway is activated when there is mitochondrial swelling and/or increased permeability of the mitochondrial membrane, which leads to the release of cytochrome C and cleavage of pro-caspase to caspase 9 (Fesik and Shi, 2001). The extrinsic pathway is activated upon ligand receptor interactions: FasL binding to Fas, or TNFα binding to TNF receptors (Wajant, 2002). STIM/Orai-mediated SOCE has been shown essential in regulating cellular apoptotic pathways with most studies demonstrating its proapoptotic characteristics although there is also evidence for STIM/Orai mediated inhibition of apoptotic signaling (Khadra et al., 2011; Liu et al., 2011; Kondratska et al., 2014).

In a human hepatocarcinoma cell line (HepG2), Yan and colleagues found that ethanol increased intracellular Ca^{2+} level and caused cell damage in a dose-dependent manner (Liu H. et al., 2012), and was associated with increased STIM1 and Orai1 protein levels. In addition, either a SOC inhibitor or a siRNA targeting STIM1 attenuated ethanol induced hepatotoxicity. Subsequent experiments from the same group showed that knockdown of STIM1 and Orai1 significantly restored the mitochondrial membrane potential, decreased cytochrome C release, and attenuated ethanol induced apoptosis (Cui et al., 2015). In a model of hepatic ischemia/reperfusion (I/R) injury, mice lacking STIM1 exhibited an attenuated cellular inflammation and apoptosis compared to controls (Li et al., 2018). In neuronal cells STIM/Orai has also been shown to regulate apoptosis. Rao *et al.* showed that, in hippocampal HT-22 cells, application of hydrogen peroxide (H_2O_2) led to significant Ca^{2+} overload and mitochondrial dysfunction, which was attenuated by an SOC inhibitor or a siRNA knockdown of STIM1 (Rao et al., 2013). In a traumatic brain injury model, Hou *et al.* also found that knockdown of STIM1 significantly inhibited apoptotic cell death (Hou et al., 2015), suggesting a role for STIM1 in regulating apoptosis and cell death signaling.

Orai-mediated apoptosis has also been studied in a variety of pathophysiological settings. For example, Flourakis *et al.* identified, that Orai1 was the main source for Ca^{2+} influx in prostate cancer cells (Flourakis et al., 2010). They reported that knockdown of Orai1 protected cells from apoptosis induced by TNFα or Cisplatin whereas Orai1 rescue re-established the normal rate for apoptosis in these cancer cells. It is important to note that although STIM1 expression remained stable when apoptosis was induced, STIM1-Orai1 coupling was required for the pro-apoptotic effects. Given the importance of Orai1 in regulating immune cell function the majority of studies have focused on the role of Orai1 in mediating immune cell apoptosis (Feske, 2009). Using an Apolipoprotein E knockout mouse model, Liang *et al.* demonstrated that silencing Orai1 led to decrease apoptosis in macrophages, which resulted in less foam cell formation and decreased vascular inflammation (Liang et al., 2016). Kim *et al.* showed reduced mitochondrial Ca^{2+} uptake and altered proapoptotic/antiapoptotic gene expression in CD4+ T cells from Orai1-KO mice and provided evidence that NFAT-mediated cell death pathway was the main downstream target for Orai1 mediated Ca^{2+} influx in T cells (Kim et al., 2011). In addition, Orai1 deficient T cells showed increased survival following adoptive transfer to host. Collectively these studies suggest that STIM1/Orai1 mediated SOCE plays an essential role in regulating the intrinsic/mitochondrial pathway for apoptosis.

In contrast, other studies have demonstrated an anti-apoptotic role for STIM1/Orai1, mainly via the extrinsic apoptotic pathway. For example, in Panc1 pancreatic adenocarcinoma cell line

knockdown of STIM1 and/or Orai1 increased apoptosis induced by 5-FU or gemcitabine (Kondratska et al., 2014). They also reported that 5-FU and gemcitabine increased SOCE via upregulation of Orai1 and STIM1. Knockdown of Orai1 was also shown to increase apoptosis in glioblastoma cells (Liu et al., 2011).

The apparent contradiction between the pro and anti-apoptotic effects of STIM/Orai-mediated SOCE could be explained in part by the fact that different cancer cell types have varying expression levels of Orai1 and STIM1. In addition, depending on the specific types of stimuli, different intracellular signaling pathways regulated by STIM1/Orai1, may be triggered thereby resulting in different outcomes. In noncancerous cells the anti-apoptotic characteristics of STIM1/Orai1 were also reported. Khadra and colleagues performed a series of experiments showing that, in response to activation of the death receptor CD95, Orai1, and STIM1 colocalize with CD95 and recruit PKCβ2 to the death receptor inducing signaling complex, thus preventing caspase activation and apoptosis (Khadra et al., 2011). In dopaminergic neurons, knockdown of STIM1 led to increased ER stress and apoptosis through PKB inhibition (Selvaraj et al., 2012). In addition to STIM1/Orai1, other STIM/Orai homologues may also play a role in regulating apoptosis. For example, Sobradillo and colleagues investigated Ca^{2+} related mechanisms for colon cancer and found that STIM2 expression was significantly decreased in cancer cells. They also showed that in normal mucosal cells STIM2 knockdown increased resistance to apoptosis (Sobradillo et al., 2014). Tu *et al.* showed in cultured cardiomyocytes that STIM2 expression was significantly increased following I/R injury; whereas knockdown of STIM2 preserved mitochondrial function and attenuated the activation of apoptotic signaling in response to I/R (Tu et al., 2020). There is also evidence for Orai3 mediated regulation of apoptosis, primarily in cancer cells. For example, in breast cancer cells and tissue, Orai3 expression was significantly higher and that knockdown of Orai3 led to cell cycle arrest and apoptosis (Faouzi et al., 2011). Dubois and colleagues introduced a novel channel consisting of Orai1/Orai3 heterodimer and demonstrated its role in prostate cancer cell proliferation (Dubois et al., 2014). They found that prostate cancer cells can undergo an "oncogenic switch." The increase in Orai3 expression and alterations of tumor microenvironment leads to an increased heteromerization of Orai1 and Orai3, which contributes to the phenotypic transition from SOCE, which is pro-apoptotic, to an Orai1/Orai3 channel that is pro-proliferative. Future studies are needed to decipher the mechanism(s) underlying Orai/STIM mediated regulation of programmed cell death under different conditions.

Unlike apoptosis, necrosis is by in large not a process of programmed of cell death. Although mechanistic studies are lacking, there is evidence that STIM/Orai-mediated SOCE may also be involved in necrotic cell death. Gombedza and colleagues investigated the effect of the internalization of stone-forming calcium crystals on Ca^{2+} signaling in human proximal tubular cells (Gombedza et al., 2019). Amongst other findings, they observed increased cellular necrosis that was accompanied by increased SOCE. They also generated a STIM1 transgenic mouse model in which STIM1 was overexpressed in the skeletal muscle, which increased both SOCE and necrosis in the myofibers of the transgenic mice (Goonasekera et al., 2014). To determine the role of Orai1 in pancreatic acinar cell injury and acute pancreatitis, Wen *et al.* transfected Orai1 into human and mouse acinar cells and found that the application of Orai1 inhibitors prevented acinar cell necrosis (Wen et al., 2015), suggesting a role of Orai1-mediated Ca^{2+} overload in acute pancreatic cell necrosis. Although it is well known that Ca^{2+} overload can lead to not only apoptosis, but also necrosis (Rizzuto et al., 2003; Shaheen et al., 2011), it remains unclear how STIM/Orai-mediated SOCE contributes to necrotic processes. One way in which STIM/Orai may contribute to necrotic processes could be through opening of the mitochondrial permeability transition pore (mPTP). It is well established that necrosis is associated with the opening of the mPTP. It has been shown by He et al., that siRNA-mediated knockdown of STIM1 in H9C2 cardiomyocytes resulted in reduced mPTP opening and reduced ROS (He et al., 2017). However, a definitive role for STIM and Orai members in regulating mPTP opening has yet to be established and would shed additional light on mitochondrial-ER Ca^{2+} regulatory mechanisms.

6.2 Autophagy

Autophagy is a tightly regulated physiological process by which cellular components are degraded and recycled, mainly through a lysosome-dependent mechanism (Feng et al., 2014). There are several forms of autophagy, namely macroautophagy, microautophagy, chaperone-mediated autophagy, and crinophagy. As the most well-studied form of autophagy, macroautophagy is a process in which cellular components are covered within a double membrane prior to its fusion with an lysosome, whereas in microautophagy, cellular targets are directly taken up by the lysosome via membrane invagination (Li et al., 2012). Unless otherwise stated, macroautophagy is referred to as autophagy in this section. Studies have shown that Ca^{2+} regulates autophagy through multiple mechanisms (Smaili et al., 2013). In 2007 Hoyer-Hansen *et al.*, demonstrated for the first time that in MCF-7 cells an increase in cytosolic Ca^{2+} induced by various calcium mobilizing agents was a potent activator of autophagy (Hoyer-Hansen et al., 2007). Specifically, they found that thapsigargin, which results in ER Ca^{2+} depletion, the first step in activating STIM1-mediated SOCE, was a potent activator of autophagy via inhibition of mechanistic target of rapamycin (mTOR) in a calcium/calmodulin-dependent protein kinase kinase (CAMKK) dependent manner. On the other hand, Medina *et al.*, concluded that lysosomal calcium was responsible for activation of the Ca^{2+} dependent phosphatase calcineurin, the subsequent dephosphorylation of Transcription factor EB (TFEB) a master transcription factor for autophagy leading to its nuclear localization (Medina et al., 2015). It is worth noting however, that they also showed that thapsigargin was sufficient to induce TFEB nuclear localization, suggesting that ER Ca^{2+} release was sufficient to activate autophagy. These pioneering studies clearly demonstrated a key role for

Ca^{2+} in regulating autophagy. While they did not identify the specific Ca^{2+} signaling pathways that were involved, they both showed that ER Ca^{2+} release, an essential step in activating STIM1-mediated SOCE, was sufficient to initiate autophagy.

Zhu *et al.* provided the first direct evidence for SOCE involvement in autophagy, in pancreatic acinar cells (Zhu et al., 2018). In a mouse model of acute pancreatitis induced by Ca^{2+} overload they observed puncta-like colocalization of STIM1 and Orai1 and an increase in SOCE. They observed that the increase in SOCE led to the activation of calcineurin leading to NFAT and TFEB nuclear localization, and subsequent initiation of autophagy. In endothelial progenitor cells, oxidized LDL induced autophagy was accompanied by increased STIM1 leading to activation of CaMKK2 and inhibition of mTOR (Yang et al., 2017). On the other hand, resveratrol a natural polyphenol, activated autophagic cell death in prostate cancer cells, which was associated with reduced STIM1 expression and SOCE (Selvaraj et al., 2016). Inhibition of SOCE had similar effects to resveratrol whereas overexpression of STIM1 reversed the effects. Similarly, knockdown of Orai1 or pharmacological inhibition of SOCE in HepG2 hepatocarcinoma cells potentiated 5-FU induced autophagy whereas overexpression of Orai1 attenuated 5-FU induced autophagic cell death (Tang et al., 2017), suggesting that STIM1 and Orai1 play significant roles in cell death processes including autophagy.

While most of the studies on STIM/Orai related to autophagy have been in the context of cancer, there is also evidence that STIM/Orai is involved in the process of autophagy in normal cells. In neonatal rat cardiomyocytes, the putative STIM1 inhibitor ML9 induced cell death by inducing lysosomal dysfunction and disrupting autophagic flux (Shaikh et al., 2018). However, interpretation of these findings needs to be considered carefully since it is well known that ML9 inhibits several different protein kinases, including myosin light-chain kinase (MLCK), PKA, and protein kinase C (PKC) (Hidaka and Kobayashi, 1992; Takahashi et al., 1997; Smyth et al., 2009). Angiotensin II (Ang II) had been shown to induce cardiomyocyte hypertrophy in an SOCE-dependent manner (Hunton et al., 2002), and more recently it was reported to induce autophagy in neonatal cardiomyocytes in an SOCE and Orai1-dependent manner (Zheng et al., 2021). In the same study, *in vivo* Ang II infusion was shown to increase autophagic flux in the heart and this was attenuated by decreasing Orai1 levels following treatment with an AAV-Orai1-siRNA1.

It is now readily accepted that Ca^{2+} plays a role in regulating autophagy (Kondratskyi et al., 2018) and while some have suggested that TRP channel family of Ca^{2+} channels contribute the regulation of autophagy (Sukumaran et al., 2015; Sukumaran et al., 2016) there is growing support that it is mediated via a STIM/Orai-dependent SOCE pathway. Studies examining the extent to which both STIM1 and Orai1 contribute to the regulation of autophagy are warranted. Nevertheless, it is clearly context dependent since SOCE appears to both activate and attenuate autophagy depending on cell type and the specific stimulus.

7 REDOX REGULATION OF STIM AND ORAI PROTEINS

Several studies have suggested that STIM and Orai proteins are sensitive to and are regulated by changes in redox status and these changes will be discussed in this section. S-nitrosylation is a significant regulator of redox signaling, which is mediated through increases in nitric oxide (NO) and subsequent covalent attachment of NO to cysteine (Cys) thiols on proteins. Interestingly, it has been shown that neuronal nitric oxide synthase (nNOS), which generates NO, is expressed in the SR (Xu et al., 1999), where STIM1 also resides. It was recently shown by Gui *et al.*, (Gui et al., 2018), that STIM1 undergoes S-nitrosylation on Cys49 and Cys56 and that S-nitrosylation of STIM1 inhibits its oligomerization and reduces SOCE. The same study also showed that genetic and pharmacological reductions in nNOS reduce S-nitrosylation of STIM1 and reversed changes in SOCE. The authors speculated that STIM1 activity and SOCE increase during heart failure because of a reduction in NO bioavailability; however, this has yet to be determined. In addition to S-nitrosylation, STIM1 has also been shown to undergo S-glutathionylation, where it has been shown to have an opposite effect on STIM1 activity and SOCE compared to S-nitrosylation. Hawkins *et al.*, have shown that STIM1 can be S-glutathionylated on Cys56, which increases both the activity of STIM1 and also increases SOCE (Hawkins et al., 2010). In the same study, it was shown that S-glutathionylation of STIM1 modulated mitochondrial bioenergetics and Ca^{2+} handling (Hawkins et al., 2010). One would hypothesize that changes in the balance between s-nitrosylation and S-glutathionylation of STIM1 could perhaps contribute to cardiovascular pathologies although this remains to be determined.

It has also been shown that STIM2 is subject to oxidative modification. For example, Gibhardt *et al.*, (Gibhardt et al., 2020), showed that STIM2 has an additional ten cytosolic cysteine residues in comparison to STIM1. They also showed that upon the induction of oxidative stress the oxidation of Cys313 on STIM2 is modified promoting a reduction in SOCE through the prevention of STIM2 oligomerization. It has also been shown that in response to the NO donor, nitrosoglutathione, STIM2 was S-nitrosylated at cysteines 15, 53, and 60 in HEK cells which was required for the stabilization of STIM2 (Novello et al., 2020). Like STIM isoforms, Orai isoforms are also subject to redox regulation. Of interest, it has been postulated and shown in HEK cells that Orai1 is redox-sensitive and that Orai3 is redox-insensitive (Alansary et al., 2015) this is largely due to the lack of the redox sensor, Cys195 in Orai3. Mutations in the Orai1 redox sensor, Cys195, have been shown to inhibit SOCE in response to the knockdown of the sodium/calcium exchange in HEK293 cells (Ben-Kasus Nissim et al., 2017). Alansary *et al.* have shown that treatment of HEK293 cells with H_2O_2 reduces Orai1-mediated SOCE which was shown to be the result of oxidation of Cys195 on Orai1 (Alansary et al., 2016). It has also been shown in HEK293 cells that hydrogen sulfide (H_2S) treatment inhibits Orai3-mediated SOCE but not Orai1 and Orai2-mediated SOCE. The authors determined that this difference was due to the presence of Cys226 and Cys232 in Orai3 both of which were

absent in other Orai isoforms and were shown to mediate the reduction in SOCE in response to hydrogen sulfide (H_2S) (Fresquez and White, 2021). It is possible that STIM1 coupling with either Orai1 or Orai3 could well depend on cellular redox status and perhaps act as a redox sensor although this remains to be determined. At present, it remains unclear as to the extent to which Orai2 is regulated by redox and oxidative stress; therefore, future studies should be aimed at determining redox modulation of Orai2 and impact on resultant SOCE. In addition, it remains unclear based on these studies how these redox modifications of STIM and Orai isoforms and splice variants impact the function of different cell types and organ systems, which needs to be established moving forward.

8 PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES OF STIM/ORAI IN THE CARDIOVASCULAR SYSTEM

It is increasingly clear that STIM-Orai signaling is an important regulator of cardiovascular physiology and homeostasis as well as playing a significant role in cardiovascular disease processes, such as hypertrophy, ischemia/reperfusion (I/R), and heart failure. In this section, we will discuss the current knowledge concerning the canonical and non-canonical functions of STIM and Orai proteins in the heart during instances of cardiac pathology and during normal physiology.

8.1 STIM1

Until the early 2000s, it was believed that cardiac TRP channels played a key role in the underlying Ca^{2+} signaling associated with cardiac hypertrophy and heart failure (Nakayama et al., 2006). However, upon the discovery of STIM1 and Orai1 as key regulators of SOCE and with STIM1/Orai1-mediated SOCE being associated with cardiac hypertrophy (Ohba et al., 2009; Voelkers et al., 2010; Hulot et al., 2011; Luo et al., 2012), there was a growing appreciation of this signaling mechanism in the heart. In these studies, STIM1, Orai1, and resultant SOCE were all shown to be increased and participate in the activation of calcineurin and the nuclear translocation of NFAT to activate hypertrophic signaling (Ohba et al., 2009; Voelkers et al., 2010; Hulot et al., 2011; Luo et al., 2012). Of note, the study by Luo *et al.*, (Luo et al., 2012), showed that STIM1L, which was very low in healthy adult cardiomyocytes, was significantly increased in response to the hypertrophic agonist, phenylephrine (PE). The authors speculated that this increase in STIM1L was a stimulus for the induction of the fetal gene program. Despite this, little remains known regarding the function of STIM1L in the heart. Since the establishment of a role for STIM1/Orai1 in cardiac hypertrophy, several additional studies have tried to examine the mechanisms driving the increase in STIM1 during hypertrophy. One such study in neonatal cardiomyocytes treated with PE showed that a decrease in expression of miR-223 was responsible for an increase in STIM1 expression and subsequent increased hypertrophic signaling, which was shown to also involve changes in GSK3β, β-catenin, and SRY-box transcription factor 2 (SOX2) (Zhao et al., 2018). Increased phosphorylation of STIM1 by Fam20c Golgi associated secretory pathway kinase (Fam20c) was also shown to contribute to increased STIM1 expression and increased SOCE during pressure-overload (Pollak et al., 2018), suggesting that several signaling mechanisms are contributing to the increase in STIM1 during pressure overload.

Rare, heterozygous gain of function mutations in STIM1 in humans result in complex neuromuscular phenotypes, including some cardiac involvement (Walter et al., 2015; Harris et al., 2017). Studies of patients undergoing cardiac catheterization found SNPs in the STIM1 gene, which correlated with metabolic defects, ER stress, and an increase in mortality (Kraus et al., 2015). More recently, mutations have been shown that impact STIM1 expression. For example, it has been shown that a variant upstream of STIM1, named rs3061890, has been associated with coronary artery disease and has been shown to repress STIM1 in an ELF1-dependent manner (Zhang et al., 2021). Collectively, these studies suggest that STIM1 has an important role in mediating cardiovascular disease although the regulatory mechanisms governing STIM1 expression and activity in the heart need to be further established.

After the establishment of its role in cardiac hypertrophy, there was significant controversy in the field as to the relevance of STIM1/Orai1-mediated SOCE in the non-diseased heart due to the predominant regulation of cardiac Ca^{2+} handling on a beat-by-beat basis by voltage-gated Ca^{2+} handling; therefore, the field began to focus their attention on determining the physiological role of both STIM1 and Orai1 in the heart. We performed the initial phenotyping of the constitutive cardiomyocyte-specific STIM1-KO mouse in 2014 (Collins et al., 2014). We showed that cardiomyocyte STIM1 was an essential regulator of ER/SR and mitochondrial function as STIM1-KO mice had a progressive dilated cardiomyopathy associated with significant ER stress, the presence of abnormally shaped and distributed mitochondria, and changes indicative of increased mitochondrial fission. Using the same mice, we later showed that cardiomyocyte STIM1 was also an important regulator of cardiac metabolism as KO mice had significant perturbations in both cardiac glucose and fatty acid oxidation (Collins et al., 2019).

STIM1 has been further linked to regulating ER stress and mitochondrial-mediated apoptosis in the heart. Using Cardiomyocyte-specific STIM1-KO and overexpression mice treated with doxorubicin, Zhu *et al.*, have shown that mice lacking STIM1 have increased cardiac injury in response to doxorubicin treatment which was associated with increased apoptosis and increased GRP78-mediated ER stress, and was essentially reversed in the STIM1 overexpression model (Zhu et al., 2021). Interestingly, in the same study, doxorubicin treatment of wild-type mice suppressed STIM1 expression and SOCE, suggesting that STIM1 and SOCE could contribute to doxorubicin-mediated cardiotoxicity although this currently remains undetermined. Several additional transgenic mouse studies have been performed to examine the function of cardiomyocyte STIM1. One of these studies by Parks *et al.*, (Parks et al., 2016), examined the impact of inducible cardiomyocyte-specific STIM1-KO in mice and showed a phenotype very similar to the constitutive cardiomyocyte-

specific STIM1-KO mice (Collins et al., 2014). However, unlike earlier studies, this study showed that KO mice had a blunted hypertrophic response to pressure overload. Ohba *et al.* (Ohba et al., 2017) examined the impact of heterozygous STIM1-KO and found that these mice were essentially normal at baseline, but increased mortality was observed in the KO in response to 4-weeks TAC despite lack of hypertrophy and induction of fetal gene expression. The authors concluded that a partial lack of STIM1 resulted in abnormal responses to cardiac stress; however, this model was a whole-body STIM1 heterozygous model rather than cardiomyocyte specific. The Houser lab also showed that STIM1-mediated Ca^{2+} influx during hypertrophy contributed to action potential prolongation, SR Ca^{2+} overload, Ca^{2+} sparks, and CaMKII-mediated cell death determined using a combination of pharmacological inhibition of STIM1 with BTP2 or a dominant-negative Orai1 construct in cardiomyocytes from banded felines (Troupes et al., 2017). These studies clearly suggest an important role of STIM1 in cardiovascular disease processes.

In addition, changes in STIM1, STIM1L, and Orai1 have been observed in a pulmonary hypertension model of monocrotaline-induced RV-hypertrophy in which STIM1 expression was reduced but the expression of both STIM1L and Orai1 were increased and this was associated with significant changes in Ca^{2+} handling (Sabourin et al., 2018). Examination of cardiac overexpression of STIM1 has also shed light on the physiological role of STIM1. Studies by Molkentin and colleagues (Correll et al., 2015) showed that cardiomyocyte-specific overexpression of STIM1 not only resulted in increased Ca^{2+} entry but also promoted cardiac hypertrophy, decreased cardiac function, increased mortality, and increased fetal gene expression which was associated with mitochondrial ultrastructural abnormalities and significant alterations in Ca^{2+} handling (i.e., spontaneous Ca^{2+} transients, increased Ca^{2+} spark frequency, increased diastolic Ca^{2+}, and remodeling of the L-type Ca^{2+} channel (LTCC) current). Like the studies in KO mice, the responses to hypertrophic stimuli in these mice were significantly exacerbated. Given that it appears too much or too little cardiomyocyte STIM1 results in similar cardiovascular phenotypes, it is likely that STIM1 levels are tightly regulated and play significant roles in the precipitation of cardiovascular diseases.

STIM1 has also been implicated in contributing to cardiomyocyte injury. For example, reductions in STIM1 levels in hypoxia/reoxygenated cardiomyocytes have been shown to mediate reduced mitochondrial Ca^{2+} overload, in addition to reduced mPTP opening and reduced ROS generation (He et al., 2017). The protective effects of resveratrol following hypoxia/reoxygenation experiments in isolated neonatal rat cardiomyocytes (NRCMs), which included reduced apoptosis and reduced Ca^{2+} overload were also attributed to inhibition of STIM1 (Xu et al., 2019); however, given that resveratrol has multiple cellular affects its direct link to STIM1 inhibition should be considered with caution. Although it should be noted that increasing STIM1 levels was shown to exacerbate the injury in the same model (Xu et al., 2019).

STIM1 has also been shown to have additional roles in the heart other than its roles in hypertrophic signaling and regulation of ER/SR-mitochondrial function. One of these roles is in regulating cardiac electrophysiology. For example, the Rosenberg group showed that STIM1 is expressed in the sinoatrial node (SAN) where it regulates SAN function through modulation of SOCE and LTCC and the regulation of heart rate and cholinergic responsiveness (Zhang et al., 2015a). The same group using STIM1 reporter mice subsequently showed that STIM1 regulates conduction from the SAN to the coronary sinus (Zhang et al., 2020). They showed that STIM1 was an important regulator of atrial function, interatrial conduction, and arrhythmic activity as mice lacking STIM1 in coronary sinus cardiomyocytes showed reductions in conduction and increased arrhythmogenesis. In addition, Bonilla *et al.*, (Bonilla et al., 2019), have shown that spontaneous Ca^{2+} sparks in the setting of cholinergic stress were reduced through STIM1 inhibition with SKF-96365 and in STIM1-KO cardiomyocytes. These observations were similar to those seen in hearts of mice with catecholaminergic polymorphic ventricular tachycardia. We have also shown that hearts from cardiomyocyte-specific STIM1-KO mice have altered heart rates and significant QT prolongation which could be the result of changes in the downstream targets of the cardiomyocyte kinome, leading to potential crosstalk with existing ionic channels that regulate the cardiac action potential such as the LTCC (Collins et al., 2022). Recently, it was hypothesized that the increased and early mortality reported in cardiomyocyte-specific STIM1-KO mouse models could be due to increased arrhythmogenic activity. In support of this, it was shown that hearts from inducible cardiomyocyte specific STIM1 knockdown mice had increased arrhythmic activity and discordant action potential alternans (Cacheux et al., 2019). It is likely that these arrhythmias are due to STIM1 regulating existing action potential currents such as the LTCC although this needs to be determined. Clearly, further studies are required to fully determine the role of STIM1 in regulating cardiac electrophysiology and how STIM1 interacts with other ionic channels known to regulate the electrical activity of the heart and to determine additional non-conical roles of STIM1.

8.2 Orai1

Studies in Zebrafish were amongst the first to indicate a significant role of Orai1 in the heart, where Orai1 deletion resulted in the development of heart failure and significant ultrastructural defects (Volkers et al., 2012). Despite this, conflicting results have been observed in mouse models. Cardiomyocyte-specific KO of Orai1 did not give rise to a significant phenotype at baseline as contractile function and cardiac hemodynamics were both normal; however, when subjected to the hypertrophic agonist, Ang II, cardiomyocyte size and fibrosis were increased contributing to exacerbated Ang II-dependent cardiac remodeling (Segin et al., 2020). Interestingly, this exacerbation was associated with reductions in both STIM1 and Orai3 expression. Interestingly, conflicting results were observed by Bartoli *et al.* who used cardiomyocyte specific Orai1 mutant mice with a mutation in the Orai1 pore and found that inhibition of Orai1 activity during TAC resulted in preserved cardiac function and preserved Ca^{2+} handling (Bartoli et al., 2020). It has also been shown that Orai1 may contribute to

hypertrophy associated with diabetic cardiomyopathy through the regulation of Drp-1-dependent mitochondrial fission (Wu et al., 2021). Specifically, inhibition of Orai1 was shown to reduce cardiac hypertrophy and improve mitochondrial function through reductions in Drp-1, calcineurin, and ERK1/2 activities. Like STIM1, these studies suggest that Orai1 has a significant role in maintaining cardiac homeostasis; however, in the same respect as STIM1, additional studies are required to determine to fully appreciate the physiological role of Orai1 in the heart.

8.3 Other STIM/Orai family members

For several years, the focus has been on the roles of both STIM1 and Orai1 in the physiological and pathological regulation of the heart, with little to no focus on other members of the STIM/Orai families. However, in recent years, STIM2, Orai2, and Orai3 isoforms have also been shown to be present in the heart and appear to have important regulatory roles. Of these additional isoforms, Orai3 has been the most interrogated in the heart. Saliba *et al.* showed that Orai3 activity was increased in hypertrophic cardiomyocytes (Saliba et al., 2015). Specifically, the authors showed that Orai3 was the preferred partner of STIM1 over Orai1 during established hypertrophy and that increased Orai3 activity was responsible for an increase in an arachidonic acid activated Ca^{2+} channel activity during hypertrophy. In a later study by the same group, they showed that the store-independent channel activity mediated by Orai3 during hypertrophy was largely driven by inflammation mediated by TNFα and CD11b/c cells (Keck et al., 2019). More recently, the physiological role of Orai3 has been interrogated in the heart using a cardiomyocyte-specific deletion. Gammons *et al.* (Gammons et al., 2021) showed that both constitutive and inducible cardiomyocyte-specific deletion of Orai3 develop a phenotype consistent with dilated cardiomyopathy, with increased fibrosis, increased mortality, ultrastructural changes in mitochondria, increased mitochondrial fission, and abnormal sarcomeric structure; highlighting a potentially important regulatory role of Orai3 in the heart.

A recent study examining the expression of STIM and Orai isoforms in human failure samples indicated that in addition to the expression of STIM1 and Orai1 being increased and decreased in the left ventricles of heart failure patients, respectively, the expression of STIM2, Orai2, and Orai3 remained unchanged (Cendula et al., 2019). The reduction in Orai1 levels was restricted to male patients, suggesting that sex differences could exist in the expression of STIM-Orai family members, which have not been examined in detail and could contribute to documented sex differences that exist in heart failure progression. Interestingly, in this same study, the STIM2 splice variant STIM2.1, shown previously to have an inhibitory effect on SOCE in T-cells (Miederer et al., 2015), was shown to be significantly reduced in LV of HF patients with a reduction in the ratio of STIM2.1/STIM2. The authors proposed this was indicative of a switch to the stimulatory form of STIM2, STIM2.2. In a cell culture model of cardiomyocyte I/R injury it has been shown that STIM2 expression is upregulated without change in STIM1 expression, and STIM2 knockdown was associated with reduced levels of apoptosis, reduced mitochondrial Ca^{2+} overload, and preserved mitochondrial function (Tu et al., 2020). In skeletal muscle STIM2 colocalizes and interacted with calsequestrin to modulate diastolic Ca^{2+} and Ca^{2+} buffering; however, it remains to be determined whether this also occurs in cardiomyocytes (Jeong et al., 2021). It is possible that targeting STIM2 isoforms could be an important therapeutic strategy in models of cardiomyocyte injury.

While there is growing evidence for a role of STIM2 and Orai3 in the heart future studies are required to establish their physiological roles in the heart and how they are involved in regulating cardiac hypertrophy and I/R injury. Moreover, our understanding of the importance of STIM2 splice variants is in its infancy. In addition, even though Orai2 is present in cardiomyocytes there have yet to be any studies examining the physiological or pathophysiological role of Orai2 in the heart. Clearly future studies are required to better elucidate the cardiovascular functions of all STIM/Orai isoforms and splice variants and their roles in mediating cardiovascular disease.

8.4 Roles of STIM and Orai proteins in non-cardiomyocyte cells in the heart

In addition to cardiomyocytes, STIM and Orai proteins are also widely expressed in other cell types present in the heart, including endothelial cells, vascular smooth muscle cells (VSMCs), and fibroblasts, where they have different effects on the functionality of each cell type. For example, it has been shown that Orai1-mediated SOCE is important for endothelial cell function since endothelial cells with Orai1 knockdown or inhibition have been shown to have reduced tube formation and migration (Li et al., 2011). In this study Orai1 disruption also reduced VEGF-mediated Ca^{2+} entry. Interestingly, STIM/Orai signaling is increased in conditions of high glucose (i.e., 25 mM) in endothelial cells where STIM1, STIM2, Orai1, Orai2, and Orai3 are all increased in addition to an increase in SOCE through calcineurin/NFAT-dependent mechanisms (Daskoulidou et al., 2015). On the other hand, coronary endothelial cells from diabetic mice had lower STIM1 levels, which was linked to impaired endothelial relaxation; this was reversed by partially restoring with adenoviral STIM1 vector (Estrada et al., 2012). These studies suggest that STIM and Orai proteins are important for endothelial cell function in the heart; however, more studies are required to examine the precise functions of STIM/Orai proteins and their respective splice variants in these cells.

STIM1/Orai1-mediated SOCE has been implicated in regulating VSMC function in the heart. Studies by Guo *et al.*, (Guo et al., 2012), have shown that siRNA silencing of both STIM1 and Orai1 not only reduced SOCE but also prevented Ang II-mediated cell proliferation and reduced Ang II-mediated neointimal growth in response to balloon injury. This reduction could be the result of changes in the expression of Orai1 interacting partners, such as SOCE-associated regulatory factor (SARAF) and Homer. It was recently shown that the expression of SARAF was increased in balloon injured arteries along with STIM1 and Orai1. SARAF was shown to specifically

regulate Orai1-mediated VMSC proliferation in response to balloon injury (Martin-Bornez et al., 2022). Homer has also been shown to colocalize with STIM1/Orai1 and regulate Orai1-mediated VSMC proliferation and neointimal growth in the setting of balloon injured arteries (Jia et al., 2017). Furthermore, VSMC remodeling in response to hypertension is associated with a decrease in L-type Ca^{2+} channels (LTCC) and a reciprocal upregulation of STIM1 and Orai1 (Johnson et al., 2020). In addition, inhibition of LTCC was found to activate STIM1/Orai1 Ca^{2+} entry potentially contributing to a proliferative phenotype; however, the mechanism by which this occurs is currently unknown (Johnson et al., 2020). Interestingly, smooth muscle cell-specific KO of STIM1 resulted in smaller myocardial infarct size following coronary artery occlusion and reperfusion (Mali et al., 2018), which was associated with reductions in ER stress, reductions in both p38 and ERK1/2 signaling, reduced apoptosis, reduced fibrosis, and reduced inflammation (Mali et al., 2018). However, the mechanisms by which smooth muscle cell STIM1 contributes to these processes following myocardial I/R remain to be determined. Mice with smooth muscle cell-specific KO of STIM1 have also been shown to have reduced myogenic tone with higher plasma levels of catecholamines and significant dysregulation of the cytoskeleton (Pichavaram et al., 2018). Recently, it was shown that mice with inducible VMSC-specific STIM1-KO had reductions in colocalization of Ca^{2+} clusters between the SR and PM. In addition, these mice were shown to be hypotensive with reduced contractility in resistance arteries (Krishnan et al., 2022). Collectively, these studies highlight the importance of STIM/Orai signaling in VMSCs which should be expanded upon in future studies including determining the roles of other isoforms (i.e., STIM2, Orai2, and Orai3) and their splice variants.

Evidence suggests that STIM/Orai-mediated SOCE may regulate cardiac fibroblast activity during induction of cardiac hypertrophy and the development of heart failure. Increased SOCE has been observed in fibroblasts from failing hearts which was associated with an increase in Orai1 expression, increased colocalization with STIM1, and associated with increased fibrosis (Ross et al., 2017). In addition, Zhang *et al.*, (Zhang et al., 2016), have shown that upregulation of fibronectin, connective tissue growth factor, smooth muscle α-actin, and smad2/3-dependent signaling seen in response to Ang II treatment could be blocked independently using both the SOCE inhibitor, SKF-96365, and STIM1/Orai1 knockdown in cardiac fibroblasts. Collagen synthesis and fibroblast proliferation have both been shown to be reduced in the setting of reduced Orai-mediated SOCE in human cardiac fibroblasts (Chen et al., 2021). This study suggests that STIM1/Orai1 may regulate cardiac fibroblast activity and activation. It has been shown that SOCE may increase in aged human cardiac fibroblasts which was associated with a reduction in the expression of pro-fibrotic sprouty homologue 1 (Spry1) possibly contributing to senescence-mediated fibrosis in the heart; however, in these studies expression levels of STIM/Orai were not changed (Mohis et al., 2018) so the catalyst driving the increase in SOCE in this study remains unclear. Moving forward, it will be important better understand the different functions and roles of STIM and Orai isoforms in the various cell types of the heart and whether these STIM/Orai isoforms contribute to crosstalk between these cell types during physiological and pathophysiological instances.

9 STIM/ORAI IN NEURODEGENERATION

In the adult mouse brain, STIM1 exhibits the highest expression in cerebellum and relatively lower expression in the cerebral cortex, whereas STIM2 is predominantly expressed in the cortex (Moccia et al., 2015). In addition, all three Orai isoforms can be detected in the mouse brain (Moccia et al., 2015). Orai1 appears to be expressed at low levels across multiple brain regions. Orai2, on the other hand, exhibits increased expression levels in the cerebellum as well as the hippocampus. Orai3 is strongly expressed in the cerebellum (Lein et al., 2007). In 2009, Venkiteswaran and Hasan demonstrated that STIM1 and Orai1 were necessary for normal flight and rhythmic firing of the flight motoneurons in *Drosophila* (Venkiteswaran and Hasan, 2009). Following this study there was growing evidence that STIM/Orai plays an important role in neuronal physiology. Interestingly, there are a couple of studies showing that STIM1 regulates Cav1.2, a voltage-gated calcium channel ubiquitously expressed in neurons, cardiac muscles, and smooth muscle cells (Park et al., 2010; Wang et al., 2010). Neuron-specific roles for STIM/Orai include regulating axonal growth, maintaining synaptic plasticity, as well as modulation of memory formation. The role of SOCE in neuronal function has been extensively reviewed elsewhere (Kraft, 2015; Moccia et al., 2015). Here, we review current understandings of STIM/Orai proteins in neurodegenerative processes with a specific highlight on transgenic models.

9.1 Trauma induced neurodegeneration

Two phases of damage can be caused by trauma to the brain. Primary damage occurs at the time of injury whereas secondary damage can last hours to months following the initial impact (Algattas and Huang, 2013). Our current understanding of the mechanism(s) underlying secondary traumatic brain damage emphasizes increased Ca^{2+} influx induced by substantial release of excitatory neurotransmitters, primarily glutamate (Weber et al., 1999). Previous studies have demonstrated the significance of both voltage-gated calcium channels and store-operated calcium entry in mediating trauma-induced cell damage in the brain (Weber, 2012). In an *in vivo* diffuse axonal injury model, neuronal STIM1 protein levels showed a time-dependent increase peaking at 12 h after injury (Li Y. et al., 2013). The authors speculated that this increase in STIM1 might contribute to neuronal necrosis via an increase in SOCE. This concept was supported in an *in vitro* traumatic neuronal injury model by Hou *et al.* who showed that following traumatic injury induced by stainless steel punch cut, STIM1 expression in mouse cortical neurons significantly increased and peaked between 6 and 12 h (Hou et al., 2015). The role of STIM1 in neuronal injury in this model was confirmed by the observation that knockdown of

STIM1 decreased neuronal apoptosis, increased viability, and attenuated glutamate receptor 1 (GluR1)-mediated increase of Ca^{2+} in the cytoplasm (Hou et al., 2015). On the other hand, Rao *et al.* showed that in an *in vivo* cortex injury model STIM2 expression was increased up to 12 h following injury whereas, STIM1 expression remained unchanged (Rao et al., 2015). Moreover, they showed that knockdown of STIM2, but not STIM1, provided protective roles in neuronal survival following injury (Rao et al., 2015). These findings are similar to reports that STIM2 rather than STIM1 contributes to ischemia-induced neuronal injury (Berna-Erro et al., 2009). The differences between these studies may be in part due to different *in vitro* and *in vivo* models for inducing neuronal injury. However, different cell distribution as well as potentially different functions of STIM1 and STIM2 may also be contributing factors. For example, STIM2 has been implicated in regulating basal neuronal Ca^{2+} levels, whereas it has been suggested that STIM1 is an important regulator of mGluR1-mediated Ca^{2+} signaling (Zhang and Hu, 2020). Similar to the cardiomyocyte studies, targeting the balance between STIM1 and STIM2 levels should be explored in more detail. Clearly, more studies are warranted to further understand how STIM1 and STIM2 are involved in trauma induced brain damage.

9.2 Ischemia induced neurodegeneration

Cerebral ischemia can be induced by a variety of events that cause inadequate oxygen delivery to the brain, including thrombotic stroke, embolic stroke, or systemic hypoperfusion. Low oxygen delivery to the brain leads to an increase in glutamate release and subsequent stimulation of glutamate receptors, primarily NMDARs, with the resulting Ca^{2+} overload leading to neuronal death. In a transient cerebral ischemia model where the middle cerebral artery was occluded for 1 h, infarct volume in mice lacking STIM2 was significantly lower than that of control mice 24 h later (Berna-Erro et al., 2009). Consistent with the *in vivo* data, they also reported that *in vitro* culture of brain tissues from STIM2 knockout mice exhibited increased survival following hypoxia. These protective effects were not observed in primary neuron cultures from either STIM1-deficient mice or Orai1-deficient mice. Interestingly, however, in wildtype mice that were transplanted with STIM1-deficient bone marrow, there was a 70% reduction in infarct size following the same 1-h middle cerebral artery occlusion, suggesting that the protective roles for STIM1 in ischemia-induced brain damage are likely due to its function in hematopoietic cells rather than neurons (Braun et al., 2009). Similar protective results were observed in Orai1-deficient bone marrow chimeras (Varga-Szabo et al., 2008).

Zhang *et al.* showed in a rat model of global cerebral ischemia model that STIM1 and Orai1 expression significantly increased following injury and peaked on day 4. Additionally, STIM1 siRNA injection significantly improved neurological functions and decreased neuronal Ca^{2+} levels following ischemia, suggesting a role for STIM1 in mediating ischemia-induced brain damage (Zhang M. et al., 2014). Orai2-deficient mice also exhibited diminished Ca^{2+} signals following oxygen deprivation and were protected from neuronal damage resulting from transient middle cerebral artery occlusion (Stegner et al., 2019). Some of the injury that occurs with this model involves T-cell mediated inflammation and Orai2 is known to regulate Ca^{2+} influx in T-cells. However, bone marrow transplant studies showed that the protective effects of a lack of Orai2 was independent of T cells (Stegner et al., 2019), suggesting a protective role for Orai2 in this setting.

While most reports link increased STIM/Orai levels to neuronal injury, Secondo *et al.*, reported that STIM1 and Orai1 expression levels decreased both in an *in vitro* hypoxia/reoxygenation model and an *in vivo* and focal ischemia model of stroke (Secondo et al., 2019). In addition, an ischemic preconditioning protocol prevented STIM1 and Orai1 downregulation. Moreover, siRNA knock down of either STIM1 or Orai1 attenuated the protection associated with ischemia preconditioning. It was proposed that the neuroprotection resulting from increased STIM1 and Orai1 was due to maintaining ER Ca^{2+} homeostasis thereby reducing ER stress (Secondo et al., 2019). There is increasing evidence of a role for STIM/Orai protein in ischemic injury; however, as with traumatic neuronal injury there are discordant results regarding the role of specific isoforms. The use of global KO models has a potentially confounding effects, given the different cell types in the brain. The development of inducible cell type specific STIM/Orai transgenic and KO models will be needed to improve our understanding of their specific roles in ischemic brain injury.

9.3 Alzheimer's Disease

Alzheimer's disease is a progressively worsening neurodegenerative disease that accounts for over 60% cases of dementia. While most cases of Alzheimer's disease are sporadic and have a relatively late onset, around 1–2% of cases are a result of an autosomal dominant genetic disease. In these familial cases onset of disease is significantly earlier (early 50s vs. over 65) (Lane et al., 2018). Although the pathological processes between sporadic forms and familial Alzheimer's disease are similar, different disease associations do exist. For instance, ApoE2 allele is associated with decreased risk of sporadic Alzheimer's disease whereas individuals with ApoE4 allele have increased disease risk. The familial form of Alzheimer's disease is associated with several proteins, including amyloid beta precursor protein, presenilin 1 and presenilin 2 (Lane et al., 2018).

Over the past two decades or so, numerous studies have demonstrated the involvement of Ca^{2+} homeostasis and intracellular signaling in the development of Alzheimer's disease (Woods and Padmanabhan, 2012; Tong et al., 2018). By comparing intracellular Ca^{2+} levels in hippocampal neurons isolated from young and mid-aged mice, Raza *et al.* provided evidence that aging neurons have significantly higher basal levels of intracellular Ca^{2+}, suggesting that altered Ca^{2+} homeostasis may be a mediator for aging related neuronal deficits (Raza et al., 2007). Ca^{2+} dysregulation has been associated with cascading events of Alzheimer's disease with evidence that it can precede detectable pathological changes (Chakroborty et al., 2009; Muller et al., 2011). Specifically, inositol triphosphate and ryanodine receptor mediated Ca^{2+} signaling was shown to play pivotal roles in AD of transgenic mouse models as well as human cells (Stutzmann et al., 2007; Cheung et al., 2010). There is growing

evidence for decreased SOCE and downregulated STIM/Orai proteins in both the sporadic form and the familial form of Alzheimer's disease. For example, there was up to 70% decrease in STIM1 levels in brain tissues from sporadic Alzheimer's disease patients compared to control (Pascual-Caro et al., 2018). STIM1 knockout in a neuroblastoma cell line showed that although STIM1 was not required for neuronal cell differentiation, it was required for cell survival (Pascual-Caro et al., 2018). In the familial form of Alzheimer's disease, SOCE was found to be attenuated and endogenous presenilin 1 interacted with STIM1 in the ER (Tong et al., 2016). These results suggest that STIM1 may be a therapeutic target for Alzheimer's disease which should be interrogated further. Of note, a recent study from Niemeyer and colleagues identified a splice variant of STIM1, STIM1B, and showed it was significantly decreased in both familial and sporadic forms of AD (Ramesh et al., 2021). Although possessing higher expression level in the cortex and hippocampus, the role of STIM2 in the disease progression of Alzheimer's disease has been less studied. A body of work from Bezprozvanny and colleagues, however, provided insights to the role of STIM2 in disease models for Alzheimer's (Zhang et al., 2015b; Popugaeva et al., 2015). They showed both *in vitro* and *in vivo* that overexpression of STIM2 attenuated mushroom spine loss induced by Ab42 oligomers and provided evidence for STIM2-mediated maintaining of calmodulin kinase II activity. These studies provided support for targeting STIM proteins in Alzheimer's disease; although, several key questions remain such as the role of Orai proteins in Alzheimer's disease. There is evidence that overexpression of both STIM2 and Orai1 increased neuronal Ca^{2+} level; however, no sign of neurodegeneration was observed, suggesting a dissociation of SOCE machinery with Alzheimer's disease progression as determined by amyloidogenesis and immunohistochemistry (Majewski et al., 2020). Of note, another study showed, in a cell model of Alzheimer's disease, that downregulation of Orai2 increases SOCE and decreases amyloid-beta accumulation, which suggests a potential benefit for Orai2 knockdown in Alzheimer's disease (Scremin et al., 2020). In addition, although the majority of current evidence suggest relationships between STIM proteins and Alzheimer's disease progression, more work needs to be done to decipher the roles of STIM/Orai-mediated calcium entry as well as STIM/Orai independent-SOCE in Alzheimer's disease.

9.4 Huntington's and Parkinson's Disease

Huntington's disease is a progressive neurodegenerative disorder that typically presents as chorea, depression, and dementia. It is caused by an expansion of CAG trinucleotide repeats in the resulting in mutant huntingtin (mHTT) gene. It is well acknowledged that overstimulation of glutamate receptors as well as the downstream effects on Ca^{2+} signaling plays a major role in neuronal death in Huntington's disease (Mccolgan and Tabrizi, 2018). Studies have shown that SOCE is involved in the pathogenesis of Huntington's disease and that abnormal SOCE leads to dysregulated synaptic response (Wu et al., 2011; Wu et al., 2016; Wu et al., 2018). Of note, STIM2 appears to play an important role in the disease process. Wu *et al.* showed increased activity of IP_3/IP_3R1 pathway and overexpression of STIM2 in neurons of a mouse model of Huntington's disease (Wu et al., 2016). Upregulated STIM2 senses ER Ca^{2+} content and leads to further dysregulation of SOCE (Wu et al., 2016). Efforts have been made to investigate the possibilities of targeting SOCE in Huntington's disease. Wu and colleagues showed that the SOCE inhibitor, 6 amino 4 (4-phenoxyphenethylamino) (EVP4593), quinazoline normalized motor behavior in a fly model of Huntington's disease. In addition, the application of EVP4593 provided protective effects in a glutamate toxicity assay in culture (Wu et al., 2016). Importantly, *in vivo* administration of EVP4593 in mice rescued age-dependent striatal spine loss (Wu et al., 2011). Ongoing efforts are directed to understanding the molecular targets of EVP4593. Interestingly, efforts have been made to elucidate SOCE machinery in Huntington's disease using patient specific iPSC derived neurons. iPSCs were made from fibroblasts of Huntington's disease patients and healthy donors and were subsequently differentiated into GABA-ergic neurons. SOCE was significantly enhanced in neurons derived from Huntington's disease patients (Vigont et al., 2018). The same group of researchers later demonstrated that in a juvenile form of Huntington's disease, there was increased SOCE that was mediated by STIM2 (Vigont et al., 2021). It is important to note that in this study all iPSCs were derived from a single donor of juvenile Huntington's disease so additional studies are required.

Parkinson's disease is currently the second most common neurodegenerative disease affecting more than 10 million people worldwide. Although currently the mechanisms for the loss of dopaminergic neurons in the substantia nigra remains unclear, it has been shown that mitochondrial dysfunction and alterations in Ca^{2+} homeostasis play a large part in the process. Two studies by Singh and colleagues demonstrated the functional role of SOCE in dopaminergic neurons in the substantia nigra. Interestingly, however, they reported that SOCE in these neurons were mediated by STIM1 and TRPC1 rather than Orai1 (Selvaraj et al., 2012; Sun et al., 2017). Dopaminergic neurons in the substantia nigra utilize Cav1.3 as the subunit for L type voltage gated calcium channel and following TRPC1 activation, L type Ca^{2+} current as well as the open probability of Cav1.3 were reduced (Sun et al., 2017). They also reported that silencing STIM1 and TRPC1 led to increased Cav1.3 current. Further, application of the neurotoxin that mimics Parkinson's disease, 1-methyl-4-phenyl-1,2,3,6-tetrahyrdro-pyridine (MPTP), led to increased activity of Cav1.3, and decreased expression of TRPC1 and inhibited thapsigargin mediated STIM1-Cav1.3 interactions (Sun et al., 2017). Zhou *et al.* found that in fibroblasts from idiopathic Parkinson's disease patients SOCE was impaired, but there was no change in STIM1 or Orai1 protein levels; however, the levels of PLA2g6 a Ca^{2+} independent phospholipase, which activates SOCE were significantly reduced (Zhou et al., 2016). Interestingly, mutations in PLA2g6 are associated with familial Parkinson's disease and they found that knockout of PLA2g6 in mice resulted in Parkinson's disease like symptoms. Loss of PLA2g6 was associated with dysfunctional autophagy in dopaminergic neurons, which was similar to that seen in Orai1-KO mice

(Zhou et al., 2016). This study indicated that defects in PLA2g6 mediated SOCE, possibly via decreased Orai1 activity, could be a novel mechanism contributing to Parkinson's disease.

Currently, most studies on STIM/Orai in Huntington's and Parkinson's disease have originated from only very few research groups and clearly much remains to be learned about the role of STIM and Orai proteins these diseases. For example, it remains unclear why STIM2 appears to play a predominant role in Huntington's, whereas the STIM1-TRPC1 interactions have been identified in models of Parkinson's disease. Moreover, it is surprising that there have been no reports of potential roles of Orai proteins in these diseases, even though STIM-Orai interactions are more commonly recognized as mediating SOCE. Future studies on iPSCs from Huntington's and Parkinson's disease patients represent a potentially valuable area of future investigation.

10 ROLE OF STIM AND ORAI PROTEINS IN CELLULAR AGING

While dysregulation in Ca^{2+} homeostasis is a hallmark of many age-related diseases such as cardiovascular and neurodegenerative diseases, the effect of aging itself on Ca^{2+} homeostasis has not received extensive investigation (Angenendt et al., 2020). Progerin, which is caused by a mutation in the Lamin A/C gene causes a premature aging syndrome called Hutchinson-Gilford progeria syndrome, and may also play a role in normal aging and in age related diseases (Ashapkin et al., 2019). Overexpression of progerin in myoblasts, resulted in the increased colocalization of STIM1 and Orai1 and enhanced SOCE (Wang et al., 2020), suggesting a potential link between aging and altered regulation of STIM and Orai mediated SOCE. STIM and Orai function have been widely studied in relation to the immune system and an age-related decline in immune system function is well established across many species (Nikolich-Zugich et al., 2012). Age-related decreases in Ca^{2+} signaling have been linked to dysfunction in aged lymphocytes. However, although the main Ca^{2+} signaling pathway in lymphocytes is mediated by STIM-activated Orai channels, little was known about the role during lymphocyte aging. Angenendt *et al.*, recently reported reduced SOCE in quiescent and activated lymphocytes from 18 to 24-month-old mice compared to 3–6-month-old mice (Angenendt et al., 2020). This reduction in SOCE was associated with reduced mRNA and protein levels of STIM1 and STIM2 under both conditions. On the other hand, in an *ex vivo* long term cell culture model designed to mimic aspects of aging, such as oxidative stress and DNA damage, the amplitude of SOCE was unchanged in aged lymphocytes although the Ca^{2+} dynamics following stimulation were altered (Rivet et al., 2016). The authors concluded that these changes in Ca^{2+} signaling were potentially a consequence of increased thiol oxidation of STIM1.

Aging is known to lead to vascular dysfunction in part by altered Ca^{2+} homeostasis and signaling in both VSMC as well as endothelial cells (Harraz and Jensen, 2021). However, even though STIM1 has been shown to be essential for regulating smooth muscle cell Ca^{2+} homeostasis and growth (Mancarella et al., 2013), little appears to be known about the role of STIM or Orai proteins in the aging process. Aortic medial degeneration, which is feature of both aortic dissection and aortic aneurysm is closely associated with aging (Nesi et al., 2009). Interestingly, microarray studies on human samples suggested that this might be due in part to lower STIM1 expression (Butt et al., 2016). In a murine model of aortic medial degeneration, inhibition of STIM1 exacerbated the development of medial degeneration (Hong et al., 2019). Of note, the effects of aging on VSMCs function appears to be dependent on specific vascular beds. For example, in 22-month-old rats, SOCE-induced vasoconstriction was enhanced in mesenteric arteries compared to 3-month-old rats; in contrast, it was decreased in the aorta. These changes in vasoconstriction were paralleled with changes in STIM1 and Orai1 protein expression in the different arterial beds (Yang et al., 2015). Despite the limited number of studies on the role of STIM1 on aging of the vasculature system, it appears complex and variable depending on the location of the vascular beds.

In skeletal muscle there have been some contradictory reports, where SOCE was maintained in muscles from aged mice despite reduced STIM1 levels (Edwards et al., 2011) while in another study SOCE was markedly reduced in aging muscle, but with no changes in either STIM1 or Orai1 expression (Zhao et al., 2008). It has also been reported that SOCE plays no role in the decrease in fiber force that occurs in senescent mouse muscle fibers (Payne et al., 2009). On the other hand, Thornton et al., concluded that impaired SOCE contributed to the decrease in contractile force characteristic of aging skeletal muscle (Thornton et al., 2011). Tubular aggregate myopathy (TAM) is a rare disorder skeletal muscle disorder associated with gain of function mutations in both STIM1 and Orai1. Tubular aggregates have also been described in extensor digitorum longus (EDL) muscles from 24-month-old male mice compared to 4–6 month-old mice (Boncompagni et al., 2020). The increase in tubular aggregates in aged muscle was associated with an increased accumulation of STIM1 and Orai1 and this was prevented by voluntary running from 9 to 24 months of age.

While there is some evidence that STIM1/Orai1 mediated SOCE is affected during normal aging the data are somewhat contradictory and gain or loss of function with aging appears to be tissue specific. It is also unknown whether aging leads to changes in the stoichiometry of STIM/Orai oligomers and if that would lead to changes in Ca^{2+} signaling. Given the relative paucity of studies examining how STIM and Orai proteins change during normal healthy aging it is premature to draw any firm conclusions. Nevertheless, the data that is available suggests that this could be a rich and important area for future research.

11 CONCLUSION

In the years since STIM1 and Orai1 were identified as the essential mediators of SOCE, the molecular mechanisms by which they facilitate Ca^{2+} entry into the cell have been explored in detail. While the canonical roles of STIM1 and

Orai1 are broadly accepted in non-excitable cells, arguably there is much less consensus regarding the roles of their isoforms, STIM2, Orai2 and 3; moreover, the identification of an increasing number of splice variants further complicates the picture. In excitable cells, such as neurons and cardiomyocytes, the function of all STIM and Orai isoforms is much less clear; however, the use of cell-specific knockout or mutant models have clearly shown that they play an essential role in maintaining cellular homeostasis. The observations that STIM and Orai isoforms are subject to redox regulation has broad implications into how they might contribute to cellular dysfunction in the setting of oxidative stress.

Despite the rapid growth in our understanding of the role of STIM and Orai and the widely accepted notion that they are core elements of a highly evolutionarily conserved Ca^{2+} signaling pathway in all eukaryotes, numerous gaps in our knowledge remain. Such gaps include the understanding of the role of many post-translational modifications on the function (Johnson et al., 2022). In addition, considerable uncertainty remains regarding the composition of the Orai channel itself, its regulation by STIM isoforms and little is known how this stoichiometry changes in response to aging or pathology. The Orai channel has long been considered to be a hexamer composed of three Orai1 dimers with the ion pore located in the center and its activation occurring primarily as a result of STIM1 C-terminus coupling to the C-terminus of Orai1 (Derler et al., 2016a). While this has been a valuable working model it has contributed to our lack of understanding of the functions of other STIM and Orai isoform. The studies by Trebak and coworkers, which have suggested that all five STIM/Orai isoforms may work together to regulate the Ca^{2+} signaling responses (Yoast et al., 2020; Emrich et al., 2021) are intriguing and could help to explain the wide ranging differences in STIM-Orai Ca^{2+} signaling observed across different tissues and cell types. A better consensus as to the specific stoichiometry of STIM and Orai isoforms will be critical in helping to understand their role in aging and age-related diseases.

As we have described here, STIM and Orai proteins are increasing recognized as regulating cellular functions beyond the classic Ca^{2+} meditated transcription pathways. In addition to metabolic and mitochondrial regulation and cell survival, a protein complex including STIM1 is responsible for trafficking of early and late endosomes (Villari et al., 2020), consistent with its identification as a microtubule plus-end tracking protein. This could have wide ranging implications given the numerous fundamental functions of the endolysosomal system including metabolic signaling, plasma membrane remodeling autophagy and cell migration (Bonifacino and Neefjes, 2017). Clearly, given their potential role in cellular dysfunction in aging and age-related diseases, targeting STIM and Orai mediating signaling as a potential therapeutic target is of interest. However, as noted in the earlier section there are many knowledge gaps that currently limit the development of such therapeutics. However, these knowledge gaps provide considerable opportunity for future research and as they are filled will be improve our understanding of how defects in their function contribute to multiple disease processes, ultimately leading to development of novel therapeutic approaches to modulate their function.

AUTHOR CONTRIBUTIONS

HC, DZ, and JC all wrote different sections of the manuscript and all reviewed and edited the complete manuscript before submission.

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Prohibitins in neurodegeneration and mitochondrial homeostasis

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The incidence of age-related neurodegenerative disorders has risen with the increase of life expectancy. Unfortunately, the diagnosis of such disorders is in most cases only possible when the neurodegeneration status is already advanced, and symptoms are evident. Although age-related neurodegeneration is a common phenomenon in living animals, the cellular and molecular mechanisms behind remain poorly understood. Pathways leading to neurodegeneration usually diverge from a common starting point, mitochondrial stress, which can serve as a potential target for early diagnosis and treatments. Interestingly, the evolutionarily conserved mitochondrial prohibitin (PHB) complex is a key regulator of ageing and metabolism that has been associated with neurodegenerative diseases. However, its role in neurodegeneration is still not well characterized. The PHB complex shows protective or toxic effects in different genetic and physiological contexts, while mitochondrial and cellular stress promote both up and downregulation of PHB expression. With this review we aim to shed light into the complex world of PHB's function in neurodegeneration by putting together the latest advances in neurodegeneration and mitochondrial homeostasis associated with PHB. A better understanding of the role of PHB in neurodegeneration will add knowledge to neuron deterioration during ageing and help to identify early molecular markers of mitochondrial stress. This review will deepen our understanding of age-related neurodegeneration and provide questions to be addressed, relevant to human health and to improve the life quality of the elderly.

KEYWORDS

neurodegeneration, aging, nervous system, prohibitin (PHB), mitochondria

Introduction

Aging has expanded the prevalence of neurodegenerative diseases (ND), which have become one of the greatest challenges in public health and are the leading cause of disability in the world (GBD 2019 Ageing Collaborators, 2022). Since the diagnosis of most of ND only occurs when the nervous system is considerably damaged and the symptoms become evident, the study of the early processes of neurodegeneration will be an important focus of research in the next years, especially for the identification of new therapeutic targets and molecular markers of disease. Among the several ND, their etiology is usually classified based on their symptomatology and the area of the nervous

system where the neurodegeneration occurs (Dugger and Dickson, 2017; Erkkinen et al., 2018). For example, loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies cause Parkinson's disease (PD), characterized by tremor and bradykinesia and progressive rigidity (Ye et al., 2022). Similarly, a progressive degeneration starting from the cortical area and spreading to other brain's structures, including amygdala and hippocampus, and the presence of senile plaques are characteristic of Alzheimer's disease (AD) (DeTure and Dickson, 2019). Moreover, loss of neurons in the cortex and striatum due to expansion of CAG repeats in the huntingtin gene causes chorea, dementia and psychiatric problems in Huntington's disease (HD) (Hong et al., 2021). In amyotrophic lateral sclerosis (ALS), degeneration of motor neurons in cortex, brainstem and spinal cord cause muscle weakness and atrophy (Grad et al., 2017). Finally, neurodegeneration in temporal and frontal cortices develop in behavioral and locomotor problems that are associated with frontotemporal dementia (FTD) (Bang et al., 2015). However, the cause(s) of the neurodegeneration in the above and other CNS diseases may be very different but converge to mitochondria. Indeed, environmental, genetic and/or spontaneous alterations in mitochondrial homeostasis may cause mitochondrial stress, which consist in damage in DNA, proteins and/or lipids that trigger quality-control pathways to overcome the damage (Youle and van der Bliek, 2012). While ND and their subtypes have different etiologies, the beginning of the neurodegeneration process in most of them share pathways related with early mitochondrial stress (Lin and Beal, 2006).

Striking progress have been obtained in the past decades when it comes to elucidating mitochondrial function and its relationship with neurodegenerative diseases and aging. In PD, environmental exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or pesticides cause mitochondrial disfunction, as well as genetic factors such as mutations in the PINK1, parkin, DJ-1, POLG, LRRK2, and SNCA genes, which are involved in mitochondrial pathways [reviewed in Borsche et al. (2021)]. In AD, mitochondrial disfunction also contributes to development of neurodegeneration, although is still not clear if an initial mitochondrial and bioenergetic alteration causes protein aggregation and further problems in the neuronal physiology, or whether upstream pathologies including aggregation trigger mitochondrial stress and progression of the disease [reviewed in Swerdlow (2018)]. In HD, despite its cause is purely genetic, the interactions of the mutated huntingtin protein with mitochondria is one of the earliest events in the development of the disease, and cause mitophagy, synaptic degeneration, defective mitochondrial transport, excessive mitochondrial fragmentation and failure to remove dead mitochondria [reviewed in Sawant et al. (2021)]. In ALS and FTD, the impairment of mitochondrial function has been widely studied, and many genes are involved in mitochondrial stress, dynamics, structure, bioenergetics and calcium buffering [reviewed in Smith et al. (2019) and Anoar et al. (2021)]. One of the major regulators of mitochondrial homeostasis is the prohibitin (PHB) complex (Lin and Beal, 2006; Artal-Sanz and Tavernarakis, 2009a). The PHB complex is formed by two prohibitin isoforms, prohibitin 1 (PHB1) and prohibitin 2 (PHB2), which form a heterodimeric ring-shaped complex in the mitochondrial inner membrane (Steglich et al., 1999; Nijtmans et al., 2000; Artal-Sanz et al., 2003). PHB-1 and PHB-2 subunits are interdependent for protein complex formation, leading the absence of one of them to the absence of the whole PHB complex (Berger and Yaffe, 1998; Nijtmans et al., 2000; Artal-Sanz et al., 2003). The PHB complex is strongly evolutionarily conserved among eukaryotes and is ubiquitously and abundantly expressed (Nijtmans et al., 2000; Coates et al., 2001). Prohibitins play important roles in many physiological events, including energy production, intracellular signaling, aging, metabolism, and apoptosis, however, their exact biochemical function remains to be clarified. Two predominant views have emerged for the function of the PHB complex; as a membrane-bound chaperone-like complex (Nijtmans et al., 2000; Nijtmans et al., 2002), and as a lipid scaffold-like complex (Merkwirth et al., 2008; Osman et al., 2009; Merkwirth et al., 2012). Although more work is needed to clarify its exact molecular function, evidences show a direct impact of the PHB complex on mitochondrial functionality. Indeed, imbalance in the cytoplasmic/mitochondrial ratio of protein levels by disruption of mitochondrial protein synthesis with thiamphenicol causes upregulation of prohibitin expression (Coates et al., 2001). This role in protein metabolism has been suggested to be carried out by the PHB complex binding directly to the newly synthetized products, avoiding their degradation and conferring stability (Nijtmans et al., 2002; Artal-Sanz and Tavernarakis, 2009a). On the contrary, lack of PHB complex induces the mitochondrial-specific unfolded protein response (UPR^{mt}) (Hernando-Rodriguez and Artal-Sanz, 2018; Hernando-Rodriguez et al., 2018) and alters lipid metabolism, specially cholesterol synthesis and alters cardiolipin acylation (Merkwirth et al., 2008; Richter-Dennerlein et al., 2014; Hernando-Rodriguez and Artal-Sanz, 2018; Lourenco et al., 2021). Furthermore, deregulation of the PHB complex and mitochondrial dysfunction have been associated with many physiological processes like cancer [reviewed in Koushyar et al. (2015)], liver injuries [reviewed in Barbier-Torres and Lu (2020)], obesity and adipocyte-immune cell cross-talk in diabetes [reviewed in Ande et al. (2014) and in Mishra and Nyomba (2017)], degenerative disorders [reviewed in Signorile et al. (2019)], sex-based immune diseases [reviewed in Mishra and Nyomba (2019) and in Zi Xu et al. (2018)], ageing [reviewed in Thuaud et al. (2013)] and cell survival and apoptosis [reviewed in Peng et al. (2015)]. Importantly, in the recent years, PHB proteins have been proposed to have a role in several ND, including PD, AD, HD, ALS, FTD and others (Merkwirth et al., 2012; Belser and Walker, 2021). However, the cellular and molecular

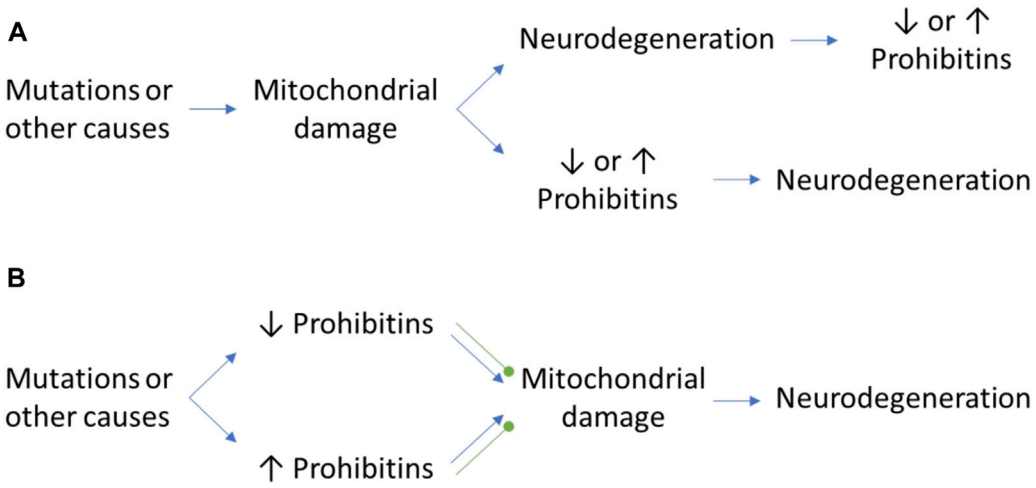

FIGURE 1
The controversial role of PHB in neurodegeneration. **(A)** Mitochondrial damage may cause neurodegeneration and a differential expression of PHB as a consequence of the neurodegenerative process or the activation of mitophagy pathways. Inversely, the mitochondrial damage itself may cause an alteration of PHB expression levels which may trigger the neurodegenerative process. **(B)** The alteration of PHB levels in normal conditions due to mutations in other proteins may have beneficial and detrimental effects that will cause the protection against mitochondrial damage or the promotion of it, respectively. Green round arrows: protection against mitochondrial damage, which prevents neurodegeneration.

underpinnings of PHB function in the nervous system and in the context of ageing are still poorly understood. Here, we review the latest advances in neurodegeneration and mitochondrial homeostasis associated with PHB in the nervous system and propose important questions to be addressed in future research.

Prohibitins in vertebrates

The study of PHB in the nervous system of vertebrates has been limited by the complexity of animal models and the short number of human samples. It has focused mainly on the correlation of molecular cues involved in mitochondrial homeostasis observed in cell cultures, and the study of protein expression and cellular viability under certain conditions.

Mitochondrial function and complex assembly

One of the crucial aspects of neuronal survival is the correct assembly of mitochondrial complex I. Several cases of ALS and FTD are related with lower metabolic activity and energy production. For example, in a liquid chromatography tandem mass spectrometry study of ALS and FTD patients, Iridoy and colleagues observed that both PHB1 and PHB2 where downregulated, and Western blot (WB) analysis further confirmed a reduction of ≈15%–20% and ≈40%, respectively, in the spinal cord (Iridoy et al., 2018). On the other hand, haploinsufficiency of C9orf72, a protein closely related with ALS and FTD, causes a ≈50% degradation of the translocase TIMMDC1, and a ≈30% blockage of complex I assembly, as measured by two-dimensional BN/SDS–PAGE analysis (Wang et al., 2021a). Wang and colleagues demonstrated that in mice, the PHB complex is recruited by C9orf72 in the mitochondrial intermembrane space, which block the degradation of the translocase TIMMDC1 and facilitates complex I assembly (Wang et al., 2021a). Another protein involved in ALS and FTD, CHCHD10, have been related with PHB and motor neuron degeneration. In a recent study by Genin and colleagues, the authors observed in patient fibroblasts and mice by immunolabelling that in 48.1% of dying spinal motor neurons, PHB aggregates with Stomatin-Like Protein (SLP2) in the absence of CHCH10, which participate in the stability of the PHB complex and regulates motor neuron death (Genin et al., 2022). Since a normal level of PHB is required for the correct functioning of mitochondria, a decrease of PHB levels in specific tissues can be a consequence of reduced cell numbers due to the neurodegeneration process rather than an actual downregulation of PHB expression. Therefore, data should be analyzed with caution as further studies analyzing PHB expression levels are required to elucidate their role on the different stages of the neurodegenerative process and ageing (Figure 1). Additionally, PHB's interactome may be cell specific. This could be the case of another major contributor to ALS and FTD, the transactive response DNA-binding protein 43 (TDP-43), which also recruits PHB2 in mitochondria, as observed in a proteomic screening of mice cortex lysates and confirmed by

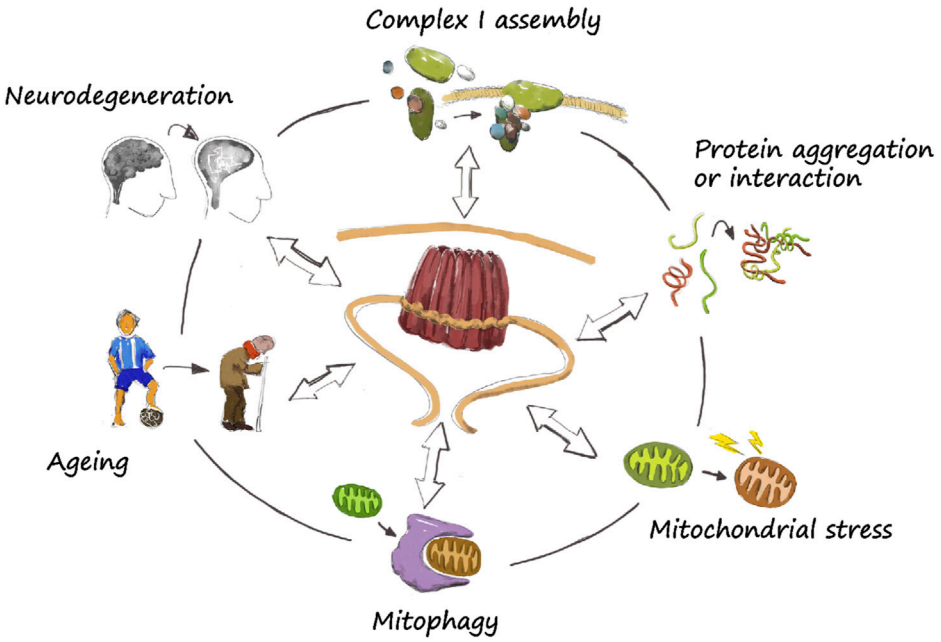

FIGURE 2
The prohibitin wheel. The role of PHBs in mitochondrial homeostasis is diverse. They directly participate in specific functions such as complex I assembly, protein aggregation and turnover, mitochondrial stress and mitophagy. They have been also described to have a role in ageing and neurodegeneration, although the exact mechanism by which they regulate lifespan or neuroprotection is still unclear. Nevertheless, a correct functioning of mitochondria is necessary, so a dysregulation of basic mitochondrial functions may lead to neurodegeneration.

immunoprecipitation by Davis and colleagues. However, the authors could not find a direct effect on mitochondrial bioenergetics in overexpression or downregulation of TDP-43 in HEK cell cultures (Davis et al., 2018).

Prohibitins in neurodegenerative diseases

PD is another neurodegenerative disease related with PHB. Prohibitins have been associated with neuroprotection in substantia nigra (SN) and ventral tegmental area (VTA). In healthy human and mice brains, PHB levels are higher at VTA than in SN, as measured by WB (1.5- and 2-fold increase respectively), qRT-PCR (3-fold increase in mice) and immunofluorescence (≈2-fold increase in human brain) (Dutta et al., 2018). Interestingly, both regions show decreased PHB protein expression in PD human brains. However, in PD induced (MPTP treated) mice brains, the number of dopaminergic neurons is more decreased in SN as compared with VTA (Dutta et al., 2018), which suggest that higher levels of PHB in VTA may protect against neurodegeneration in this area. Indeed, in the SN of MPTP-treated mice the PHB levels increase 2-folds after 3 days of treatment, but decrease after 7 days, suggesting that PHB upregulation upon mitochondrial stress may be an early mechanism of neuroprotection. Similarly, in a 6-hydroxidopamine (6-OH)-induced PD rat model, PHB expression is upregulated in SN, and more in particular, in dying dopaminergic neurons, where PHB interacts with the mitochondrial complex I NADH-ubiquinone oxidoreductase 30 kDa subunit (NDUFS3), suggesting a possible role of PHB in neuroprotection of dopaminergic cells against mitochondrial dysfunction (Park et al., 2010). However, the role of PHBs in neurodegeneration is still unclear. In another study performed in mice by Triplett and colleagues, the authors performed a 2D-electrophoresis proteomic analysis in Ser/Thr kinase PTEN-induced kinase 1 (PINK1) deficient mutants, a mitochondrial gene associated with early-onset PD. They observed an overall decrease of PHB expression in PINK1 mutants (0.464-fold) as compared with control animals (Triplett et al., 2015). This goes in line with the growing evidences that PHBs regulate PINK1-PRKN/Parkin-dependent mitophagy (Wei et al., 2017; Yan et al., 2020). PHBs help to stabilize PINK1 in the outer mitochondrial membrane, who recruits Parkin to ubiquitinate membrane proteins and causing membrane rupture. This exposes PHB2 from the inner mitochondrial membrane, which interacts with phagophores to initiate mitophagy. It remains unclear though how the inhibition of mitophagy by lack of PINK1 could lead to a decrease of PHB expression. This suggest that the mechanisms regulating PHB expression in brain may be different depending on the tissue or the molecular pathways that promotes it. For example, protein quality control in mitochondria is a key component of

TABLE 1 Summary of the studies involving PHB expression or function in the nervous system, ageing, and disease.

Subject	Related gene or disease	Treatment	Effect	Reference
Vertebrates				
Human	ALS and FTD	None	↓PHB1	Iridoy et al. (2018)
			↓PHB2	
Mice	*C9orf72*$^{(-/-)}$ (ALS and FTD)	None	Loss of PHB recruitment—mitochondrial complex I assembly	Wang et al. (2021a)
Human fibroblast/mice	CHCHD10$^{(-/-)}$ (ALS and FTD)	None	SLP2 aggregation and dysregulation of motor neuron death	Genin et al. (2022)
Mice	TDP-43$^{(-/-)}$ (ALS and FTD)	None	PHB2 recruitment in mitochondria	Davis et al. (2018)
Human	Healthy	None	PHBs levels in VTA > SN	Dutta et al. (2018)
	PD patients	None	↓ PHBs in VTA and SN	
Mice	PD-induced	MPTP	Degeneration in SN > VTA	
Rat	PD-induced	6-OH	↑PHBs in SN	Park et al. (2010)
		6-OH and Phb knockdown	↑Toxicity	
Mice	PINK1$^{(-/-)}$ (PD)	None	↓ PHBs in brain	Triplett et al. (2015)
MEF	*HtrA2*$^{(-/-)}$ (PD)	None	↑ PHBs	Goo et al. (2014)
HEK cells	PD	*HtrA2* knock-down		
Mice	TG2$^{(-/-)}$ (PD and HD)	None	TG2—PHB interaction to facilitate mitochondrial complex I assembly	Battaglia et al. (2007)
Mice neuroblastoma cells and primary cortical neurons	Healthy	*Phb* overexpression	Neuroprotection against hypoxia and cellular stress	Korwitz et al. (2016), Anderson et al. (2020)
Primary cortical neurons	Healthy	Glucose and oxygen deprivation	PHB interaction with NO—Neuroprotection	Qu et al. (2020)
Mice	Healthy	Intense exercise	↓PHB in neurons of dentate gyrus	So et al. (2017)
	Healthy	Moderate exercise	↑PHB in neurons of dentate gyrus	
Rat primary neurons	Aged	Purine derivative drug treatment	Interaction with PHB to improve cognitive deficits	Guyot et al. (2020)
Human fibroblast cells	*FBXO7* (PD)	None	Protein aggregation aggravated by PHB1	Zhou et al. (2015)
Mice	Healthy adult (Hearing loss)	None	↓PHB and ↓Mitophagy in cochlea	Yu et al. (2021)
Rat—PC12 cells	Healthy (AD)	$A\beta_{42}$ treatment	↓PHB2	Sinclair et al. (2021)
Neuronal and glial mouse cell cultures	Healthy	Paroxetine	↓PHB	McHugh et al. (2008)
Neuroblastoma SH-SY5Y cells	Healthy	Morphine	↓PHB	Mouledous et al. (2005)
Mouse hippocampal HT22 cells	Healthy	Ochratoxin A	↓Viability	Yoon et al. (2009)
			↑PHB	
Dopaminergic SH-SY5Y cells	Healthy	*Phb* overexpression and MPP^+ treatment	↑Neuroprotection	Wang et al. (2021b)
Rat—PC12 cells	Healthy	*Phb* overexpression and rotenone treatment	↑Neuroprotection	Zhou et al. (2012), Anderson et al. (2018)
Human	Schizophrenia	None	↑PHB in oligodendrocytes of right dorsolateral white matter	Bernstein et al. (2012)
Mouse NSC-34	Healthy	Enterovirus 71—infected	Cell surface PHB2 mediates virus internalization/ Mitochondrial PHB2 facilitates virus replication	Too et al. (2018)
Invertebrates				
Drosophila	*U6atac* (Splicing factor)	None	↓PHB2	Pessa et al. (2010)
Planarian stem cells	Healthy	Knockdown of *Phb2*	Blockage of regenerative capabilities	Rossi et al. (2014)
Insects	Healthy	Dengue virus—infected	PHB2 mediates virus internalization	Kuadkitkan et al. (2010)
C. elegans	Healthy	*Phb* knockdown	Decreases lifespan	Artal-Sanz and Tavernarakis (2010)
		Phb knockdown + *daf-2* knockout	Increases lifespan	

mitochondrial homeostasis, and defects in these pathways can cause mitochondrial malfunction and neurodegeneration. Indeed, deficiency of the mitochondrial protease HtrA2 has been implicated in PD and causes motor neuron degeneration in mice. Goo and colleagues demonstrated using immunohistochemistry and luminescence assays that HtrA2 directly interact with PHB, which is overexpressed in HtrA2$^{-/-}$ mutants, causing higher ROS production (≈3-fold increase) and abnormal mitochondrial membrane potential (≈40% decrease) (Goo et al., 2014). As in ALS and FTD, the correct assembly of mitochondrial complex I has also been associated with proteins involved in HD and PD, such as transglutaminase 2 (TG2). Mutant mice lacking this protein are more vulnerable to neurodegeneration of nigrostriatal cells upon treatment with disruptors of mitochondrial complex I and II. In a proteomic analysis by Battaglia and colleagues, and confirmed by SDS–PAGE and WB, they found that TG2 directly interacts with PHB, participating in the regulation of the respiratory chain by generating post-translational modifications on the PHB complex which facilitates the assembly of mitochondrial complex I (Battaglia et al., 2007).

Protective role of prohibitin in the nervous system

On the other hand, in mice neuroblastoma cells and primary cortical neurons, overexpression of PHB protects the nervous system against hypoxia and cellular stress by fully reducing cytochrome *c* release, possibly by stabilization of cardiolipin in mitochondria and regulation of the AAA-protease OMA1 (Korwitz et al., 2016; Anderson et al., 2020). In another study, also in primary cortical neurons, PHB interacts with NO under glucose and oxygen deprivation to mediate neuroprotection, promoting a 50% increase of cell viability, which is not observed under NO deprivation (Qu et al., 2020). On the contrary, intense exercise in mice decreases PHB expression in neurons of dentate gyrus (≈40% decrease), while a moderate exercise promotes it (≈25% increase), as observed in quantification of immunohistochemistry images (So et al., 2017). However, it is not clear if the decrease is caused by a hypoxic environment or other exercise-associated causes. Similarly, it is not clear whether the increase of PHB is caused by promotion of cell proliferation and migration promoted by moderate exercise or by local induction of PHB expression (So et al., 2017; Davis et al., 2018). This data further highlights the need of deeper knowledge in PHB, as depending on the mitochondrial parameter of the study and the cause of a mitochondrial disfunction, the up or down regulation of PHB can be either beneficial or detrimental (Figure 1).

The protective role of PHB in apoptosis and oxidative stress has position this molecule as a strong candidate as a therapeutic target for ND and ageing. For example, Guyot and colleagues reported that in rat primary neuron cultures and mice, a purine derivative drug targets PHB1 and PHB2 with a binding affinity (k_D) of $9.50 \times 10^{-6} \pm 4.60$ and $1.29 \times 10^{-6} \pm 1.16$ M, respectively, to improve cognitive deficits in ageing by regulating apoptosis and ROS production, as well as transcription of factors involved in synaptic function, neuroplasticity, and inhibition of neuronal Tau phosphorylation. Additionally, they reported that this purine drug reduces ≈2-folds the IL-β expression as measured by analysis of fluorescent images, *via* interaction with PHB, participating in the inhibition of neuro-inflammation (Guyot et al., 2020). Inversely, the role of PHB1 as a mitochondrial protease inhibitor may cause a more deleterious effect in protein aggregation. This is the case of mutations in F-box protein 7 (FBXO7), which causes juvenile PD. Mutations in FBXO7 cause a more severe aggregation in mitochondria, leading to toxicity and increased mitophagy, which can be further aggravated by PHB1 (Zhou et al., 2015). Considering that neurodegenerative diseases such as PD and AD implicate the misfold and aggregation of proteins, among other factors, it would be of interest to study the effect of PHB in protein aggregation under those backgrounds.

Another age-related disease is hearing loss, where ROS production induces mitochondrial damage and decreases its function. This causes a reduced mitophagy that is associated with higher cellular damage at aged cochlea and increased hearing loss. Interestingly, PHB2 expression is reduced (2.5-fold) along other mitophagy factors such as PINK1, Parkin and TOMM20 in mice with age-related hearing loss, which suggest a possible role of this protein in aging (Yu et al., 2021). Similarly, in a proteomic study by Sinclair and colleagues, PC12 cell cultures were treated with $A\beta_{42}$, which caused a reduction in PHB2 expression (−1.591-fold decrease), suggesting a possible role for PHB in AD (Sinclair et al., 2021).

The role of PHB in neurodegeneration must be taken not only from a therapeutic point of view, but also as secondary risk factor of drug treatments for other diseases. For example, the antidepressant paroxetine has been shown to cause a decrease of PHB expression (0.47-fold decrease) in neuronal and glial mouse cell cultures (McHugh et al., 2008). Similarly, in SH-SY5Y cell cultures expressing the human mu-opioid receptor, a long term exposure to morphine causes a reduction of PHB expression of 38.4% ± 6.4% (Mouledous et al., 2005). On the contrary, commonly encountered natural products, such as mycotoxins in contaminated foods or chemical toxins in pesticides, can also cause an alteration in PHB expression. Indeed, Ochratoxin A (OTA), a mycotoxin present in mold particles from contaminated food causes a reduction of viability in mouse hippocampal HT22 cells, and the upregulation (3.5-fold increase) of PHB (Yoon et al., 2009). This suggest that PHB upregulation could be used as a protective mechanism against toxic insult, just as observed in neurons of PD-induced models (Park et al., 2010; Dutta et al., 2018). In dopaminergic SH-SY5Y cells treated with MPP^+, overexpression of PHB restores

mitochondrial membrane potential (≈2-fold increase as compared to control treated samples), decreases ROS (≈2-fold decrease) and reduces cytochrome c release (≈−0.8-fold decrease) (Dutta et al., 2018; Wang et al., 2021b), while knockdown of PHB increases the toxic effect (≈1.6-fold) of 6-OH as measured by cell viability (Park et al., 2010). Rotenone, another neurotoxin involved in mitochondrial complex I disruption, have been also associated with a protective role of PHB in PC12 cell cultures and rat primary neurons. Indeed, upregulation of PHB causes a decrease of ROS production (≈40% decrease after 15 min of rotenone induction) and restore mitochondrial complex I activity (≈10% increase) under rotenone treatment (Zhou et al., 2012; Anderson et al., 2018). Considering the wide role of PHB in regulating mitochondrial homeostasis, further studies on their inducibility are necessary, as well as elucidating their molecular interactome to develop more precise and targeted therapeutic treatments.

However, it is important to not place PHB as only a neuronal therapeutic target. For example, in human brains diagnosed with Schizophrenia, the right dorsolateral white matter area shows a higher density of prohibitin-expressing oligodendrocytes (Bernstein et al., 2012). In mice, PHB has been associated with dysfunction of mitochondria in Schwann cells and demyelination. PHB1 and PHB2 play diverse roles during developmental myelination and myelin maintenance in the peripheral nervous system (Poitelon et al., 2015). Depletion of PHB1 in Schwann cells also reduces levels of PHB2 protein, in agreement with their interdependence for PHB protein complex formation. First, PHB1 depletion activates mTORC1 and c-Jun in Schwann cells, which participate in the demyelination process (Della-Flora Nunes et al., 2021a). Della-Flora and colleagues also proposed that PHB2 has extra-mitochondrial activities during development, which are necessary for proper radial sorting, while both PHB1 and PHB2 are required in mitochondria for long-term myelin maintenance (Della-Flora Nunes et al., 2021b).

In summary, despite the important advances in the understanding of PHB's function in mammals, their regulatory pathway and how they protect mitochondria from stress are still poorly understood. This is partly due to the fact that many studies have followed different approaches to study protein expression, going from proteomic screening to WB or qRT-PCR, and in many times, lack of molecular mechanisms is striking. Although the information provided by these results is of interest to the scientific community, a systematic approach should be followed to evaluate whether expression levels of PHBs, especially in downregulation, is actually caused by changes in cell proliferation or neurodegeneration rather than by regulation of PHB expression. Furthermore, the study of PHB's interactome would be crucial to understand their role in neurodegenerative diseases, specially in those where protein aggregation is a particular characteristic, and where it seems that PHB may play a protective role. More studies are necessary to evaluate PHB as a potential therapeutic target to avoid possible secondary effects.

Prohibitins in invertebrates

Drosophila

The study of Prohibitins in the nervous system of invertebrate models is less abundant, although interesting progress has been achieved over the past few decades. In flies, the mechanisms regulating mitochondrial aggregation may be similar to those observed in mammals. The overexpression of human wild type FBXO7 in dopaminergic neurons of *Drosophila* also leads to neuron degeneration and FBXO7 aggregation (Zhou et al., 2015). If these effects can be aggravated by PHB1 in human cell lines, it would be interesting to study how PHBs participate in protein aggregation in simpler animal models such as *Drosophila*. More advances have been achieved with regard to the expression of PHB2 and its role in metabolic pathways *via* mitochondrial homeostasis. CG15081/l(2) 03706 is the human ortholog of PHB2 in flies, and contains a U12-intron that is recognised by the splicing machinery, which plays an important role in gene regulation and mRNA splicing. Mutations in the splicing factor U6atac leads to downregulation of PHB2 (−1.29-fold), which may lead to the downregulation of other metabolic genes, including enzymes, nucleases, cytochrome P450 or detoxification-related transferases causing larval lethality (Pessa et al., 2010). It remains unclear though, whether these changes in the genetic profile caused by PHB2 downregulation could be also observed in a tissue-specific manner.

Caenorhabditis elegans

In *C. elegans*, the study of PHB-1 and PHB-2 has focused on their effect on longevity, which is dependent on their metabolic state or other genotypes (Artal-Sanz and Tavernarakis, 2010). Knockdown of each of the subunits shorten lifespan in these animals (17 and 18 days, respectively, versus 20 days in wild type), however, in genetic backgrounds with mutations in signalling growth factors, mitochondrial homeostasis or metabolic proteins, PHB depletion has an opposing effect, dramatically extending lifespan to even three times longer than in wild type (Artal-Sanz and Tavernarakis, 2009a; b; 2010). Interestingly, neuronal knockdown of *cco-1*, a member of the electron transport chain, induces the UPR^{mt}, increasing mitochondrial HSP-6 expression (10-fold increase) in the intestine and extending lifespan (23.8 mean lifespan) (Durieux et al., 2011). The UPR^{mt} is also involved in lifespan regulation upon PHBs depletion (Yoneda et al., 2004; Gatsi et al., 2014), opening the possibility that neuronal manipulations of PHB could also have a systemic effect. Similarly, neuronal expression of an aggregation-prone polyglutamine (PolyQ40) induces the UPR^{mt} in the intestine (≈1.33-fold increase) and affects whole-animal physiology (Berendzen et al., 2016). It is unknown whether PHBs functions cell-autonomously and if depletion of PHBs in neurons or specific tissues may have an

impact in the lifespan of these animals or in the development of neurodegenerative diseases.

Other invertebrates

In planarians, interesting results have been observed also for PHB2, although its particular role in neurodegeneration is less studied in these organisms. However, they may reveal important features of PHB in stem cells considering that cell therapies are emerging strategies for the treatment of ND. Indeed, Rossi and colleagues knocked-down the *Phb2* ortholog in planarians, *DjPhb2*, and observed a blockage of regenerative capabilities of the stem cells, which eventually lead to death, suggesting that DjPhb2 is also involved in cell cycle proliferation and mitochondrial morphogenesis (Rossi et al., 2014). A role of PHB in neurogenesis has also been suggested in the sea urchin, where an expression of *Lv-prohibitin* has been observed in tissues where neurons of the pyloric and anal sphincters originate, although a neuronal role of *Lv-prohibitin* in this organism still needs to be proven (Slota et al., 2019). In insects, PHB2 acts as a receptor to mediate the internalization of the dengue virus (Kuadkitkan et al., 2010). Similarly, the enterovirus 71 (EV71), involved in neurological diseases, uses PHBs located at the cell surface to penetrate into the neurons and mitochondrial PHBs for viral replication (Too et al., 2018). This is an interesting characteristic of PHBs that would also be important to study in viruses targeting the central nervous system and may open the possibility of PHBs as targets for drug delivery.

In summary, research in PHB's with invertebrate models has further increased our knowledge about their role in mitochondrial stress and ageing. The good use of these models can be taken to promote future research lines to explore interesting characteristics of these molecules, such as drug delivery, cell proliferation, or to study in deep their molecular mechanism.

Future perspectives

The characterization of PHBs has enormously advanced in the recent years, and progress has been achieved in key points of its role upon mitochondrial homeostasis. Their biological function may be directly involved in specific roles, including complex I assembly, protein aggregation, induction of mitochondrial stress, and mitophagy, which are relevant in the ageing and neurodegenerative process (Figure 2). However, there is still much to learn about these proteins and its controversial role upon regulation of lifespan and neurodegeneration, although it seems to be an overall tendency to neuroprotection upon PHB upregulation (Table 1). Interestingly, a similar controversial characteristic is observed for PHB1 in liver injury and cancer, where it can be pro- or anti-tumorigenic, although the overall tendency is also protective [reviewed in Barbier-Torres and Lu (2020)]. It remains unclear though whether PHB2 has the same role in liver than his partner. Considering that neurodegenerative diseases are usually diagnosed at an advanced status, when symptoms become evident, PHB's may stand as an early marker of diseases. Thus, a better understanding of the molecular mechanisms of PHBs in the regulation of mitochondrial homeostasis in ageing and neurodegeneration would be an important advance in the field (Figure 2). It remains unclear the global effect that neuronal PHBs may have in a whole organism or the central nervous system. Phb depletion has been shown to induce the UPR^{mt} (Gatsi et al., 2014; de la Cruz-Ruiz et al., 2021). Interestingly, mitochondrial stress in neurons induce the UPR^{mt} in other unrelated tissues, possibly mediated by neuropeptides (Durieux et al., 2011; Shao et al., 2016; Zhang et al., 2018). Advances in these important questions will be beneficial for human health and for the development of alternative therapeutic treatments.

Author contributions

JF-A and MA-S wrote the manuscript which was drafted by JF-A.

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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.

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Periodic ethanol supply as a path toward unlimited lifespan of *Caenorhabditis elegans* dauer larvae

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The dauer larva is a specialized stage of worm development optimized for survival under harsh conditions that have been used as a model for stress resistance, metabolic adaptations, and longevity. Recent findings suggest that the dauer larva of *Caenorhabditis elegans* may utilize external ethanol as an energy source to extend their lifespan. It was shown that while ethanol may serve as an effectively infinite source of energy, some toxic compounds accumulating as byproducts of its metabolism may lead to the damage of mitochondria and thus limit the lifespan of larvae. A minimal mathematical model was proposed to explain the connection between the lifespan of a dauer larva and its ethanol metabolism. To explore theoretically if it is possible to extend even further the lifespan of dauer larvae, we incorporated two natural mechanisms describing the recovery of damaged mitochondria and elimination of toxic compounds, which were previously omitted in the model. Numerical simulations of the revised model suggested that while the ethanol concentration is constant, the lifespan still stays limited. However, if ethanol is supplied periodically, with a suitable frequency and amplitude, the dauer could survive as long as we observe the system. Analytical methods further help to explain how feeding frequency and amplitude affect lifespan extension. Based on the comparison of the model with experimental data for fixed ethanol concentration, we proposed the range of feeding protocols that could lead to even longer dauer survival and it can be tested experimentally.

KEYWORDS

dauer larvae, lifespan extension, metabolic network, mathematical model, ethanol, periodic feeding

1 Introduction

Caenorhabditis elegans is a well-known free-living nematode studied as a model organism to address a broad range of biomedical questions from genetics, cell biology, and human disease conditions to nematode control Sydney (1974); Corsi et al. (2015). In the context of how organisms may adapt to stressful environmental conditions, *C. elegans* larval stage called "dauer" is of particular interest Riddle (1988); Hu (2007); Erkut and Kurzchalia (2015). A developing *C. elegans* larva at the L1 stage can turn into an alternative

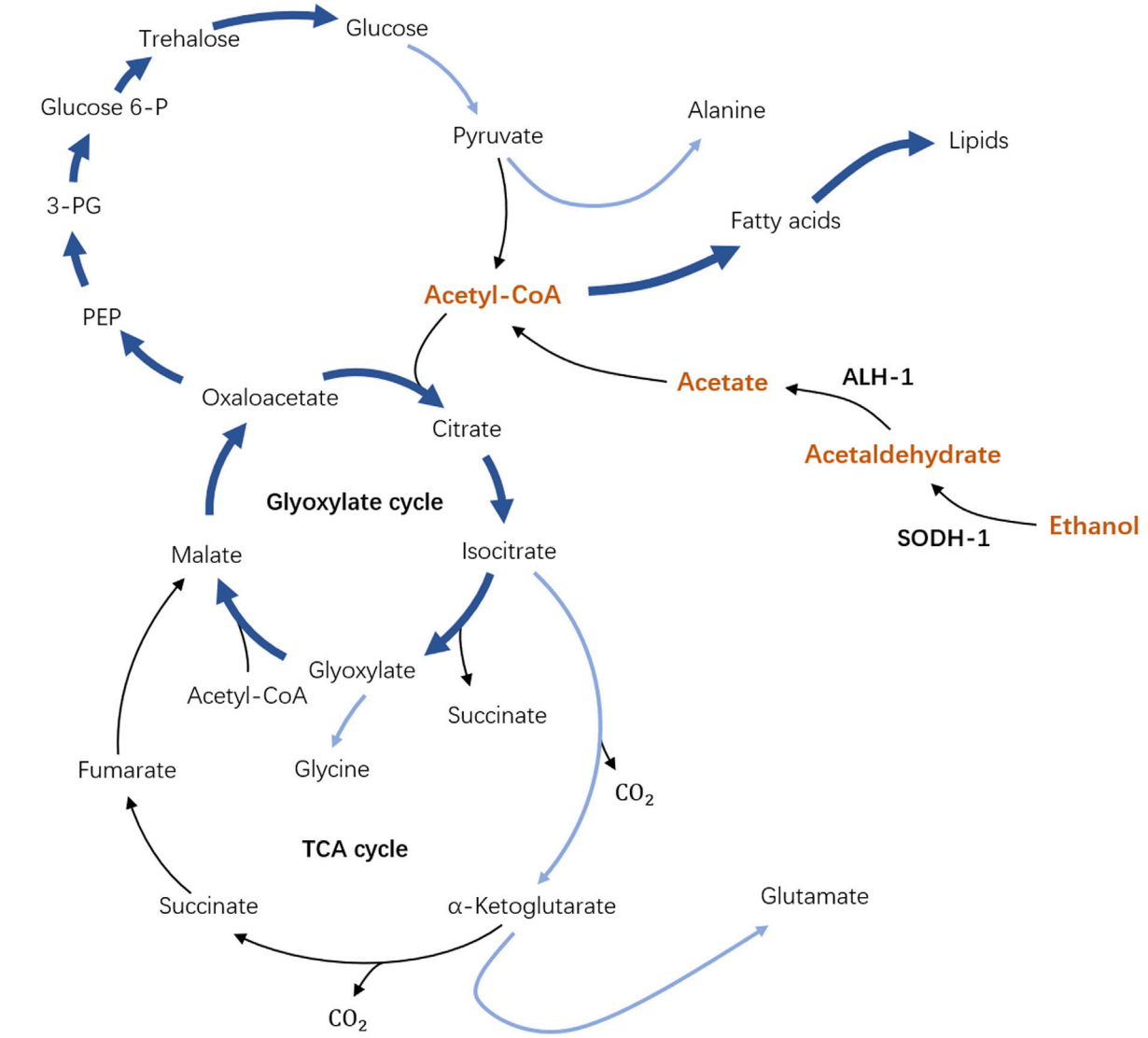

FIGURE 1
Schematics of the metabolic pathway of dauer larvae in the presence of external ethanol. In dependence on SODH-1 and ALH-1 enzymes, external ethanol is broken up and activated to Acetyl-COA (part of the pathway shown in orange) which is the key element of the original unperturbed metabolic pathway. Importantly, the arrows now pointing from Acetyl-CoA to fatty acids show the possibility of lipid storage accumulation in the presence of ethanol.

dauer larva developmental stage under harsh environments such as lack of food or high population density Riddle (1988); Hu (2007); Erkut and Kurzchalia (2015). To be able to survive these conditions, *C. elegans* dauer develop a strong cuticle that covers its whole body, such that most of the matter exchange across its body boundary shuts down. As a result, it was long believed that *C. elegans* dauer survive solely on stored lipids and are not able to uptake any carbon source from their environment Riddle (1988); Hu (2007); Erkut and Kurzchalia (2015). However, our recent findings Kaptan et al. (2020) showed that *C. elegans* dauer can utilize ethanol as an external carbon source, see Figure 1. Remarkably, at optimal concentrations, ethanol could expand the lifespan of dauer larvae twofold for a wild type and up to fourfold for some mutants. Ethanol can penetrate across the cuticle and thus gets channeled in the metabolic pathways of *C. elegans* dauer larvae. The enzymes responsible for the first metabolic steps are SODH-1 and ALH-1 which transform ethanol to acetate which can be activated into acetyl-COA and enters the major metabolic pathways of the TCA cycle, glyoxylate shunt, gluconeogenesis, and lipid metabolism, thus augmenting the metabolic pathways that dauers use for energy production Kaptan et al. (2020); Erkut and Kurzchalia (2015); Penkov et al. (2020); Burnell et al. (2005); Wadsworth and Riddle (1989). SODH-1 and ALH-1 are found to be upregulated in the presence of ethanol, whereas in *sodh-1* mutant, the ethanol is no longer incorporated and does not affect the lifespan of dauer. Experiments with radioactively-labeled ethanol have shown that it can be utilized for the production and accumulation of stored lipids, thus providing an effectively unlimited source of energy to dauer larvae in case of permanent ethanol supply Kaptan et al. (2020).

This led us to the question of why even in the presence of this energy source dauers do exhibit a longer lifespan but then eventually die. To help to answer this question, we proposed a mathematical model describing the relation between the lifespan of *Caenorhabditis elegans* dauer and the supplied ethanol based on the known metabolic pathway of dauer larvae. We assumed that the dauer dies either due to the lack of energy or due to the accumulation of some not yet identified toxic compound(s) Kaptan et al. (2020) that could resemble the so-called "lipotoxicity" factors in mammalian systems Burnell et al. (2005); Schooneman et al. (2013); DeFronzo (2010). As experimentally observed, the death of worms was preceded by the deterioration of mitochondria. We also assumed that these two mechanisms led to mitochondria damage and then to death. This model was very successful in explaining experimental data on the lifespans of dauer and various mutants in the presence of and without ethanol.

While identifying the exact toxic component that limits the lifespan of dauer is still an ongoing research project, we were interested in exploring whether or not the lifespan could be extended even further. To this end, we assume there are two self-recovery mechanisms, namely, regeneration of mitochondria and detoxification, and we test what they lead to. These two mechanisms alone still result in dauer's death if the feeding protocol is constant. However, when we use a periodic supply of ethanol in the model, an unlimited lifespan can emerge according to the numerical simulation. By comparing model predictions with existing data on constant feeding, we also suggest feeding protocols that can be directly tested in future experiments on dauer.

Certainly, the unlimited lifespan for the wild-type *C. elegans* dauer larvae even under the proposed feeding protocol sounds unfeasible and is a regime predicted by an idealized theoretical model that proposes two recovery mechanisms. However, testing this model prediction in experiments will provide crucial insights into the metabolism of dauers if there is *any* significant extension of the lifespan for periodic feeding that would support the hypothesis of the existence of recovery mechanisms that could be further explored.

2 Methods

2.1 Mathematical model

A minimal model of the metabolic network of *C. elegans* dauer larvae was introduced in Kaptan et al. (2020) and accurately reproduced the lifespans of dauer with and without ethanol for wild-type worms and various mutations. The framework of the model follows the largely coarse-grained metabolic pathway of dauer. All the chemical components falling into the category of "available energy" are combined and called "acetate", which is the central representative component of this category. Similarly, the components corresponding to "stored energy" and "consumed energy" are denoted as lipids and carbohydrates, respectively. Acetate and lipids could transform into each other as the balance between free and stored energy. At the same time, acetate continuously transforms into carbohydrates unidirectionally to support the main functions of an organism including mitochondria. If the production of carbohydrates drops below a certain minimal threshold, mitochondria start to get damaged and the dauer dies. In the presence of ethanol, acetate gains an influx proportional to its concentration. During the process of releasing stored lipids, toxic compounds are produced as a side product and as the second major reason that causes damage to the mitochondria alongside the lack of carbohydrate production.

Our model also included the effect of genes that were identified as regulatory factors through genetic experiments with loss-of- or reduction-of-function mutations Kaptan et al. (2020). We use *daf-2(e1370)* strain as a proxy for the control strain in our current study. The differences between wild-type dauers and *daf-2* mutants were previously discussed in Kaptan et al. (2020). Specifically, this strain has a Daf-c (dauer constitutive) phenotype due to a conditional, temperature-activated mutation that induces dauer formation even on ample food and at a low population density. It undergoes controlled dauer formation upon temperature switch and provides two advantages compared to the wild-type: i) *daf-2* animals enter dauer state more synchronously and, thus, the age variations are minor; ii) as they enter dauer state under unrestricted food supply, they might be able to store more lipids prior to entering dauer arrest. Otherwise, there is a well-documented agreement in the field that *daf-2* dauer larvae recapitulate to a high degree the signaling and metabolic processes in wild-type worms undergoing dauer formation Hu (2007). Loss-of-function mutation in the *aak-2*/AMPKα in *daf-2(e1370);aak-2(gt33)* double mutants causes an enhanced lipolysis rate, which leads to a reduced lifespan compared to the control strain under both feeding conditions with and without ethanol Penkov et al. (2020); Narbonne and Roy (2009). A reduction-of-function allele in the class I PI3-kinase *age-1(hx546)* (single mutant), on the other hand, is supposed to have a reduced lipid synthesis rate. This assumption is based on experimental results with the mutant *age-1* in dauer state Kaptan et al. (2020). Its lifespan is similar to control dauer when there is no ethanol supply but has a large increase when the ethanol is supplied Kaptan et al. (2020).

The goal of this work is to identify potential ways of how the dauer could survive even for a longer time. Thus, here, we consider mechanisms by which the model will be able to produce an unlimited lifespan while still remaining consistent with the results of the previous experiments. There are two essential and rather natural mechanisms that have been omitted in the original model Kaptan et al. (2020) while having potential for lifespan extension: detoxification Xu et al. (2005); Chen (2011) and a possibility for mitochondria to regenerate Palikaras and Tavernarakis (2014); Ni et al. (2015); Chan (2012) (see green arrows in Figure 2). We will show in the following that the model containing these two mechanisms predicts the possibility for lifespan extension under periodic supply protocol of ethanol.

To demonstrate this, we first formalize the schematics in Figure 2 into the system of ordinary differential equations that describe the chemical reaction network of ethanol metabolism Fromm and Hargrove (2011):

$$\frac{da}{dt} = -(k_1 + k_4)a + k_2 l + j_{\text{in}}, \tag{1}$$

$$\frac{dl}{dt} = k_1 a - k_2 l, \tag{2}$$

$$\frac{dc}{dt} = k_3 k_2 l - k_c c, \tag{3}$$

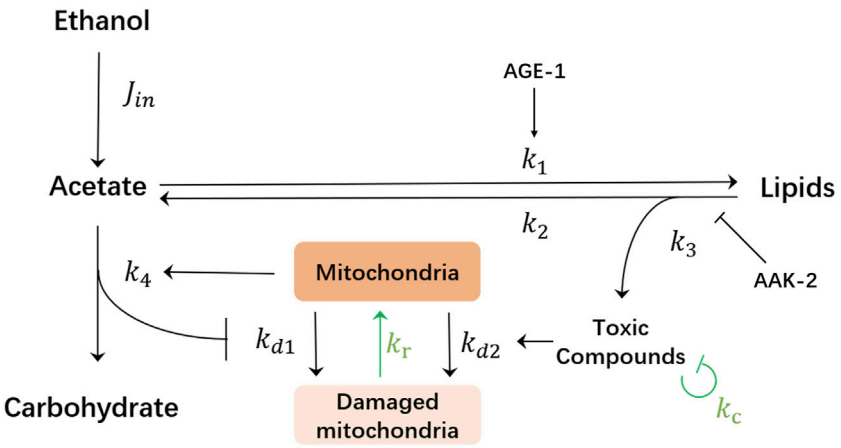

FIGURE 2
Schematics of the mathematical model of the metabolic network of *C. elegans* dauer larvae. Externally supplied ethanol is transformed into the acetate creating its influx j_{in}, which can be either constant or varies in time depending on the respective supplied ethanol concentration. Acetate (ch. acetyl-CoA in Figure 1) is then used either for energy production and carbohydrate synthesis or can be stored in lipids. Mitochondria undergo damage from lack of carbohydrate production or accumulation of toxic compounds as the product of lipolysis. Aak-2 kinase exerts the inhibitory effect on the lipolysis, thus the *aak-2* mutation leads to an enhanced lipolysis rate. Age-1 kinase stimulates lipid synthesis and thus *age-1* mutation would lead to the reduction of lipid synthesis. Green arrows indicate two hypothesized mechanisms of recovery by degradation of toxic compounds with the rate k_c and mitochondria regeneration with the rate k_r.

$$\frac{dm}{dt} = -k_{d1}\Theta(k_4 a - j_m)m - k_{d2}\Theta(c_h - c)m + k_r(1 - \Theta(k_4 a - j_m))(1 - \Theta(c_h - c))(1 - m). \quad (4)$$

Here "a" and "l" denote the concentrations of the acetate and lipids, respectively, while "c" represents the concentration of toxic compound(s), and m designates the wellbeing of mitochondria. The consumption of acetate for simplicity is assumed to be unidirectional (not explicitly modeled in the system) with the rate k_4, but acetate can also be stored in lipids, see Eq. 1. In the presence of external ethanol, an influx j_{in} of acetate is included as the source. This influx j_{in} is assumed to be proportional to the external ethanol concentration. Lipids get created from acetate with rate k_1 while they are released through the lipolysis process with rate k_2, Eq. 2. The toxic compound(s) c is produced as the side product of lipolysis with proportionality factor k_3 and spontaneous degradation rate k_c, Eq. 3. The variable m ranges from 1 to 0 and denotes the wellbeing of mitochondria, where $m = 1$ means a fully functional mitochondria and $m = 0$ means a fully damaged one. Mitochondria can be damaged with a rate k_{d1} if the carbohydrate production $k_4 a$ falls below the minimal required "energy" flux j_m, or with a rate k_{d2} when the toxic compound c accumulates above a certain threshold concentration c_h (Θ in the equation is the Heaviside step function). There are many known mechanisms of mitochondria surveillance and maintenance Palikaras and Tavernarakis (2014); Ni et al. (2015); Chan (2012). Here, for simplicity, we suggest a phenomenological law of mitochondria recovery. When mitochondria do not suffer any damage (i.e., there is enough ethanol and the toxic compound is below the critical threshold) they can regenerate. The regeneration rate is proportional to the current damage level of mitochondria ($1 - m$) with a rate constant k_r. This term ensures that the value of m recovers toward one from any state of $0 < m < 1$.

While most of the reaction rates in the above equations are considered constant for simplicity, some rates do depend on variables. The first example is the linear dependence of k_4 on m, which assumes that the energy production requires functional mitochondria:

$$k_4 = \tilde{k}_4 m, \quad (5)$$

where $\tilde{k}_4$ is a constant. Another non-constant rate is k_1 quantifying acetate-to-lipids conversion. It reflects the fact that each dauer has a storage limit capacity l_s (it cannot accumulate unlimited amounts of lipids):

$$k_1 = \tilde{k}_1 \frac{l_s - l}{l_1 + (l_s - l)}. \quad (6)$$

Here, l_1 is a characteristic lipid concentration at which the conversion starts to saturate and $\tilde{k}_1$ is a constant. Finally, we also assume that k_2 has the functional form of Michaelis-Menten reaction Fromm and Hargrove (2011).

$$k_2 = \tilde{k}_2 \frac{1}{l_2 + l}, \quad (7)$$

where $\tilde{k}_2$ and l_2 are constants. If in the above equations, we set $k_r = 0$ and $k_c = 0$, we will recover the system studied in Kaptan et al. (2020).

The model including the self-recovery mechanism, however, should also reproduce the lifespan of dauers with and without ethanol, as well as different mutants as was observed experimentally Kaptan et al. (2020). This also means that this model should result in a finite lifespan under a constant ethanol supply. However, the novel possibility for lifespan extension may now emerge for non-constant feeding, where the supplied ethanol concentration varies in time, for instance, according to a periodic sinusoidal protocol. We next show by using numerical simulations that the model reproduces experimental observations under constant feeding and predicts the lifespan extension under periodic feeding protocol.

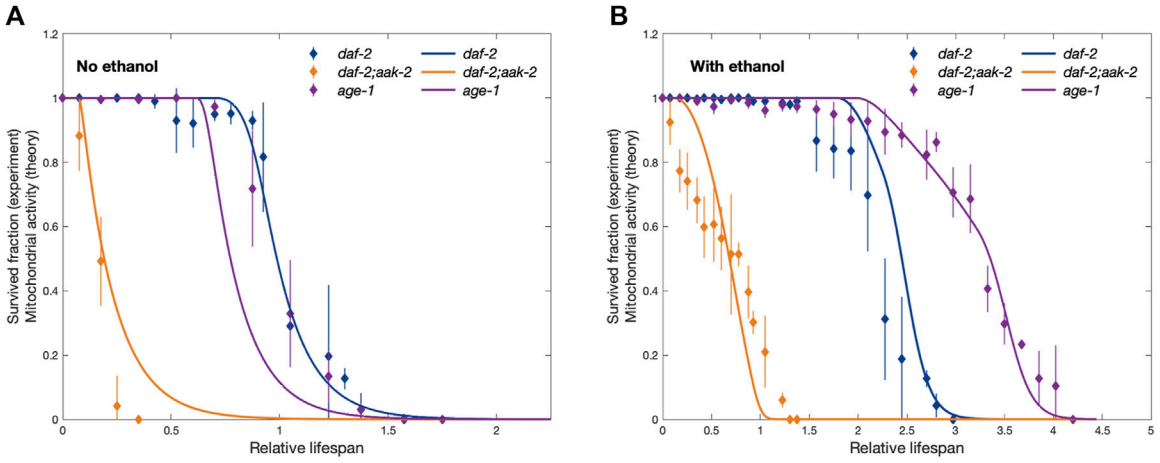

FIGURE 3
Comparison between experimentally observed lifespan of dauers of different strains (dashed lines) showing the fraction of survived dauers as a function of time and the corresponding simulation results (solid lines) showing the activity of mitochondria. Panels **(A)** and **(B)** correspond to no ethanol and provided ethanol conditions, respectively. The time axis is rescaled for the experimental curves by the time at which the fraction of survived *daf-2* strain dauers used as a control drops to 0.5 (50%) in no ethanol condition (t =40 days). Similarly, for the simulation results, the time axis is rescaled by the time at which the activity of mitochondria in control conditions with no ethanol drops to 50%. This rescaling allows for direct theory-experiment comparison even if we do not link simulation time to real-time units. Experimental data is taken from Kaptan et al. (2020), and error bars show standard deviation.

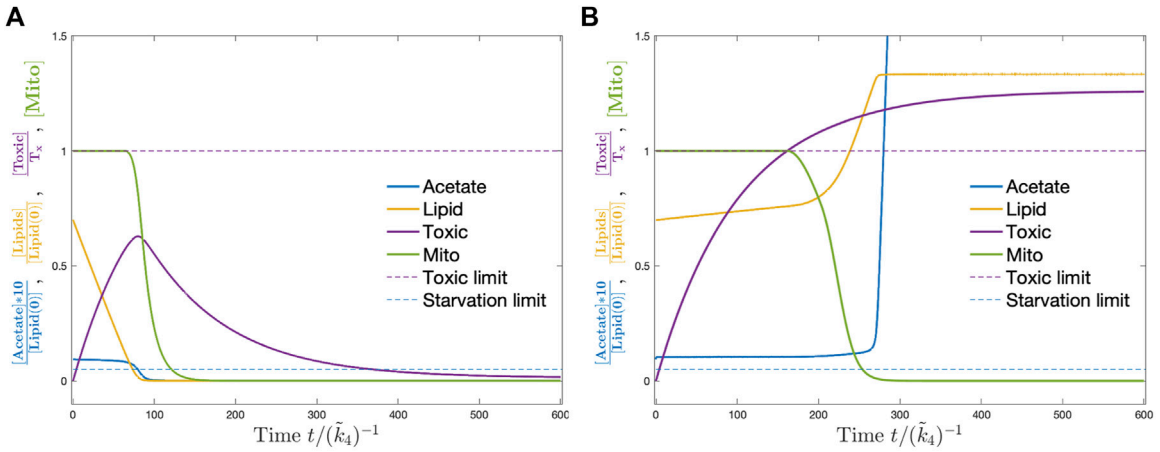

FIGURE 4
Dynamics of the model corresponding to control strain without **(A)** and with ethanol **(B)**. Without ethanol supply, the dauer consumes the stored lipids (yellow) which keep acetate levels (blue) and mitochondria (green) at constant levels. Toxic compounds accumulate (magenta). When lipids are used up, the acetate level drops below the critical threshold (dashed blue line) and mitochondria start to get damaged till the larvae die due to starvation. With ethanol supply, acetate never ends and mitochondria start to damage after the toxic compounds go above a critical threshold (dashed magenta line). Then, dauer dies due to the accumulation of toxic compounds.

3 Results

3.1 Constant ethanol supply

We first check if the model with the self-recovery can recapitulate experimental observations with a constant ethanol supply. Parameters used in the simulations were chosen by checking whether the lifespan ratios between mutants (*daf-2*, *daf-2;aak-2*, and *age-1)* with and without ethanol generated by simulations fit the previous experimental results Kaptan et al. (2020). When one set of parameters is considered the control strain without ethanol feeding, the corresponding parameter set of *daf-2;aak-2* mutant is defined by increasing the value of $\tilde{k}_2$ while keeping other parameters unchanged. Similarly, *age-1* mutant has a reduced $\tilde{k}_1$ constant. Three strains under two feeding conditions give rise to a total of six sets of parameters including the starved control strain as the baseline parameter set. We can call these sets a parameter collection. A parameter collection of the model is said

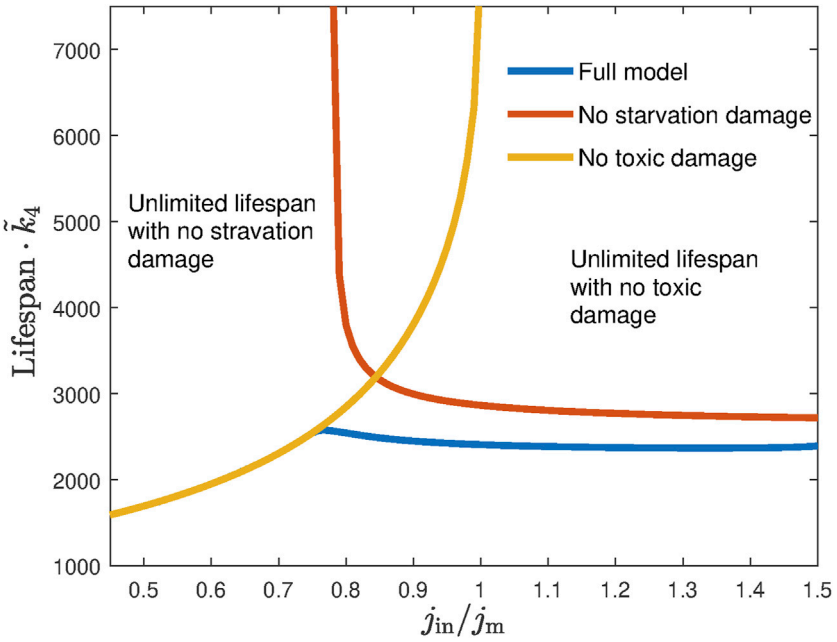

FIGURE 5
No lifespan extension is possible when larvae are fed with constant ethanol influx. The red and yellow lines show the lifespan as a function of the ethanol influx (normalized by minimal energy flux j_m required for mitochondria wellbeing) in the absence of toxic damage and no starvation damage, respectively. While both cases have regions of influx supporting infinite lifespan, they do not overlap, leading to the overall final lifespan when both damages are present (blue line).

to reproduce the experimental observation if all the ratios of lifespans produced by its parameter sets reproduced the experimentally observed values. Figure 3 shows the reproduction of the observed lifespan ratios by the model with self-recovery.

The detailed dynamics of one of the parameter sets for control strain with and without feeding is shown as an example in Figure 4. When there is no feeding, dauer breaks down storage lipids to keep its acetate level and thus the carbohydrate production rate. As the lipids run out, the mitochondria are damaged for lack of carbohydrate production which results in the death of dauer. When ethanol is supplied at a sufficient level, starvation no longer becomes a concern. However, the toxic compound continuously accumulates and as it goes beyond the threshold at some point, the mitochondria start to take damage and finally the larvae die. The details of the simulation including the numerical methods Fromm and Hargrove (2011); Murray (1989); Strogatz (2000) and parameters are provided in Supplementary Appendix S1.

As the above two examples show, starvation or accumulation of toxic compounds is the reason for mitochondria damage and the resulting death of larvae. We can demonstrate more generally the condition for the finite lifespan of dauers for a constant ethanol concentration.

Figure 5 shows the lifespan of dauer as a function of the external ethanol influx. If either starvation damage k_{d1} or toxic damage k_{d2} is removed, the dauer may have an infinite lifespan when the ethanol concentration is sufficiently high or low, respectively. If, however, these two lifespans vs. influx curves intersect at some value of j_{in}, lifespan will always remain finite, and as for any given ethanol concentration and the corresponding influx, there will be at least one reason that the dauer dies within the limited time determined by k_{d1} or k_{d2}.

If the two curves (with each of the damage removed) do not intersect, they would form boundaries of a domain in between where the value of j_{in} would support an infinite lifespan. As we mentioned above, in experiments, the dauer always survives the finite time in the presence of ethanol, thus defining for us the parameter range that has to be chosen in simulations.

3.2 Periodic ethanol supply

The above results show that ethanol supply keeps mitochondria operational, but the accumulating toxic compounds damage the mitochondria. Here, we hypothesize that periodic ethanol supply might be the key to an unlimited lifespan of dauer. While periods of supply might be used to replenish lipid storage and repair mitochondria, periods of no feeding can be used to degrade the accumulated toxic compounds. We now test this hypothesis numerically. For simplicity, we use a sinusoidal feeding protocol with a feeding amplitude A, feeding frequency ω_E, and a positive baseline value j_0:

$$j_{in} = j_0 + A \sin(\omega_E t + \phi). \quad (8)$$

With a proper parameter choice, the numerical simulations of the model show that the mitochondria damage and regenerate periodically until the end of simulations no matter how long these last.

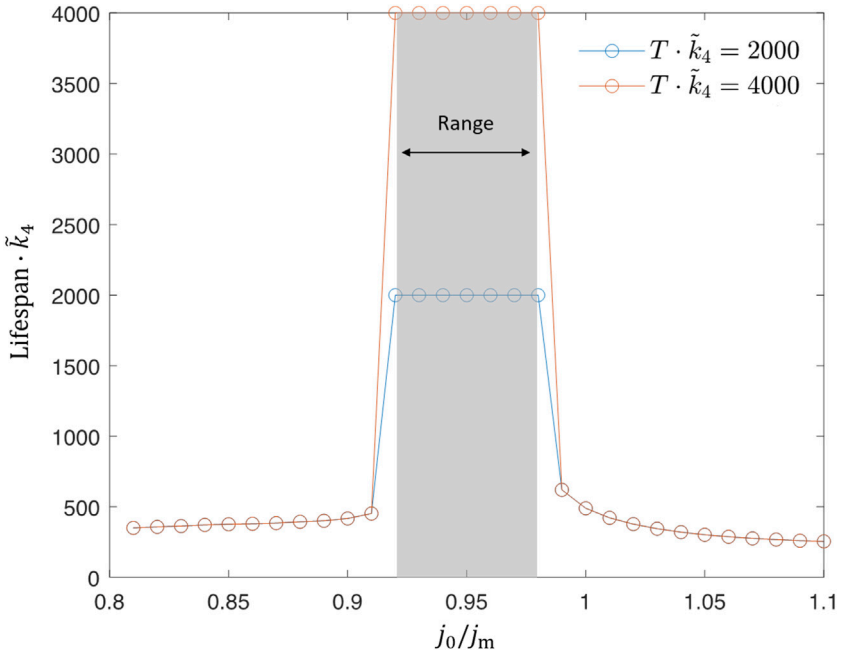

FIGURE 6
Example of a lifespan extension as a result of periodic feeding. The baseline value j_0/j_m =0.8 while $A = j_0$. The feeding frequency ω_E is the same as the oscillatory frequency of acetate level *a* shown in blue. The inset shows oscillations of toxicity near but below the toxic limit, while mitochondria are almost fully active.

Indeed, this situation becomes possible when parameters are tuned such that the periodic feeding permits the worm to accumulate toxic compounds while intaking ethanol and fuelling mitochondria but then remove them with a diet at the cost of some mitochondria damage, which can, however, be regenerated during the next intake cycle. These simulations suggest that the periodic feeding protocol does provide a theoretical possibility of an unlimited lifespan extension (Figure 6). We next investigate in more detail how this effect depends on model parameters.

Numerically, an unlimited lifespan can be defined as survival until the end of the simulation regardless of the simulation time. However, in practice, the time for which we can observe the system is always limited. Thus, we set a certain threshold value $T_{\max}$ for the survival time. If a dauer survives until $T_{\max}$ in simulation, we say the lifespan of the dauer is unlimited under this set of parameters. Our analytical considerations also suggest that there may exist a true infinite lifespan given a certain set of parameters in the model.

3.3 Effect of feeding parameters

A single simulation does not reflect the whole picture but only indicates a possibility. To quantify the robustness of lifespan extension, we defined a new value "range" w as the size of the interval within which the baseline influx j_0 may vary so that the dauer exhibits an unlimited lifespan, see Figure 7. The range w of this baseline interval thus quantifies the ability of a certain set of parameters (ω_E, A) to support the extension of lifespan. We note that the lifespan seems to go through a sharp transition from a finite to an infinite value, first when approaching from the side of low baseline value and second when approaching from the side of large baseline levels. The transition at low influx seems to be a jump-like switch (as we could only test numerically). The high influx condition is amenable to analytical analysis and we could show that it has a shape of a logarithmic divergence (see Supplementary Appendix S3). By quantifying the survival ability with the value of w and studying its dependence on feeding parameters, ω_E and A would eventually help us to identify the optimal experimental conditions where the lifespan extension of dauer could be tested.

We next plot the range w as a function of feeding amplitude A and frequency ω_E, see Figure 8.

According to the simulation results, we see that lifespan extension is possible when the feeding frequency is within a certain interval. The experiment with the feeding frequency ω_E corresponding to the maximal range w is expected to give the highest chance to observe the effect. The simulations also suggest that the range w grows with feeding amplitude A in an almost linear way if the feeding frequency is high enough. This is because, at a high-frequency region, the range w is proportional to the oscillation amplitude of acetate, which can be explained by an approximate analytical solution. (For details of the analysis, see Supplementary Appendix S2).

The range-frequency curves can potentially help us to identify suitable feeding frequencies and amplitudes for which it is most likely to observe lifespan extension in experiments. To do so, we still need to connect our mostly dimensionless equations to realistic parameters. This is not too straightforward since not all

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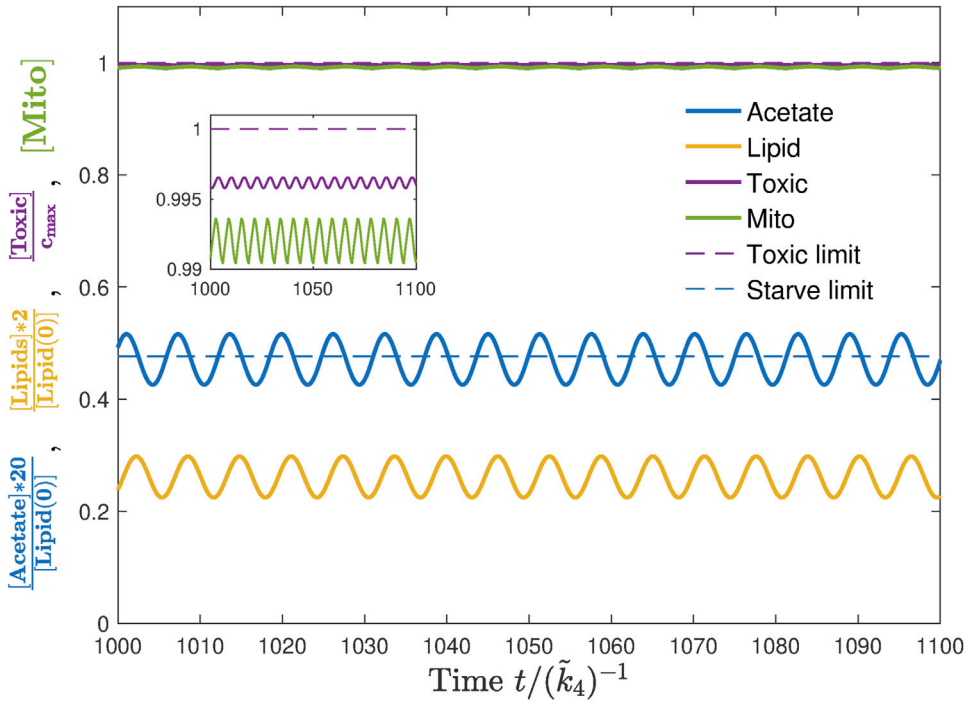

FIGURE 7
Range w is the size of an interval of j_0 where the dauer experiences lifespan extension. Its value is obtained numerically by scanning over the baseline value j_0 while keeping ω_E and A fixed. For each j_0, we run multiple simulations corresponding to various initial lipid storage $l(0)$ and take the largest w from those simulations as the range w corresponding to this j_0. The baseline value j_0 can be smaller than the minimal energy flux j_m as the oscillations with large enough amplitude A make j_{in} larger than j_m for a certain fraction of the feeding period. Here, we also show that the range does not strongly depend on the chosen threshold, $T_{max} \cdot \tilde{k}_4 - 2000, 4000$, which is approximately 20 and 40 times greater than the survival time of control strain without ethanol.

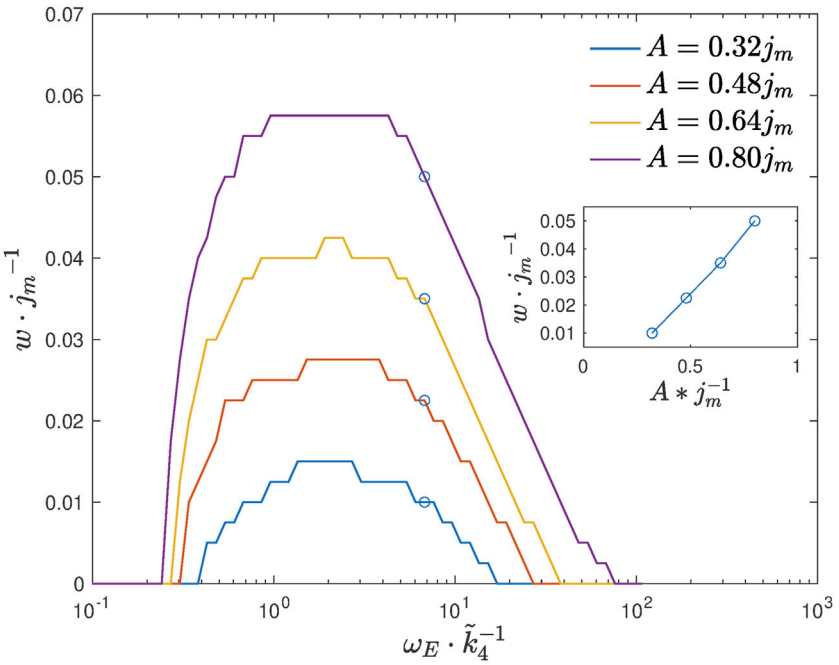

FIGURE 8
Range w as a function of the feeding frequency ω_E for different values of amplitude A. The inset shows a linear-like dependence of the range on amplitude in the region of high-feeding frequencies.

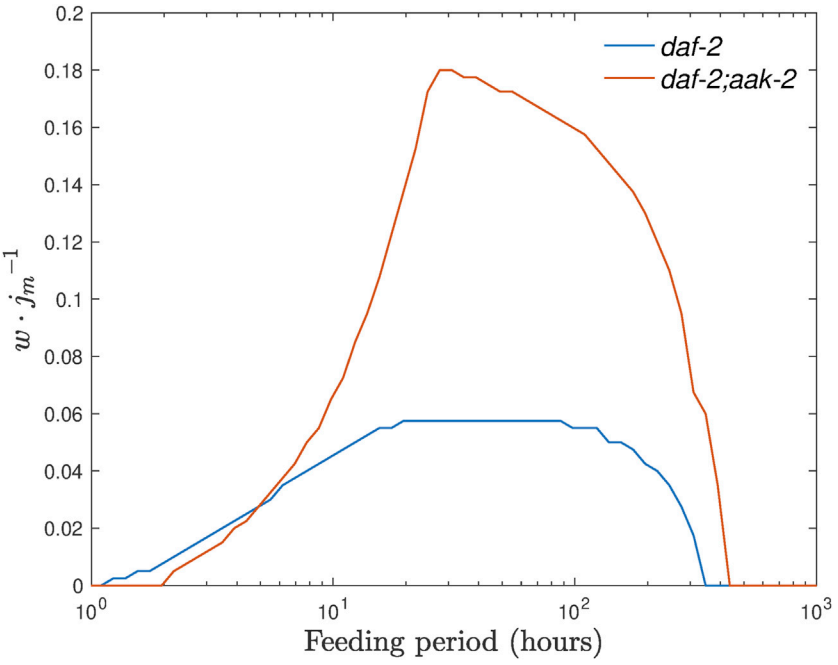

FIGURE 9
Range of the baseline ethanol supply levels where we expect to see unlimited survival of dauer larvae shown for control and *daf-2;aak-2* strains as a function of the feeding period $T = 2\pi/\omega_E$. Here, it is assumed that the amplitude A takes the value of $\min[j_0]$.

parameters of the enzymatic kinetics as well as chemical concentrations in the dauer were measured yet. However, for the case of the feeding frequency, we may take a short-cut where we can determine the timescale by equalling the control lifespan without ethanol in the model defined as the time where m falls to, for example, 0.5, compared to that in experiment defined as respective 50% survival and restore all reaction rates in real-time units. Also, the feeding amplitude is simply set as large as possible (see below) so there is no more information needed. Figure 9 shows the range vs. period (given in hours) relation for control strain and *daf-2;aak-2* mutants under maximal feeding amplitude. The maximal feeding amplitude is defined as $A = \min[j_0]$ (i.e., the smallest j_0 among all j_0 used in scanning, such that the influx j_{in} is always positive). Another definition $A = j_0$ for all j_0 is also possible and leads to similar results.

These simulations not only suggest an optimal feeding period for both strains but also indicate that *daf-2;aak-2* mutant is a better option for the experiment, not only for the larger range value but also for the smaller optimal feeding period. It requires a feeding period of the order of 10 h (so the media for larvae can be changed once a day) and the effect should be seen much earlier, as the original lifespan of *daf-2;aak-2* is much shorter, and thus overall a shorter experiment could be carried out.

4 Discussion and conclusion

Previously, we have shown that the lifespan of *Caenorhabditis elegans* dauer larvae can be greatly extended due to the metabolism of externally provided ethanol. With the help of the mathematical model of this metabolic pathway, we proposed that the lifespan remains limited due to the accumulation of toxic compounds resulting from the process of lipolysis. So far, however, we have neglected the possibility of mechanisms that help dauer to recover from this damage. Therefore, two biological self-recovery mechanisms, namely, detoxification and mitochondria regeneration, were introduced into the model. Importantly, despite self-recovery mechanisms for constant ethanol supply, the model reproduces the experimental observations of extended but limited lifespan.

However, when the feeding protocol is periodic, an unlimited lifespan can emerge. The possibility of an unlimited lifespan can be explained by the switch between two feeding phases, where the first one at high ethanol concentration repairs the mitochondria at the cost of toxic compounds' accumulation, while the second one, at low ethanol concentration, has the toxic compounds degraded but also damages the mitochondria slightly. For this process to keep the dauer surviving, both mitochondria regeneration and toxic compounds detoxification mechanisms are required to function.

Specifically in the context of dauer larvae survival in the wild Schulenburg and Félix (2017); Frézal and Félix (2015); Félix and Duveau (2012), our results might indicate that the time-varying availability of ethanol supply (when feeding phases are interspersed with starvation and toxic compound degradation) might be more beneficial for lifetime extension. One could speculate that fluctuating environmental conditions might be more common than a steady and abundant ethanol supply.

To characterize the unlimited lifespan predicted by the model systematically, we defined a range of baseline feeding fluxes, which quantifies the ability of a certain set of feeding parameters to

support the unlimited lifespan. The dependence of this range on feeding frequency and amplitude was studied numerically with some supporting analytical arguments. This dependence combined with previous data helped us to suggest a suitable feeding period and amplitude that can now be tested experimentally.

While an unlimited lifespan is not uncommon in nature and is exemplified in worms by some planarian flatworm species Tan et al. (2012), observing this for the short-lived wild-type *C. elegans* dauer larvae is not likely. Our model, proposed and experimentally tested for constant ethanol supply, suggests that under the assumption of the existence of two recovery mechanisms (mitochondria repair and degradation of toxins) in a rather broad range of parameters, an unlimited lifespan is theoretically possible. Any significant extension of the lifespan for periodic feeding in the experiment would support the hypothesis of the existence of recovery mechanisms. Furthermore, an extended but limited lifespan would trigger the search for other yet unknown mechanisms that serve as limiting factors. Importantly, it also highlights the importance of ultimately identifying candidates of toxic compounds which, in the model, are the main cause of death when starvation is overcome by ethanol. This study treats the identity of toxic compounds openly and does not specify the concrete mechanisms of mitochondria recovery and detoxification. Ultimately, for our comprehensive understanding of dauer larvae lifespan extension mechanisms and the generalization of those to other organisms, we need to push toward identifying the exact biological players of toxicity and recovery competition.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

XZ investigation, methodology, writing–original draft preparation. SP methodology, writing—review and editing. TK conceptualization, writing–review and editing. VZ conceptualization, writing—review and editing, supervision. All authors contributed to the article and approved the submitted version.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fragi.2023.1031161/full#supplementary-material

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The untapped potential of targeting NRF2 in neurodegenerative disease

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Since its initial discovery almost three decades ago, the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) has been shown to regulate a host of downstream transcriptional responses and play a critical role in preventing or promoting disease progression depending on the context. Critically, while the importance of proper nuclear factor erythroid 2-related factor 2 function has been demonstrated across a variety of pathological settings, the ability to progress NRF2-targeted therapeutics to clinic has remained frustratingly elusive. This is particularly true in the case of age-related pathologies, where nuclear factor erythroid 2-related factor 2 is a well-established mitigator of many of the observed pathogenic effects, yet options to target this pathway remain limited. Along these lines, loss of nuclear factor erythroid 2-related factor 2 function has clearly been shown to enhance neuropathological outcomes, with enhancing nuclear factor erythroid 2-related factor 2 pathway activation to prevent neurodegenerative/neurological disease progression continuing to be an active area of interest. One critical obstacle in generating successful therapeutics for brain-related pathologies is the ability of the compound to cross the blood brain barrier (BBB), which has also hampered the implementation of several promising nuclear factor erythroid 2-related factor 2 inducers. Another limitation is that many nuclear factor erythroid 2-related factor 2 activators have undesirable off-target effects due to their electrophilic nature. Despite these constraints, the field has continued to evolve, and several viable means of targeting nuclear factor erythroid 2-related factor 2 in a neuropathological context have emerged. In this perspective, we will briefly discuss the key findings and promising therapeutic options that have been discovered to date, as well as highlight emerging areas of NRF2-neurodegeneration research that provide hope for successfully targeting this pathway in the future.

KEYWORDS

Nrf2, therapeutics, neurodegeneration, KEAP1, oxidative stress

Introduction

The transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) is a critical regulator of cell survival, mediating vital aspects of redox, protein, and metabolic homeostasis. In fact, NRF2 target genes are involved in glutathione synthesis, peroxide reduction, xenobiotic detoxification, proteasome assembly, autophagy, transport and storage of iron, lipid catabolism, and carbohydrate metabolism (Dodson et al., 2019). Accordingly, dysregulation of NRF2 signaling has been linked to promoting disease progression, with restoration of proper NRF2 function restoring homeostasis (Cuadrado et al., 2019). Perhaps

one of the greatest ongoing frustrations in the field is that despite an ever-increasing number of compounds that beneficially activate or inhibit NRF2 in an experimental setting, very few have progressed through clinical trials to become viable therapeutics. This shortfall also holds true for neurodegenerative diseases, where loss of NRF2 is a well-established driver of neurodegenerative phenotypes, yet restoration of NRF2 signaling remains an unharnessed therapeutic possibility.

Critically, NRF2 has been shown to play an important role in mitigating the onset and progression of several neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and Multiple sclerosis (MS) (Cuadrado, 2022). Loss of NRF2 function significantly exacerbates neurodegenerative phenotypes, often resulting in increased inflammation, oxidative stress, or proteotoxicity that enhance pathogenesis of the chosen model (Brandes and Gray, 2020). Importantly, NRF2 activity has been shown to decline with age (Suh et al., 2004; Zhou et al., 2018), inferring that the greatest risk factor for developing neurodegenerative disease is associated with progressive loss of NRF2. This indicates that restoring proper NRF2 function, either by direct activation of NRF2 or blocking the mechanisms that leads to its decline, represents a feasible strategy to prevent onset and progression of these debilitating diseases. Below, we will give a brief overview of the NRF2 signaling pathway and discuss the experimental evidence supporting a role for NRF2 across different neurodegenerative contexts. Next, we will highlight the compounds identified to date that have shown the most therapeutic promise, as well as the feasibility of utilizing gene therapy-based approaches and drug delivery systems to achieve a more potent and targeted effect. Finally, we will discuss the future of the NRF2-aging field, including the key barriers that need to be overcome to progress the science from experimental evidence to actual translational applications.

Nrf2 and neurodegeneration

The Nrf2 signaling pathway

NRF2 is regulated primarily at the protein level by the Kelch-like ECH-associated protein 1-Cullin-3-RING box protein-1 (KEAP1-CUL3-RBX1) E3 ubiquitin ligase complex. Under basal, non-stressed conditions, NRF2 is targeted by this complex for proteasomal degradation (Itoh et al., 1999; Kobayashi et al., 2004; Zhang et al., 2004); however, upon the introduction of electrophilic/oxidative stress (Dinkova-Kostova et al., 2002; Zhang and Hannink, 2003), mutations in NRF2 or its degradation machinery (Singh et al., 2006; Shibata et al., 2008; Martinez et al., 2013; Ooi et al., 2013), or autophagy dysfunction (Komatsu et al., 2010; Lau et al., 2010), NRF2 accumulates in the nucleus and binds small musculoaponeurotic fibrosarcoma F/G/K (sMaf F/G/K) proteins to activate transcription of antioxidant response element (ARE)-containing target genes (Itoh et al., 1997). Along with KEAP1-dependent degradation, glycogen synthase kinase β (GSK3-β)-dependent phosphorylation of NRF2 can result in recruitment of the S-phase kinase-associated protein 1-Cullin-1-Rbx1/β-transducin repeat-containing protein (SCF/β-TrCP) E3 complex and degradation of NRF2 (Rada et al., 2011). Additionally, synoviolin-1 (also known as Hrd1) is an E3 ligase that has been shown to degrade NRF2 in the endoplasmic reticulum, particularly during liver cirrhosis (Wu et al., 2014). Along with ubiquitination, other posttranslational modifications, including acetylation (Sun et al., 2009), phosphorylation (Huang et al., 2002), methylation (Liu et al., 2016), and SUMOylation (Malloy et al., 2013) of NRF2, as well as OGlcNAcylation of KEAP1 (Chen et al., 2017), have been shown to dictate NRF2 localization and stability. It is also worth noting that NRF2 expression can be regulated at the DNA and mRNA levels. For example, methylation of the *KEAP1* or *NFE2L2*/NRF2 promoters (Wang et al., 2008; Muscarella et al., 2011; Khor et al., 2014), as well as transcriptional up or downregulation of NRF2 expression by other transcription factors (i.e., nuclear factor kappa B [NF-κB] and aryl hydrocarbon receptor [AhR]) (microRNA) (Miao et al., 2005; Liu et al., 2008), have all been shown to dictate the NRF2 response. Finally, several microRNAs (i.e., miR-27a, miR144, miR153, and miR142-5p) have been reported to suppress expression of NRF2 (Narasimhan et al., 2012; Zhao et al., 2018; Chu et al., 2019). Overall, it is clear why NRF2 dysregulation leads to disease, as the complex and interconnected nature of the NRF2 signaling cascade presents a multitude of possible points of dysfunction. Below, we will highlight evidence of NRF2 importance in the context of different age-related neurodegenerative disorders.

Parkinson's disease

Several experimental studies have demonstrated the importance of NRF2 in preventing the development of PD phenotypes. For example, on the chemical induction front, numerous studies utilized administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a well-established chemical inducer of dopaminergic neuron death and the onset of parkinsonian phenotypes, with *Nrf2*$^{-/-}$ mice exhibiting a more pronounced loss of dopaminergic function and onset of PD-related phenotypes (Burton et al., 2006; Chen et al., 2009; Innamorato et al., 2010; Rojo et al., 2010; Kaidery et al., 2013). Importantly, similar results were obtained with rotenone, 6-hydroxydopamine, and paraquat, three other notable chemical inducers of PD-relevant outcomes (Jakel et al., 2007; Wang et al., 2017; Wei et al., 2020). Supporting this notion, a series of recent studies from us and a collaborator's group showed that loss of NRF2 significantly enhanced dopaminergic neuron loss, autophagy dysfunction, inflammation, and cell death in the Thy1 mouse model of human α-synuclein overexpression (Corenblum et al., 2016; Ray et al., 2018; Anandhan et al., 2021; Anandhan et al., 2022). A similar study indicated that *Nrf2*$^{-/-}$ mice were more susceptible to PD phenotypes when stereotactically injected with adenoviral α-synuclein (Lastres-Becker et al., 2012). This infers that both genetic and chemical models of PD are enhanced by genetic ablation of NRF2, clearly indicating its importance in preventing disease progression (summarized in Figure 1, right panel).

Conversely, several studies have shown that genetic ablation/suppression of *KEAP1*, which results in constitutive upregulation of the NRF2 signaling cascade, improves neuronal survival and decreases PD phenotypes both *in vitro* and *in vivo* (Satoh et al., 2009; Williamson et al., 2012). Interestingly, specific overexpression of *NFE2L2*/NRF2 in astrocytes was shown to prevent MPTP-induced PD pathogenesis (Chen et al., 2009). Thus, an ongoing area of interest in the field is determining the cell type-specific

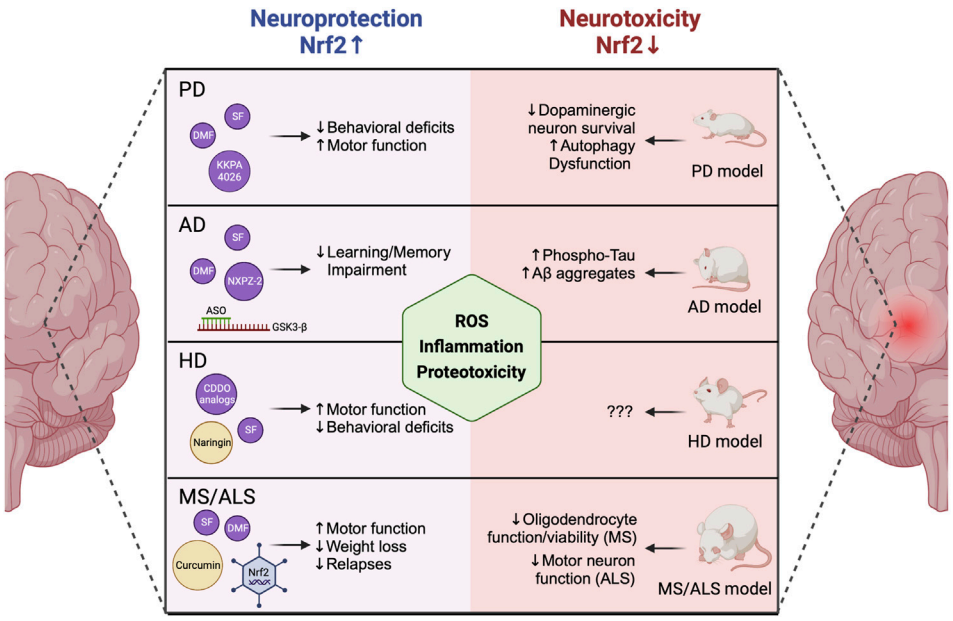

FIGURE 1
NRF2 levels mediate neurodegenerative disease progression. NRF2 activation via covalent and non-covalent pharmacological modifiers or genetic modulation of KEAP1 has been linked to decreased behavioral deficits, increased motor function, decreased learning and memory impairment, and decreased weight loss and disease relapse in animal models of PD, AD, HD, and MS/ALS. Contrastingly, loss of NRF2 enhances neurodegenerative phenotypes, including increased ROS-, inflammation-, and proteotoxicity-dependent inhibition of neuronal and glial function and viability. PD = Parkinson's disease, AD = Alzheimer's disease, HD = Huntington's disease, MS = Multiple sclerosis, ALS = Amyotrophic lateral sclerosis, ROS = Reactive oxygen species. Created with Biorender.

relevance of NRF2 in glia (i.e., astrocytes, oligodendrocytes, and microglia) versus neurons in dictating PD progression (Liddell, 2017), with much work still to be done. Much like genetic modification of *KEAP1* or *NFE2L2*/NRF2 itself, several NRF2 inducers have been shown to ameliorate pathogenic features of PD. This includes covalent (i.e., sulforaphane [SF], di-/mono-methyl fumarate [DMF/MMF], bardoxolone analogs [CDDO], and curcumin), and non-covalent (i.e., KKPA4026 and pinostrobine) inducers (Figure 1, left panel), that exert their protective effects via disrupting KEAP1-dependent degradation of NRF2, allowing it to translocate to the nucleus and activate transcription of its downstream antioxidant and anti-inflammatory target genes (Jazwa et al., 2011; Morroni et al., 2013; Johnson and Johnson, 2015; Ahuja et al., 2016; Cui et al., 2016; Zhou et al., 2016; Li et al., 2018; Kim et al., 2020). However, an important limitation to the current state of the field is that many of the anti-neurodegenerative effects that have been associated with NRF2 activation were observed in a cell culture setting, or in mouse models where a less severe phenotype is obtained, but the actual levels and form of the compound that pass the BBB and reach affected tissues is unknown. Another caveat is that many covalent inducers are electrophilic, and as such can target reactive cysteines on proteins other than KEAP1, leading to undesirable off-target toxicity that limits their applicability. However, despite this limitation, SF is currently being tested in a phase II clinical trial to determine if it improves cognition in PD patients (Clinicaltrials.gov; NCT05084365), and DMF is approved by the FDA to treat relapsing forms of MS. Along these lines, repurposing DMF to treat PD has garnered some interest (Lastres-Becker et al., 2016), as it is already FDA approved, and it was shown to prevent oxidative stress and cytotoxicity in several *in vitro* and *in vivo* models of PD (Majkutewicz, 2022). Overall, administration of DMF and its other electrophilic counterparts has shown enough benefit to warrant continued development and consideration as alternative approaches are developed and eventually implemented.

Importantly, several alternative strategies have emerged to obtain beneficial induction of NRF2 without using potentially toxic electrophilic compounds. One example is the protein-protein interaction inhibitor (PPI) KKPA4026, which was shown to prevent dopaminergic neuron cell death and ameliorate parkinsonian behavioral deficits in an MPTP model of PD (Kim et al., 2020). Liposomal delivery of resveratrol suppressed oxidative stress and enhanced circulatory function in cerebral vascular cells from aged rats in an NRF2-dependent manner (Csiszar et al., 2015), inferring that improved delivery through the BBB could also enhance the efficacy of other NRF2 inducers. Thus, while electrophilic inducers continue to represent the gold standard, efforts towards improved delivery systems, non-covalent modifiers, and gene-therapy based approaches continue to emerge as more targeted and possibly potent solutions, which will be discussed in more detail below.

Alzheimer's disease

Like PD, several studies have indicated that loss of NRF2 enhances AD phenotypes. For example, amyloid precursor protein/presenilin 1 (APP/PS1) mice lacking NRF2 exhibited autophagy dysfunction-dependent accumulation of insoluble Aβ aggregates, resulting in an increased pro-inflammatory phenotype

(Joshi et al., 2015). A similar study testing NRF2 loss in a combined model of amyloidopathy and tauopathy (AT mice) demonstrated that *AT-Nrf2*$^{-/-}$ mice exhibited increased levels of phosphorylated tau, higher levels of Aβ aggregates, and more severe learning and memory deficits than their *AT-Nrf2*$^{+/+}$ counterparts (Rojo et al., 2017). Perhaps one of the more interesting findings from this study was that *Nrf2*$^{-/-}$ mice, even in the absence of excess amyloid or tau, exhibited dysregulation of 7 of the 10 pathways associated with aging and AD progression. This important finding clearly indicates that an age-related decline in NRF2 function is a key driver of neurodegenerative disorders such as AD. Supporting the notion that NRF2 is needed to prevent Aβ-driven AD pathogenesis, mice genetically engineered to overexpress NRF2 in an AD context (*Keap1*$^{FA/FA}$;*APP*^{NLGF}) exhibited increased glutathione, decreased oxidative stress and inflammation, and improved cognition compared to wildtype, with similar benefits being obtained via administration of the isothiocyanate 6-(Methylsulfinyl)hexyl isothiocyanate (6-MSITC) (Uruno et al., 2020).

Also on the pharmacological front, several electrophilic and non-electrophilic compounds have been shown to exert anti-AD effects via activation of NRF2. DMF, SF, and curcumin, much like in a PD setting, have been shown to prevent oxidative stress, inflammation, and pathogenic protein accumulation in *in vitro* and *in vivo* models of AD (Campolo et al., 2018; Paraiso et al., 2018; Xu et al., 2019; Sun et al., 2022). Of note, protection by DMF was observed in male, but not female mice, indicating the possibility of sex-dependent effects on efficacy (Mohle et al., 2021), although further studies to clarify this effect are needed. A pair of non-electrophilic NRF2-KEAP1 PPIs, NXPZ-2 and POZL, discovered by the same group, have both been shown to ameliorate AD phenotypes in Aβ-injected or APP/PS1 mice, respectively (Sun et al., 2020; Sun et al., 2023). One interesting recent alternative to a pharmacological approach is the utilization of antisense oligonucleotides (ASOs) that target the NRF2 machinery. Along these lines, an ASO targeting GSK3-β, which can initiate β-TRCP-dependent degradation of NRF2, was shown to increase NRF2 levels, resulting in decreased oxidative stress and less severe learning and memory impairment in a *SAMP8*$^{-/-}$ AD mouse model (Farr et al., 2014). Thus, much like PD, several pharmacological and non-pharmacological means of targeting the NRF2 pathway have shown therapeutic promise in mitigating AD phenotypes.

Huntington's disease

Unlike AD and PD, where NRF2 localization is altered or its levels are low, no studies, at least to our knowledge, have shown if NRF2 levels are altered in HD patient brains. However, it has been shown that NRF2 is activated in a cell model of huntingtin overexpression, inferring that NRF2 is responsible for mitigating some of the harmful effects brought on by HD progression (van Roon-Mom et al., 2008). Pharmacologically, CDDO-ethyl amide and CDDO-trifluoroethyl amide were shown to decrease oxidative stress and improve motor performance in an N171-82Q transgenic mouse model of HD (Stack et al., 2010). Similarly, naringin, a dietary flavonoid obtained from grapefruit was also shown to activate NRF2-dependent amelioration of HD phenotypes in 3-nitropropionic acid (3-NP)-induced HD (Gopinath and Sudhandiran, 2012). SF, curcumin, and tert-butylhydroquinone (tBHQ) were also shown to protect against 3-NP-induced HD in an NRF2-dependent manner (Sandhir et al., 2014; Jang and Cho, 2016; Silva-Palacios et al., 2017), and a novel covalent modifier MIND4, and its derivative 4–17, activated NRF2 and suppressed oxidative stress in HD cell and animal models, as well as patient monocytes (Quinti et al., 2016; Quinti et al., 2017). Finally, compound 2, a non-covalent chalcone-derived NRF2 inducer, was shown to reduce oxidative stress and improve the survival of H_2O_2-treated primary astrocytes isolated from a zQ175 mouse model of HD (Moretti et al., 2021). Overall, NRF2 clearly plays a protective role in preventing HD onset and progression, and efforts continue to determine the relevance of targeting this pathway to treat patients with HD.

Other neurological diseases

Two other critical central nervous system disorders, amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS), have also been shown to involve NRF2 signaling. In the case of ALS, there are contradictory reports indicating that the spinal cord and motor cortex of ALS patients have both lower and higher mRNA and protein levels of NRF2 (Sarlette et al., 2008; Lastres-Becker et al., 2022). However, the study by Lastres-Becker et al. also tested downstream target genes, showing elevated expression of the key detoxifying target gene NADPH-quinone oxidoreductase 1 (*NQO1*), as well as the iron metabolism protein heme-oxygenase 1 (*HMOX1*). Thus, while this study infers target gene activation does occur in ALS tissues, further clarification of NRF2 levels in ALS patients is needed. Much like the MPTP-induced model of PD, the cell type-relevance of NRF2 may also be important to consider, as astrocyte-specific overexpression of NRF2 increased survival of mice overexpressing mutant superoxide dismutase 1 (*SOD1*^{G39A}), an established model of ALS (Vargas et al., 2008). Conversely, neither whole body knockout, nor targeted overexpression of NRF2 in neurons or skeletal muscle had a pronounced effect on *SOD1*^{G39A} mouse survival (Guo et al., 2013; Vargas et al., 2013). This was further supported by a later study, which investigated a possible gene therapy-based approach via adeno-associated viral delivery of *NFE2L2*/NRF2, which was able to activate NRF2 and its downstream genes *NQO1* and *HMOX1* in NSC-24 motor neuron cells and *SOD1*^{G39A} mice; however, overall mouse survival was unaffected (Nanou et al., 2013). These studies further highlight the cell type-relevance of NRF2 in different neurodegenerative contexts, and that additional experimental models of ALS may need to be considered to better correlate patient observations with lab-based studies.

Finally, NRF2 has also shown importance in MS models and patient contexts. Like ALS, NRF2 was shown to be upregulated in MS patient lesions (Licht-Mayer et al., 2015), and transgenic activation of NRF2 specifically in astrocytes prevented, whereas whole body knockout exacerbated, the oligodendrocyte loss and enhanced inflammation observed in a cuprizone-induced model of MS (Draheim et al., 2016; Nellessen et al., 2020). Mentioned briefly above, the gold standard treatment for MS, DMF (Tecfidera), has also been shown to activate NRF2 in MS patient blood and immune cells (Gopal et al., 2017; Hammer et al., 2018; Carlstrom et al., 2019). Furthermore, DMF-dependent activation of NRF2 in neurons and glia was associated with decreased oxidative stress and increased overall survival in a myelin oligodendrocyte glycoprotein-driven mouse model of MS. The protective effect of DMF was not observed in *Nrf2*$^{-/-}$ mice, although a later study using this same model

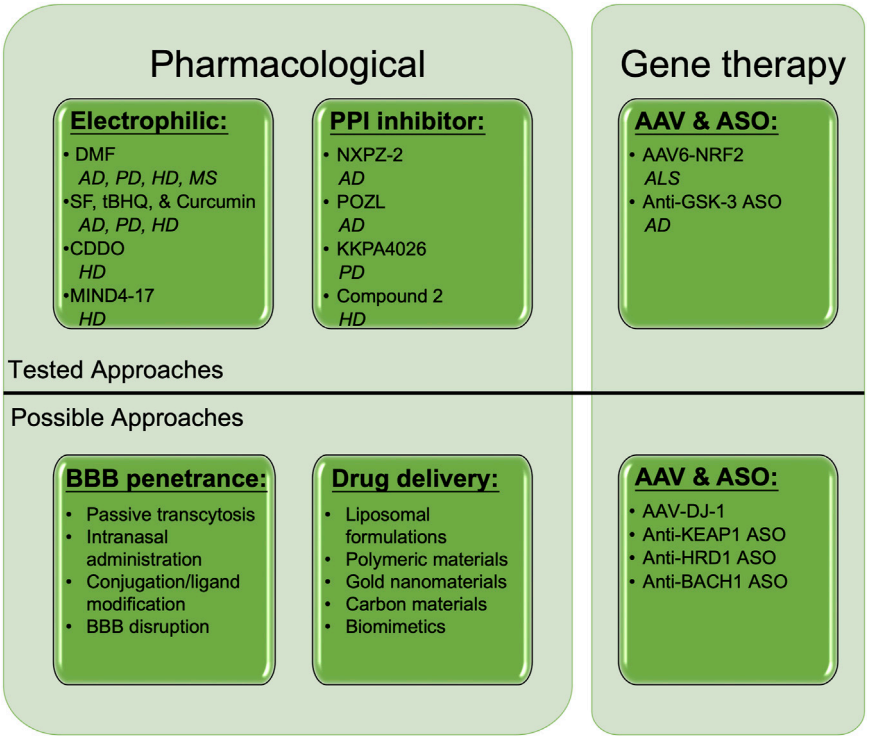

FIGURE 2
Established and putative means of NRF2 activation to treat neurodegeneration. Several pharmacological and gene therapy-based approaches have shown therapeutic promise in treating neurodegenerative diseases. Electrophilic activators, including DMF, SF, tBHQ, Curcumin, CDDO, and MIND4-17, as well as non-covalent protein-protein interaction inhibitors such as NXPZ-2, POZL, KKPA4026, and Compound 2 have all been shown to ameliorate AD, PD, HD, and MS progression in an NRF2-dependent manner. Adenovirus-associated NRF2 (AAV6-NRF2), as well as antisense oligonucleotides (ASOs) against GSK-3β, have been shown to prevent ALS and AD phenotypes, respectively. Possible untested pharmacological, drug delivery, and AAV/ASO approaches to target NRF2 in neurodegenerative disease include passive transcytosis, intranasal delivery, drug modification, membrane disruption, liposomal/nanoparticle formulations, and AAV/ASOs targeting NRF2 stabilizers (DJ-1), or its degradation machinery (KEAP1, HRD1) and transcriptional repressor (BACH1). DMF = Dimethyl fumarate, SF = Sulforaphane, tBHQ = tert-butylhydroquinone, BBB = Blood brain barrier, AAV = Adeno-associated virus, ASO = Antisense oligonucleotide. Created with Biorender.

produced contradictory results, which could be due to discrepancies in DMF dose and time of treatment (Linker et al., 2011; Schulze-Topphoff et al., 2016). In general, these studies indicate that activation of NRF2 represents a feasible strategy to treat MS progression, with DMF and its derivatives representing the best current approach.

Caveats and future considerations

Activation of NRF2 continues to represent a therapeutic strategy with promising, yet untapped potential. As loss of NRF2 clearly exacerbates the progression of experimental models of neurodegenerative disorders, it remains clear that preserving or re-establishing proper NRF2 function should mitigate disease progression and improve patient prognosis across a wide range of neuropathological contexts. While the most promising options identified to date are electrophilic (i.e., SF, DMF, and CDDO), alternative approaches, including protein-protein interaction inhibitors, adeno-associated viral (AAV)-mediated delivery, antisense oligonucleotides, and enhanced delivery systems continue to emerge as viable possibilities (Figure 2). Another interesting approach currently being tested is hybrid molecules, whereby activators of NRF2 (i.e., DMF) are coupled to molecules that inhibit its upstream repressors or co-activate its downstream effectors (i.e., GSK3- β and HMOX1) (Di Martino et al., 2020; El Ali et al., 2020). However, as discussed above, continued reliance on electrophilic compounds with known off-target effects is still likely to result in toxicity regardless of the specificity of the conjoined molecule. Another intriguing class of compounds not discussed in detail here is natural compounds (Moratilla-Rivera et al., 2023), which clearly exert beneficial effects, but often lack sufficient clarity on the mechanism of action and whether the compound itself, or a metabolite, are responsible for the observed protection. Regardless, pharmacological intervention continues to warrant further investigation, particularly in cases where no toxicity is observed.

Along with the methods already being tested in an NRF2 context, several other possible strategies for therapeutic intervention also warrant consideration. One popular approach is improving the ability of small molecules to cross the BBB. This includes mildly disrupting BBB integrity, modifying/tagging established compounds to improve their stability/penetrance, as well as utilizing intranasal administration to bypass the BBB altogether, among others (Figure 2). Much like the liposomal delivery approach discussed above, several nanoparticle-, bioengineering-, and biomimetic-based approaches have also garnered recent interest (Sun and Roy, 2021), inferring that testing these systems with NRF2-targeted therapies could also

work. Finally, based on the promise of AAV-mediated NRF2 overexpression, as well as targeted enhancement of NRF2 transcription in astrocytes in mouse models of neurodegeneration, gene therapy-based approaches that lead to brain cell type-dependent increases in NRF2 expression also appear to have significant merit. This is further supported by the beneficial, NRF2-dependent, effects observed in the presence of ASOs targeting GSK3-β, as other ASOs targeting negative regulators of NRF2 signaling (i.e., KEAP1, synoviolin [HRD1], and the transcriptional repressor or NRF2-AREs, BACH1, could all theoretically upregulate NRF2 to provide therapeutic benefit (Figure 2). AAV-mediated overexpression of DJ-1, which has been shown to stabilize NRF2 in a PD context (Clements et al., 2006; Im et al., 2012), could also be effective. Continued testing of these and other already established NRF2-based strategies promises to yield better NRF2-targeted therapies that progress to clinical trials and can eventually be used for intervention in patients suffering from these debilitating diseases.

Concluding remarks

NRF2 continues to represent a viable therapeutic target with endless possibilities. While current efforts have shown great promise, the field has continued to evolve towards more targeted, efficient, and potent possibilities. Considering the NRF2 field is still relatively young, at just over two decades old, the progress made to date regarding our mechanistic understanding of this pathway in disease, including viable means to target it even at the patient level, is remarkable. Clearly the sky is the limit in harnessing the protective potential of this pathway across the neurodegenerative disease spectrum, and only time will tell if we can finally progress from experimental promise to therapeutic reality.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MD: Conceptualization, Writing–original draft, Writing–review and editing. W-TC: Conceptualization, Writing–original draft, Writing–review and editing.

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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.

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