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Autophagy and autophagy genes in pathophysiology of human disease and ageing

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Xiaonan Dong and Zvulun Elazar

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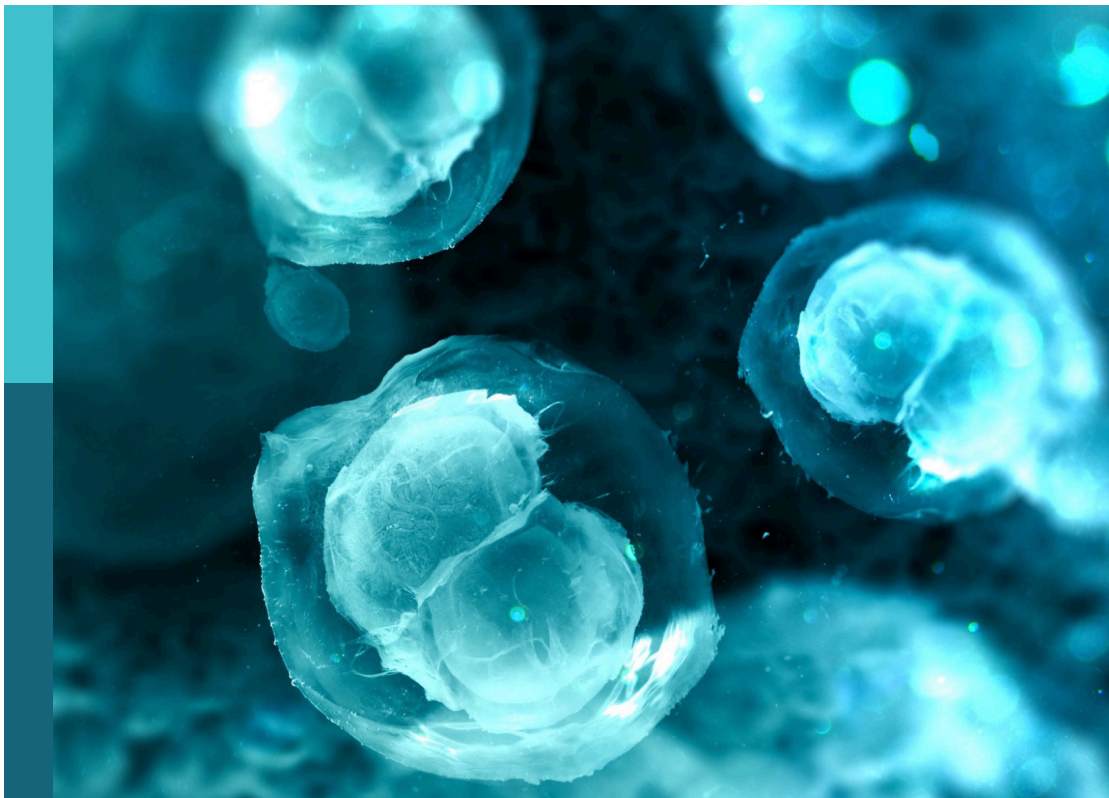

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Autophagy and autophagy genes in pathophysiology of human disease and ageing

Topic editors

Xiaonan Dong — Independent researcher, China

Zvulun Elazar — Weizmann Institute of Science, Israel

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REVIEW
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Binding Features and Functions of ATG3

*Dongmei Fang, Huazhong Xie, Tao Hu, Hao Shan and Min Li**

School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China

Autophagy is an evolutionarily conserved catabolic process that is essential for maintaining cellular, tissue, and organismal homeostasis. Autophagy-related (*ATG*) genes are indispensable for autophagosome formation. *ATG3* is one of the key genes involved in autophagy, and its homologs are common in eukaryotes. During autophagy, ATG3 acts as an E2 ubiquitin-like conjugating enzyme in the ATG8 conjugation system, contributing to phagophore elongation. ATG3 has also been found to participate in many physiological and pathological processes in an autophagy-dependent manner, such as tumor occurrence and progression, ischemia–reperfusion injury, clearance of pathogens, and maintenance of organelle homeostasis. Intriguingly, a few studies have recently discovered the autophagy-independent functions of ATG3, including cell differentiation and mitosis. Here, we summarize the current knowledge of ATG3 in autophagosome formation, highlight its binding partners and binding sites, review its autophagy-dependent functions, and provide a brief introduction into its autophagy-independent functions.

Keywords: ATG3, autophagy, binding feature, cancer, homeostasis, function, phosphatidylethanolamine, post-translational modification

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***Correspondence:**
Min Li
limin65@mail.sysu.edu.cn

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INTRODUCTION

Autophagy plays an important role in maintaining cellular energy balance, structural reconstruction, and immunity when cells respond to stress conditions, such as amino acid starvation. Based on how a cargo is transported to lysosomes, autophagy can be divided into macroautophagy, microautophagy, and molecular chaperone-mediated autophagy (Sridhar et al., 2012; Catarino et al., 2017). Macroautophagy (hereinafter referred to as autophagy) is a highly evolutionarily conserved process that sequesters cytoplasmic components, cellular organelles, invading microorganisms, and aggregated proteins into a double membrane–bound structure called the autophagosome, which then fuses with lysosomes to degrade the cargo.

The most crucial event in autophagy is autophagosome formation, which was first observed by Christian de Duve in the 1960s. A series of autophagy-related (*Atg*) genes was subsequently identified through genetic studies in yeast in the 1990s (De Duve and Wattiaux, 1966; Tsukada and Ohsumi, 1993). Currently, scientists have identified more than 40 *Atg* genes, mainly by genetic screening using model organisms, such as *Saccharomyces cerevisiae* (Morita et al., 2019). Among these *Atg* genes, one subset has been identified as the core *Atg* genes because they are required for autophagosome formation, including nucleation, elongation, and closure of the isolation membrane (Xie and Klionsky, 2007).

Two ubiquitin-like conjugation systems, the ATG12 conjugation system and Atg8/microtubule-associated protein 1 light chain 3 (LC3) lipidation system, are known to be indispensable for phagophore elongation (Shintani et al., 1999; Ichimura et al., 2000; Tanida et al., 2002a). ATG3 acts as an E2-like enzyme in the Atg8/LC3 lipidation system and is essential for the lipidation of Atg8/LC3 (Ichimura et al., 2000; Tanida et al., 2002b). Although lipidation of LC3 can occur during the non-canonical autophagic process in an ATG5/ATG7-independent manner, there is no current evidence indicating that cells deficient in ATG3 can convert LC3-I; to LC3-II (Moloughney et al., 2011; Chang et al., 2013). Although $Atg3^{-/-}$ mouse embryo fibroblast cells (MEFs) are survivable, $Atg3^{-/-}$ mice are nonviable, suggesting that Atg3 is essential for the homeostasis of the organism (Sou et al., 2008). ATG3 is conserved in eukaryotes and can interact with many proteins, such as LC3ylation (Agrotis et al., 2019). In addition, functions of ATG3 are emerging in many contexts, including the maintenance of mitochondrial homeostasis, regulation of tumor progression, and clearance of viral infection (Radoshevich et al., 2010; Altman et al., 2011; Choi et al., 2014). Recently, a few autophagy-independent functions of ATG3 were found, indicating that the roles of ATG3 might be more complex. In this review, we sought to elucidate the binding features of ATG3 in two conjugation systems and their functions in autophagy-dependent and -independent pathways.

ROLES OF ATG3 IN TWO CONJUGATION SYSTEMS

Unlike ATG4, which has four subtypes, and ATG16L1, which has three subtypes, there is only one type of ATG3 in organisms. ATG3 and its homologs are common in eukaryotes, including fungi, and higher eukaryotes, such as mammals, insects, and plants. Furthermore, their amino acid sequences in different species are highly conserved (Tsukada and Ohsumi, 1993; Xu et al., 1999; Juhasz et al., 2003; Wu et al., 2006; Hanada et al., 2009).

In 1993, Ohsumi et al. first isolated 15 mutants, *apg1–apg15*, from *S. cerevisiae*, which could not accumulate autophagic bodies in vacuoles under starvation conditions in the absence of vacuolar proteinases (Tsukada and Ohsumi, 1993). Almost simultaneously, Thumm et al. isolated several *aut* mutants of *S. cerevisiae* using the same method as Ohsumi et al. and identified aut1, which encodes 310 amino acids (Thumm et al., 1994; Schlumpberger et al., 1997). In 2000, a sequence analysis suggested that *apg3* is identical to *aut1* (both named *Atg3* for unified), and its gene product is an E2-like enzyme in the Atg8 lipidation system and the cytoplasm to vacuole targeting (Cvt) pathway (Ichimura et al., 2000; Kim et al., 2001).

During autophagosome elongation, the LC3 precursor is cleaved by ATG4B following exposure of Gly120 to form LC3-I;, which is activated by the E1-like activating enzyme, ATG7. LC3-I; is subsequently transferred to the E2-like conjugated enzyme, ATG3, and finally links to PE with the help of the E3-like ligase ATG12–ATG5 conjugate (Tanida et al., 2002a,b, 2004; Hanada et al., 2007).

ATG3 contributes to autophagosome formation by interacting with ATG7, ATG8, ATG12, and the lipid membrane. A recent study has shown that human ATG3 induces membrane aggregation *in vitro*, indicating that ATG3 contributes to vesicle restraint preceding fusion events in autophagosome elongation, which means that the function of ATG3 in autophagosome biogenesis may be more complex (Hervas et al., 2017). ATG3 is also required for Atg8/LC3 lipidation, not only for canonical autophagy but also for noncanonical LC3 lipidation (Chang et al., 2013). Translation of ATG3 also affects autophagy in mammals, *Caenorhabditis elegans*, and yeast. As reported recently, eukaryotic translation initiation factor 5A (eIF5A) is required for LC3 lipidation by assisting the ribosome via its hypusine residue to increase the translation of ATG3 at its DDG motif, which is a motif conserved in eukaryon and displays eIF5A hyperdependency. The connection of eIF5A with ribosomes is enhanced when autophagy is induced (Lubas et al., 2018). Hence, ATG3 is a key autophagy molecule worthy of further studies.

Structure of ATG3

Atg3/ATG3 is a dynamic protein lacking a rigid structure. In fact, approximately one-third of the Atg3/ATG3 sequences are missing in the crystal structure (Popelka et al., 2014). The structure of Atg3/ATG3 resembles a hammer composed of a head region (core region) and a handle region (HR). The core region with an α/β-fold is topologically similar to canonical E2 enzymes, such as ubiquitin-conjugating enzyme 9 (Ubc9), although they have little sequence homology with each other. The HR consists of a long α-helix and a partially disordered loop region. Furthermore, there is a "floating" helix C called FR at the interface between the core region and HR (**Figure 1A**; Yamada et al., 2007).

Binding Features of ATG3

Interaction Between ATG3 and ATG7

ATG3 forms an E1–E2 complex with ATG7, which is unique compared with other protein-conjugation systems. An *in vitro* pull-down assay showed that $Atg3^{HR}$ and $Atg3^{FR}$ are responsible for binding with Atg8 and Atg7, respectively (Tanida et al., 2002a; Yamada et al., 2007).

Abbreviations: Asp, aspartic acid; ATG/Atg, autophagy-related gene; Ala, alanine; BAG3, BCL2 associated athanogene 3; BLAST, Basic Local Alignment Search Tool; CTD, C-terminal domain; CLU, clusterin; Cvt, cytoplasm to vacuole targeting; Cys, cysteine; DC, dendritic cells; eIF5A, eukaryotic translation initiation factor 5A; ER, endoplasmic reticulum; FR, "floating" helix C; FLIP, FLICE-like inhibitor protein; FLIPs, death effector domains; GATA-1, a hematopoietic transcription factor; GAPCs, glyceraldehyde-3-phosphate dehydrogenases; GS, glutathione; Glu, glutamic acid; Gly, glycine; GR, glucocorticoid receptor; Hat, histone acetyltransferase; HDAC1, histone deacetylase 1; HVJ-E, inactivated Sendai virus; HR, handle region; HCC, hepatic cellular cancer; I/R, ischemia–reperfusion; Ile, isoleucine; IRG, immunity-related GTPase; LAPTM4B, lysosomal-associated protein transmembrane 4β; LC3, microtubule associated protein 1 light chain 3; LncR, long non-coding RNA; Lys, lysine; Leu, leucine; LRRK2, leucine-rich repeat kinase 2; MEFs, mouse embryo fibroblast cells; MiR, micoRNA; Mtb, *Mycobacterium tuberculosis*; NTD, N-terminal domain; NSCLC, non–small cell lung cancer; PE, phosphatidylethanolamine; PMT, posttranslational modification; PDCD6IP, ESCRT-associated protein Alix; Pro, proline; Thr, threonine; TGF-β1, transforming growth factor β1; TRAIL, TNF-related apoptosis-inducing ligand; TNF, tumor necrosis factor; TMV, tobacco mosaic virus; TNFAIP8, tumor necrosis factor α–induced protein 8; *T. gondii*, *Toxoplasma gondii*; Trp, tryptophan; Phe, phenylalanine; Ubc9, ubiquitin-conjugating enzyme 9.

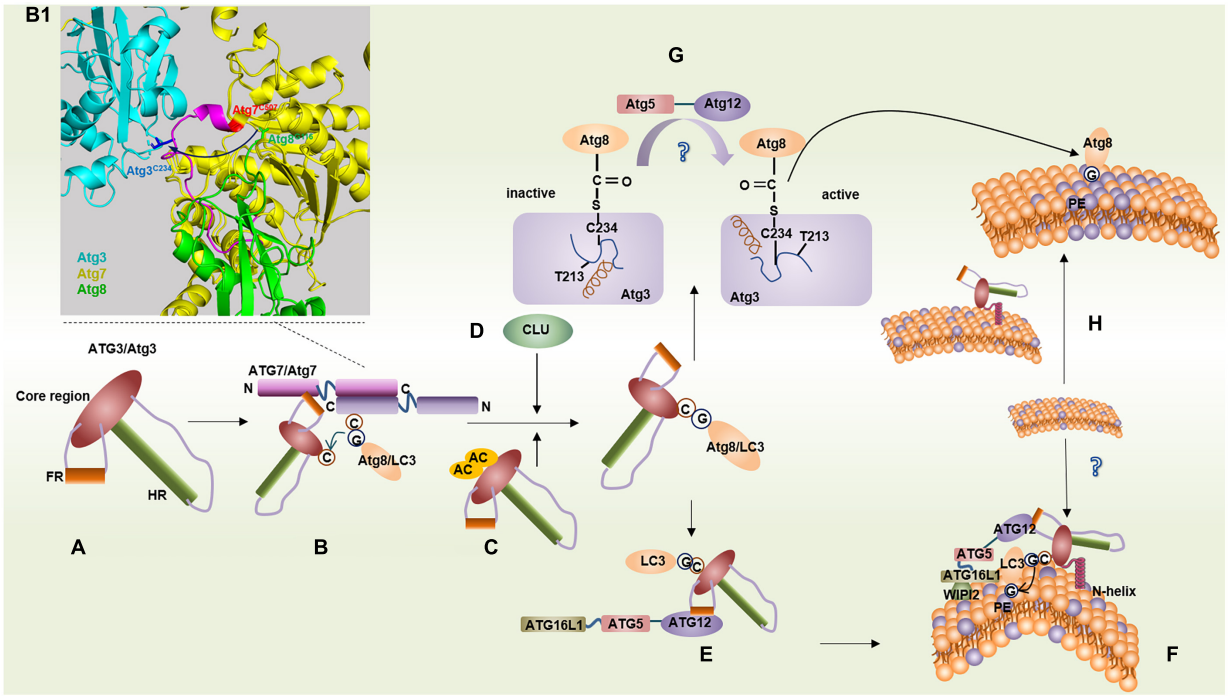

FIGURE 1 | Binding features of ATG3/Atg3 in two conjugation systems. **(A)** Structure of ATG3/Atg3. **(B)** LC3/Atg8 transfers from ATG7/Atg7 onto ATG3/Atg3 via a transmechanism. **(B1)** Model representation of the Atg7~Atg3~Atg8 complex by aligning Atg7–Atg3 (PDB ID: 4GSL) with the $Atg7^{CTD}$–Atg8 (PDB ID: 3RUI). $Atg8^{G116}$ transfers from $Atg7^{C507}$ to $Atg3^{C234}$, where Atg3 binds with another Atg7 within an Atg7 dimer. **(C)** Acetylation of K19 and K48 of Atg3 promotes Atg3–Atg8 conjugate. **(D)** Molecular chaperone clusterin (CLU) promotes ATG3-LC3 heterocomplex stability. **(E)** $ATG3^{FR}$ binds with ATG12 conjugate facilitating LC3 lipidation. **(F)** ATG3 N-helix inserts highly curved membrane mediating LC3 lipidation. **(G)** In yeast, the interaction between the loop (blue) containing the catalytic Cys234 and helix G (brown) in Atg3 makes Cys234 face away from Thr213, which suppresses Atg3 conjugase activity. Atg12–Atg5 leads to the reorientation of Cys234 by some unknown means, and then Cys234 faces to Thr213 to facilitate the Atg8 lipidation. **(H)** N-terminal domain of Atg3 can increase local PE density on the membrane.

In yeast, a short α-helix of $Atg3^{FR}$ can insert a hydrophobic groove in the N-terminal domain (NTD) of Atg7, in which the Atg3 residue, Met139, docks in a hydrophobic pocket formed by the Atg7 residues, Phe93, Lys94, Trp139, and Pro283 (Yamaguchi et al., 2012). Thereafter, Atg8 transfers from Atg7 to Atg3 and forms a thioester bond with Cys234 of Atg3 via a transmechanism. Atg7 can form a dimer by interacting with two active-site cysteine residues in the C-terminal domain (CTD). The Atg8 thioester linked to the CTD of one Atg7 transferred to Atg3 interacted with the NTD of the other Atg7 within a dimer (**Figures 1B,B1**; Komatsu et al., 2001; Hanada et al., 2007; Taherbhoy et al., 2011). A similar pattern of interaction between ATG3 and ATG7 was also found in mammals, such as mice. However, in plants such as *Arabidopsis thaliana*, $AtATG3^{CORE}$ binds to the C-terminal side of $AtATG7^{NTD}$ to form an L-shaped structure through hydrophobic interactions (Yamaguchi et al., 2012).

Interaction Between ATG3 and LC3/ATG8

LC3/Atg8 usually binds to the catalytic active sites of ATG3/Atg3 via a thioester bond (Yamada et al., 2007). In mammals, the molecular chaperone clusterin (CLU) can promote ATG3-LC3 heterocomplex stability and LC3 lipidation via direct interaction with the LC3 protein, and the ATG3-LC3 complex is significantly reduced in CLU-silent cells (**Figure 1D**; Zhang et al., 2014). In yeast, Atg3 is a substrate of the histone acetyltransferase Esa1, and acetylation of Lys19 and Lys48 of Atg3 positively regulates autophagy by promoting Atg3–Atg8 and Atg8–PE conjugates (**Figure 1C**; Hanada et al., 2009).

Atg3 can also bind to Atg8 in the absence of a thioester bond in yeast (Yamada et al., 2007). Nuclear magnetic resonance spectroscopy revealed that Atg3 directly interacts with Atg8 through the WEDL (Trp^{270}–Glu^{271}–Asp^{272}–Leu^{273}, called $Atg3^{AIM}$) sequence conserved in eukaryotes in HR. $Atg3^{AIM}$ affects the Cvt pathway and the formation of Atg8–PE but does not affect the formation of the Atg8–Atg3 thioester intermediate (Yamaguchi et al., 2010). Interestingly, $Atg3^{AIM}$ in *Toxoplasma gondii* contains an FADI (Phe–Ala–Asp–Ile) sequence and a WLLP (Trp–Leu–Leu–Pro) sequence, which is different from that of yeast $Atg3^{AIM}$ (Chen et al., 2016; Liu et al., 2018; Varberg et al., 2018). Such findings indicate that $Atg3^{AIM}$ may be species specific; thus, antitoxoplasmosis drugs and anti-*Plasmodium* drugs that target Atg8–Atg3 could be developed.

Interaction Between ATG3 and ATG12

In $Atg3^{-/-}$ MEFs, the Atg12–Atg5 complex is markedly reduced. In contrast, the overexpression of ATG3 could facilitate the formation of the ATG12–ATG5 complex in humans

(Tanida et al., 2002b; Sou et al., 2008). The yeast Atg12–Atg5 conjugate is unnecessary for Atg8–PE conjugation *in vitro* at pH 7.58 but is indispensable *in vivo* (Suzuki et al., 2001; Yamada et al., 2007). In canonical E2 enzymes, such as Ubc9, an asparagine residue promotes ubiquitin or ubiquitin-like proteins linked to E2 enzymes through a thioester bond to transfer to a lysine residue of the substrate (Wu et al., 2003; Yunus and Lima, 2006). However, in Atg3, the corresponding amino acid is a threonine residue that is conserved in all known homologs (Thr213 in *S. cerevisiae*), and its Atg3 catalytic center is rearranged, with Cys234 reoriented toward Thr213 in the presence of Atg12–Atg5; this reorientation of Cys234 enhances the conjugate activity of Atg3 (**Figure 1G**; Sakoh-Nakatogawa et al., 2013). Hence, the Atg12–Atg5 conjugate is regarded as an E3-like ligase in the Atg8 lipidation system. However, the interaction between Atg3 and Atg12 has not been reported in yeast, and how Atg12–Atg5 plays an E3-like ligase role remains unclear.

In mammals such as humans, ATG3 can communicate with the E3-like ligases, ATG12–ATG5 to ATG16L1, by forming a β-sheet between $ATG3^{FR}$ and ATG12. A 13-residue-long sequence of $ATG3^{FR}$ called $ATG3^{RIA12}$, which is highly conserved in eukaryotes except in fungi, is responsible for binding to ATG12 (**Figure 1E**; Metlagel et al., 2013). Therefore, in yeast, Atg12–Atg5 might act as an E3-like enzyme indirectly because there may not be a binding site for Atg12 in Atg3 based on the current evidence.

ATG3 can also interact with free ATG12 more preferentially than the ATG12–ATG5 conjugate, and the excess interaction of ATG3 with free ATG12 could partially inhibit the interaction between ATG7 and free ATG12, resulting in the suppression of LC3 lipidation (Tanida et al., 2002a), likely for the reason that the linear sequence of human $ATG3^{FR}$ that binds to ATG12 and ATG7 is overlapped at the 157–176 amino acids (Qiu et al., 2013; Ohashi and Otomo, 2015).

Membrane Binding and Sensitivity of ATG3

The N-terminal of Atg3 was found to be essential for binding with PE in Atg8 lipidation according to a flotation assay. However, the 1–20 amino acids within the N-terminal cannot function alone, suggesting that other regions of Atg3 are required for the interaction with PE-containing liposomes. The researchers hypothesized that Atg8 moves from Cys234 to the N-terminal of Atg3 and then combines with PE (Hanada et al., 2009). In mice and humans, the 20 N-terminal amino acids of ATG3 form an amphipathic helix that is sensitive to a highly curved membrane when the membrane has a low PE density (<30 mol%) and acts via membrane insertion (**Figure 1F**; Dancourt and Melia, 2014; Nath et al., 2014). Recently, a study suggested that human ATG3 communicates information from the N-terminal amphipathic helix to the C-terminus to catalyze LC3–PE conjugation (Ye et al., 2021). Under these conditions, LC3 lipidation can be made very efficient even in the absence of the ATG12–ATG5∼ATG16L1 complex *in vitro*; however, *in vivo*, the location of ATG3-mediated lipidation depends on the localization of ATG16L1 (**Figure 1F**; Fujita et al., 2008; Dancourt and Melia, 2014; Nath et al., 2014). The N-terminal–positive residues, Lys9 and Lys11, are essential for recognizing phospholipid-negative moieties, and the presence of anionic phospholipids, particularly anionic lipids with a negative intrinsic curvature, can suppress the preference for highly curved membranes (Hervas et al., 2017). Acetylation of ATG3 Lys19/Lys48 can also enhance its binding to PE-containing liposomes and the endoplasmic reticulum (ER), thereby promoting the lipidation process (Li et al., 2017).

Interestingly, PE density under physiological conditions ranges from 15 to 20 mol% in yeast ER and Golgi (Nair et al., 2011), which have been implicated as possible sources of the autophagosome membrane. However, whether ATG3 highly curved membrane sensing is predominant *in vivo* and how the highly curved membrane is formed remain unknown. A recent study found that the NTD of Atg3 not only serves as a membrane anchor but also attenuates lateral diffusion of PE, thereby increasing the local PE density on the membranes. Atg8 lipidation can thus bypass the Atg3 highly curved membrane sensing and conjugate to PE (**Figure 1H**; Wang et al., 2020). However, considering the assay was performed *in vitro*, whether Atg3 can also increase local PE density on the membranes *in vivo* remains unknown. Another study detected highly curved membrane formation in synapses, where the phosphorylation of the endophilin A–BAR domain by leucine-rich repeat kinase 2 served as a docking station of ATG3 and facilitated highly curved membrane formation (Soukup et al., 2016).

Nevertheless, three fundamental aspects of membrane binding and the sensitivity of ATG3 remain unclear: Can ATG3 induce a curved membrane by itself? Are proteins that can induce curved membranes, such endophilin A, present at the isolation membranes in other tissues? And how does ATG3 interact with other proteins on the isolation membrane? Further studies will provide more details to enable a better understanding of the molecular mechanism.

Switch Mechanism of ATG3

Recently, an *in vitro* assay of yeast ATG proteins identified a region called $Atg3^{E123IR}$ (Ile129-Lys142) that could autoinhibit the catalytic Cys234 residue of Atg3 within $Atg3^{FR}$. $Atg3^{E123IR}$ is relocated when binding to $Atg7^{NTD}$. Thereafter, the Atg3 catalytic core is rearranged, leading to activated Cys234 that attacks the Atg7–Atg8 intermediate, forming the Atg3–Atg8 thioester intermediate. With Atg7 leaving, $Atg3^{E123IR}$ autoinhibits the catalytic Cys234 residue again to keep the Atg3–Atg8 intermediate stable. When Atg12–Atg5∼Atg16 binds to $Atg3^{E123IR}$, the Atg3–Atg8 intermediate participates in a nucleophilic attack and Atg8–PE formation (Zheng et al., 2019).

In mammals and yeast, the catalytic cysteine residue of ATG3/Atg3 is bound to LC3/Atg8 through a stable thioester when autophagy is inactive. However, the thioester becomes transient upon autophagy stimulation, followed by the exposure of catalytic thiols, and then forms a disulfide heterodimer with ATG7/Atg7 or a glutathione adduct. This process is upregulated, which might contribute to impaired autophagy during aging, partly in aged mouse tissues (Burgoyne, 2018).

REGULATION FACTORS AND PATHOPHYSIOLOGICAL ROLES OF ATG3

Autophagy is involved in a wide range of physiological processes; for example, ATG3 can inhibit autophagy-induced apoptosis in inactivated Sendai virus (HVJ-E)–treated cells (Wang T. et al., 2018). The downregulation of mouse Atg3 expression results in compromised embryonic stem cell self-renewal, pluripotency, and differentiation (Sou et al., 2008). Its multiple physical functions are realized through a crosstalk with diverse cellular pathways via interactions among pivotal gene components. ATG3 is a key component of autophagy and is required to keep mammals alive (Sou et al., 2008). The role of ATG3 in autophagy-mediated physical functions can be regulated by its binding partners at different levels (**Table 1**).

ATG3 in Cancer

The expression of ATG3 changes significantly in various types of cancer tissues (**Table 2**), indicating that the expression level of ATG3 is closely related to cancer. For example, *ATG3* knockdown remarkably suppressed the proliferation and invasion of colon cancer cells (Huang et al., 2019). In addition, ATG3 can be modified by acetyltransferases recruited by Myc box II, a region within Myc-nick, which is a cleavage product of Myc being present in most tumor samples, leading to the upregulation of autophagy and cancer cell survival (Conacci-Sorrell et al., 2014). ATG3 can also interact with cellular and viral FLIPs (death effector domains) to suppress autophagy, resulting in tumor development (Lee et al., 2009). In general, the expression and modification of ATG3 play important roles in tumor development and progression (**Figure 2**).

A gene expression microarray study revealed that ATG3 was downregulated in myelodysplastic syndrome patients and patients progressing to leukemia (Ma et al., 2013; Wang et al., 2014). However, hematopoietic cells expressing BCR-AbI (a constitutively active oncogenic kinase) are highly sensitive to autophagy and fail to generate leukemia cells without ATG3 (Altman et al., 2011). PU.1 is a positive transcriptional regulator of *ATG3*, and low expression of PU.1 may account for the low expression of ATG3 in acute myeloid leukemia (Jin et al., 2018). For example, the overexpression of ATG3 in SKM-1 cells can induce autophagy and increase sensitivity to bortezomib treatment (Zhuang et al., 2016). However, ATG3 in human erythroleukemia cells with JAK2 V617F mutation is induced, whereby JAK2 V617F inactivates protein arginine methyltransferase 5 and promotes GATA-1 to bind with the ATG3 promoter (Harr et al., 2011). Such findings suggest that the low expression of ATG3 is essential for maintaining leukemia, and restoring autophagic activity might be beneficial in differentiation therapies. Hence, ATG3 could be a potential target for treating leukemia by increasing its expression or specifically inhibiting its degradation if specific mutations can be excluded.

In non-small cell lung cancer (NSCLC) patients, the expression level of miR-16 was significantly downregulated, whereas that of ATG3 was upregulated, with the 3'-UTR of ATG3 as the direct target of miR-16 (Wang H. et al., 2018). MiR-204-5p binds to the 3'-UTR of ATG3 to inhibit the expression of ATG3. ATG3 overexpression could reverse the effect of miR-204-5p on NSCLC cell proliferation inhibition (Kang et al., 2019). MiR-1 overexpression could improve the *cis*-platinum sensitivity of NSCLC cells by reducing the expression of ATG3, causing impaired ATG3-mediated autophagy, which provides a potential target for relieving antitumor drug resistance (Hua et al., 2018). In summary, ATG3 in patients with NSCLC is upregulated and protects NSCLS cells, such as A549, through autophagy. Hence, drugs that target the degradation of ATG3 or the reduced expression of ATG3 may be designed to suppress NSCLC cell proliferation.

ATG3 mRNA expression levels were found to be higher in hepatic cellular cancer (HCC) tissues than in adjacent nontumor liver tissues. The transcription of *ATG3* is activated by lysosomal-associated protein transmembrane-4β (LAPTM4B) to modulate apoptosis and autophagy in HCC cells (Wang et al., 2019). Moreover, long noncoding RNA (lncRNA) plasmacytoma variant translocation 1 expression is increased, which can facilitate autophagy by sponging miR-365 to target the 3'-UTR of ATG3 and upregulate ATG3 (Yang et al., 2019). Tumor necrosis factor α–induced protein 8 (TNFAIP8) can interact with ATG3 and subsequently create cellular autophagy events that promote cell survival and drug resistance (Kristensen et al., 2012; Niture et al., 2018, 2020). The Cancer Genome Atlas showed that the lncRNA nuclear enriched abundant transcript 1 is upregulated in HCC tissue and promotes HCC autophagy and sorafenib resistance by sponging miR-204 to upregulate ATG3 expression (Li et al., 2020). Some studies found that the ectopic expression of CD147-ICD causes the accumulation of ATG3 via the nuclear factor κB–TRAIL–caspase8–ATG3 axis, which increases the viability of cisplatin-treated HCC cells by enhancing autophagy in HCC cells (Wu et al., 2017). The above studies indicate that drug resistance is associated with high expression of ATG3 to some extent. In addition, the overexpression of ATG3 in HCC increases autophagic flux, which is beneficial for the growth of HCC cells. Therefore, downregulation of ATG3 or destruction of E2 enzyme activity while using drugs such as sorafenib to treat HCC could improve drug sensitivity.

ATG3 is upregulated in many other tumors. In gastric cancer tissues, a study showed that the expression of ATG3 is upregulated and acts as a favorable independent prognostic factor, as supported by overall survival analysis (Cao et al., 2016). However, another study showed that in colon cancer tissues, the expression of ATG3 was upregulated. As a result, ATG3 could promote proliferation and invasion in colon cancer, whereas the downregulation of ATG3 could suppress the progression of colon cancer (Huang et al., 2019). In prostate cancer cells, TNFAIP8 can interact with ATG3 and subsequently create cellular autophagy events that promote cell survival and drug resistance (Kristensen et al., 2012; Niture et al., 2018, 2020), thereby indicating that ATG3 may play different roles in different cancers.

ATG3 in Ischemia–Reperfusion Injury

ATG3 expression contributes to ischemia–reperfusion (I/R) injury. A recent study found that the upregulation of

TABLE 1 | ATG3 modulators and functions.

Modulated types	Modulators	Functions	References
Transcription level			
	GATA-1	Induces ATG3 in erythroleukemia	Harr et al., 2011
	LAPTM4B	Activates ATG3 in HCC	Wang et al., 2019
	GR	Increases ATG3 in folic acid deprivation cells	Sun et al., 2016
Translation level			
	MiR-16	Upregulates ATG3 in NSCLC by negative regulation	Wang H. et al., 2018
	MiR-204-5P	Reduces ATG3 in NSCLC	Kang et al., 2019
	MiR-1	Reduces ATG3 in NSCLC	Hua et al., 2018
	MiR-365	Upregulates ATG3 in HCC	Yang et al., 2019
	MiR-204	Upregulates ATG3 in HCC	Li et al., 2020
	MiR-431-5p	Upregulates ATG3 in colon cancer by negative regulation	Huang et al., 2019
	MiR-155	Downregulates ATG3 in tuberculosis	Etna et al., 2018
	HDAC1	Downregulates ATG3	Du et al., 2019
	MiR-495	Downregulates ATG3	Li et al., 2016
	MiR-23a	Downregulates ATG3	Li et al., 2018
	MiR-206	Downregulates ATG3	Kong et al., 2019
Posttranslational modification			
	Acetyltransferases	Promotes cancer cell survival	Tan et al., 2016
	FLIPs	Suppresses autophagy	Lee et al., 2009
	Caspase-3	Cleaves ATG3	Norman et al., 2010
	Caspase-6	Cleaves ATG3	Norman et al., 2010
	Caspase-8	Cleaves ATG3	Norman et al., 2010; Oral et al., 2012
	TNFAIP8	Creates cellular autophagy	Niture et al., 2020
	PDCD6IP	Distributes late endosome	Murrow et al., 2015
	Calpain 1	Cleaves ATG3	Norman et al., 2010
	Calpain 2	Cleaves ATG3	Zhao et al., 2016
	Beclin-1	Protects liver	Wang et al., 2011
	GAPCs	Regulates autophagy negatively	Han et al., 2015; Liu T. et al., 2017; Ismayil et al., 2020
	Hat1	Appressorium formation and pathogenicity	Yin et al., 2019

GATA-1, a hematopoietic transcription factor; LAPTM4B, lysosomal-associated protein transmembrane-4 beta; GR, glucocorticoid receptor; MiR, microRNA; HCC, hepatic cellular cancer; HDAC1, histone deacetylase 1; FLIP, FLICE-like inhibitor protein; NSCLC, non–small cell lung cancer; TNFAIP8, tumor necrosis factor α–induced protein 8; PDCD6IP, ESCRT-associated protein Alix; GAPCs, glyceraldehyde-3-phosphate dehydrogenases; Hat, histone acetyltransferase.

TABLE 2 | Changes of ATG3 in different types of tumors.

Tumor types	ATG3	References
Myelodysplastic syndrome	↓	Ma et al., 2013; Wang et al., 2014
Myeloid leukemia	↓	Ma et al., 2013
Erythroleukemia (JAK2 V617F mutation)	↑	Harr et al., 2011
Non–small cell lung cancer	↑	Hua et al., 2018; Wang H. et al., 2018
Hepatic cellular cancer	↑	Wang et al., 2019; Li et al., 2020
Gastric cancer tissues	↑	Cao et al., 2016
Colon cancer	↑	Huang et al., 2019
Prostate cancer	—	Niture et al., 2018

"—", not determined.

mitochondrial RNA processing endoribonuclease and the upregulation of ATG3 caused by the downregulation of miR-206 might worsen myocardial I/R injury (Kong et al., 2019). However, in fatty livers, ATG3 seems to be a protective factor against I/R injury. Another study revealed that, compared with normal livers, fatty livers are more susceptible to I/R injury because of the higher expression of calpain 2 after I/R, and amino acids 92–97 of ATG3 can be cleaved by calpain 2, causing the inhibition of autophagy. Furthermore, the *in vitro*/*in vivo* overexpression of ATG3 could enhance autophagy and reduce cell death after I/R injury in fatty liver (Zhao et al., 2016). However, the interaction between ATG3 and beclin-1 could protect the livers of old mice

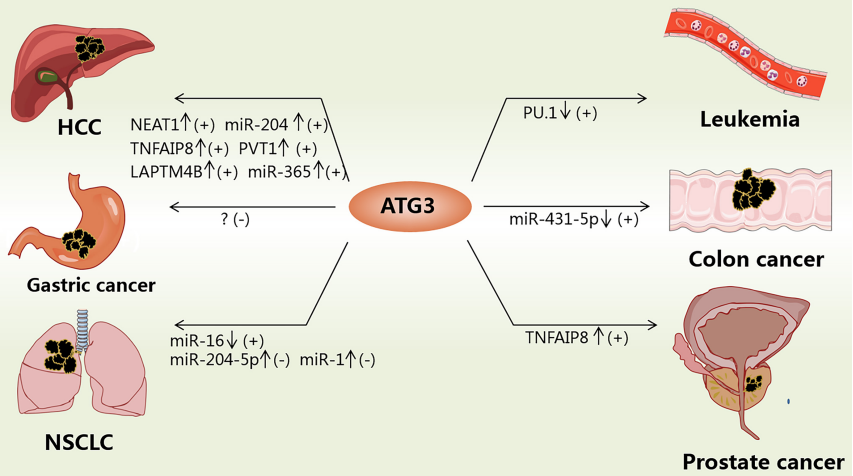

FIGURE 2 | Functions of ATG3 in cancers. ATG3 could be modulated by different factors in cancers. "↓" means the corresponding factor is downregulated; "↑" means the corresponding factor is upregulated; "+" means inducing cancers; "-" means suppressing cancers.

from I/R injury to some extent (Wang et al., 2011). Currently, the specific mechanism remains unaddressed because ATG3 is downstream of beclin-1. Based on the evidence, the effect of ATG3 on I/R appeared to differ in different organs. For example, ATG3 plays a negative role in myocardial I/R injury but plays a protective role in I/R injury in fatty livers.

ATG3 in the Clearance of Pathogens

ATG3 can aid in the clearance of pathogens by regulating autophagy. A study found that live and virulent *Mycobacterium tuberculosis* (Mtb) significantly stimulated the expression of miR-155 in dendritic cells. MiR-155 binds to the ATG3 3'-UTR to inhibit the translation of ATG3, causing autophagy inhibition and Mtb survival (Etna et al., 2018). In infected cells, the expression of ATG3 is required for immunity-related GTPase (IRG) to dock to *T. gondii* and *Chlamydia trachomatis* pathogen-containing vacuoles, possibly by activating IRG, thereby protecting host cells (Haldar et al., 2014). Hence, developing novel drugs that would boost autophagy is a new therapeutic strategy against tuberculosis, or drugs targeting miR/lncR to promote ATG3 expression might be an underlying tool to clear pathogens.

ATG3 in Organelle Homeostasis

ATG3-dependent autophagy is critical for mitochondrial homeostasis (Liu et al., 2016). A previous study showed that ATG3-depleted *T. gondii* exhibited remarkable mitochondrial fragmentation (Besteiro et al., 2011). Dysfunctional mitochondria accumulated in adipocytes lacking ATG3 due to postdevelopmental impairment of autophagy, resulting in increased lipid peroxidation, adipose tissue inflammation, systemic insulin resistance, and Nrf2 and keap1 activation (Cai et al., 2018). This accumulation of damaged mitochondria may be owing to mitophagy defects in the absence of ATG3.

ATG3 can form an ATG12–ATG3 complex with unconjugated ATG12 in mammals. The ATG12–ATG3 conjugate is produced by the autocatalytic reaction of ATG3, where the ATG12 thioester linked to the catalytic cysteine of ATG3 is transferred to the lysine residue (Lys243 of human ATG3) of the same ATG3 molecule (Tanida et al., 2002a; Radoshevich et al., 2010). The ATG12–ATG3 complex is responsible for mitochondrial homeostasis, and cells lacking ATG12–ATG3 undergo mitochondrial mass expansion and mitophagy blocking (**Figure 3A**; Radoshevich et al., 2010). Interestingly, a recent study has reported a new posttranslational modification of LC3, named LC3ylation. In this modification, LC3 can also conjugate to the ATG3 residue, Lys243, to form an LC3–ATG3 conjugate. The LC3–ATG3 conjugate can be cleaved by ATG4B, which is defined as deLC3ylation (**Figure 3B**; Agrotis et al., 2019). However, the specific mechanism and physiological function of LC3ylation should be further studied.

ATG3 plays a key role in late endosome function. In fact, ATG12–ATG3 is essential for multitudinous PDCD6IP-mediated functions, including late endosome distribution, exosome biogenesis, and viral budding (Murrow et al., 2015). PDCD6IP contains three structural domains, including an N-terminal Bro1 domain, a C-terminal proline-rich domain (PRD), and the V domain binding to the YPXnL motifs (Strack et al., 2003; Baietti et al., 2012). The YPXnL-binding site in the V domain is inhibited by intramolecular interaction of the PRD with the Bro1 and V domains. ATG12–ATG3 binds to the Bro1 and V domains to release the YPXnL-binding site and then supports multiple PDCD6IP functions (Murrow et al., 2015).

ATG3 in Plant Growth and Plant-Associated Microbiome

In *Nicotiana benthamiana* plants, ATG3 interacts with cytosolic glyceraldehyde-3-phosphate dehydrogenases (GAPCs) to negatively regulate autophagy (Han et al., 2015). In potato, the interaction of StATG3 and StGAPCs might contribute to the maintenance of tuber apical dominance, possibly by preventing

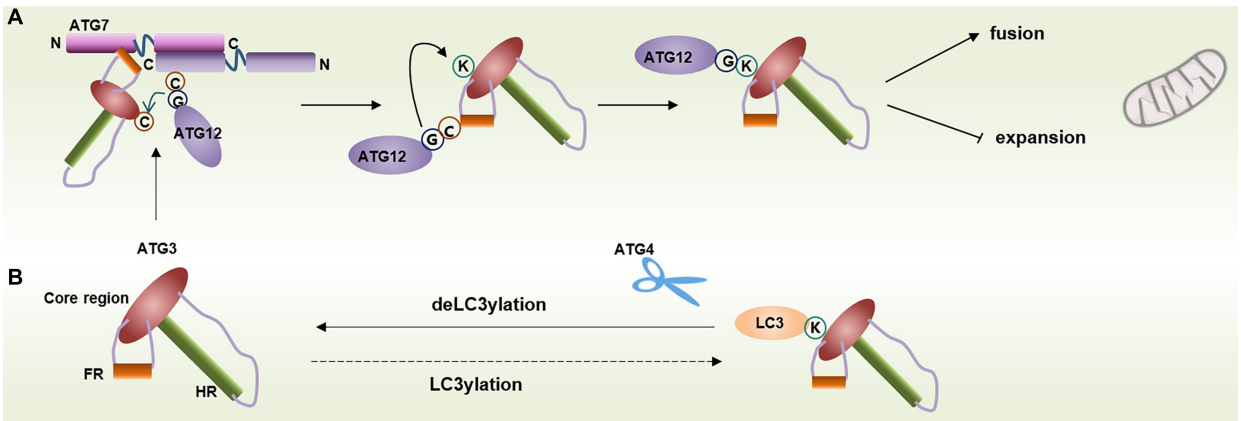

FIGURE 3 | Modification of ATG3 residue Lys243. **(A)** Lys243 within ATG3 can conjugate with free ATG12 and contribute to mitochondrial homeostasis. **(B)** Lys243 within ATG3 can also form a conjugate with LC3 via a process called LC3ylation, and ATG4 can cleave the conjugate.

cell death in the tuber apical bud meristem (Liu T. et al., 2017). Such finding suggests that the interaction between ATG3 and GAPCs is important for the growth of plants, and chemicals targeting ATG3-GAPCs could be designed and used to maintain or eliminate tuber apical dominance to improve production or control plant type.

ATG3 can relieve viral symptoms via autophagy in plants. ATG3 is a regulator of plant immunity-related cell death that limits the extent of spread of tobacco mosaic virus (TMV)–induced hypersensitive response programmed cell death (Liu Y. et al., 2005). As a result, ATG3 could protect plants from TMV infection, likely through autophagy. In cotton leaf curl Multan betasatellite, CLCuMuB βC1 can activate autophagy by disrupting GAPCs–ATG3 interactions, and mutant virus carrying βC13A, which could not induce autophagy, showed more severe viral symptoms and viral DNA accumulation (Ismayil et al., 2020). However, the acetylation of pathogenic ATG3 seemed to be an adverse factor for plants because of the activation of autophagy in pathogens. In the rice blast fungus, *Magnaporthe oryzae*, ATG3 is acetylated by Hat1 to facilitate autophagy and function in appressorium formation and pathogenicity (Yin et al., 2019). These studies suggest that autophagy in plants is facilitated or suppressed in pathogens by modulating ATG3 to alleviate viral infection.

AUTOPHAGY-INDEPENDENT FUNCTIONS OF ATG3

As a crucial gene of autophagy, several autophagy-related functions of *ATG3* have been reported; however, only a few studies have investigated its autophagy-independent functions.

ATG3 is not only a key component of autophagy but also functions in DNA damage–induced mitosis in an autophagy-independent manner. Research has shown that ATG3 is degraded via Tyr203 phosphorylation during etoposide or cisplatin treatment, causing DNA damage in cancer cell lines. However, the inhibition of ATG3 degradation during DNA damage could facilitate DNA damage–induced mitotic catastrophe, potentially via the interaction between ATG3 and BAG3 (BCL2-associated athanogene 3), which is a key protein in the mitotic process, even in the absence of ATG7 (Ma et al., 2017).

In *Fusarium oxysporum*, each wild-type hypha contains only one nucleus in one hyphal compartment; however, the *Foatg3* null mutant hyphae were found to contain more than one nucleus in one hyphal compartment, causing virulence reduction, which indicated that Foatg3 is a crucial target in the treatment of root crop dry rot disease (Liu T. et al., 2017; Khalid et al., 2019). In addition, unlike $Atg16^{-/-}$ (another essential gene in LC3 lipidation system) mice that can survive, mice without Atg3 died within 1 day of birth. $Atg3^{-/-}$ MEFs could also be subcultured, indicating that Atg3 is important for cell differentiation (Saitoh et al., 2008; Sou et al., 2008; Liu T. et al., 2017).

ATG3 might play important roles in cell differentiation and mitosis in an autophagy-independent manner. Although only limited evidence of the nonautophagic roles of ATG3 has been reported thus far, we believe that more autophagy-independent functions may be discovered through future investigations.

CONCLUSION AND PERSPECTIVES

Autophagy is crucial for maintaining cellular homeostasis and plays an essential role in infectious diseases, cancers, and neurodegenerative diseases (Choi et al., 2013). As a core autophagy-related protein, ATG3 is necessary for LC3 lipidation via its E2-like–conjugated enzyme and membrane-binding functions; however, details such as the source of highly curved membranes *in vivo*, mechanism of increased local PE density by Atg3, and the mode of Atg5–Atg12 required to affect Atg8 lipidation in yeast remain unknown. As a result, the specific mechanism requires further investigation. ATG3 can form an ATG3–ATG12 conjugate at Lys243, which is crucial for maintaining mitochondrial homeostasis (Radoshevich et al., 2010). Interestingly, a recent study has

reported that LC3B conjugation to ATG3, called LC3ylation, also occurs at Lys243 in the absence of ATG4. However, whether there are new physiological functions modulated by this conjugate remains unknown, thereby warranting further studies (Agrotis et al., 2019).

ATG3 plays a key role in all cancer types. In established solid tumors, such as NSCLC, ATG3 is significantly upregulated, resulting in increased autophagy flux, which may confer a survival advantage for solid tumor cells (Harr et al., 2011; Sun et al., 2016; Kang et al., 2019; Yang et al., 2019; Li et al., 2020). Thus, inhibitors that can downregulate ATG3 may be developed as antisolid tumor drugs for most tumors, except for gastric cancer; this is because ATG3 is a favorable independent prognostic factor of this cancer type (Cao et al., 2016). However, ATG3 is downregulated in blood tumors, such as leukemia without the JAK2 V617F mutation (Lee et al., 2009; Wang et al., 2014). Hypoxia upregulated oriental river prawn MnATG3 mRNA expression in a time-dependent manner, suggesting that autophagy could protect crustaceans from hypoxia (Sun et al., 2019). Therefore, ATG3 downregulation in blood cancer is likely because oxygen is abundant in blood vessels, which increases the proliferation rate of tumor cells. Autophagy did not occur in this process because ATG3 might regulate blood tumors in an autophagy-independent manner. Overall, ATG3 regulators could be developed to modulate tumor progression; however, the lack of an effective assay to detect ATG3 activity is a current limitation that should be addressed in a further study.

Recently, researchers have found several autophagy-independent functions of ATG3, including its promotion of DNA damage–induced mitotic catastrophe (Liu Y. et al., 2005; Ma et al., 2017; Khalid et al., 2019). Such findings suggest that the functions of ATG3 are more complex. Owing to the functions of ATG3 in parasites and plants, it could be a potential target for protecting hosts from parasites and increasing crop yields. We anticipate the discovery of more functions of ATG3 in the future. Furthermore, we believe that the novel posttranslational modifications of ATG3 might be a good focal point for excavating its new functions.

AUTHOR CONTRIBUTIONS

DF conceived the review, consulted the literature, and drafted the figures and the manuscript. HX, TH, and HS consulted the literature and helped with the drawing and manuscript revision. ML came up with the idea and wrote the final paper with the feedback from all authors.

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ORF3a-Mediated Incomplete Autophagy Facilitates Severe Acute Respiratory Syndrome Coronavirus-2 Replication

Yafei Qu[1†], Xin Wang[1†], Yunkai Zhu[2†], Weili Wang[1†], Yuyan Wang[2], Gaowei Hu[2], Chengrong Liu[1], Jingjiao Li[1], Shanhui Ren[2], Maggie Z. X. Xiao[3], Zhenshan Liu[1], Chunxia Wang[4], Joyce Fu[5], Yucai Zhang[4], Ping Li[6]*, Rong Zhang[2]* and Qiming Liang[1,7,8]**

[1] Research Center of Translational Medicine, Shanghai Institute of Immunology, Shanghai Children's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2] Key Laboratory of Medical Molecular Virology (MOE/NHC/CAMS), School of Basic Medical Science, Shanghai Medical College, Biosafety Level 3 Laboratory, Fudan University, Shanghai, China, [3] Faculty of Medicine, University of Alberta, Edmonton, AB, Canada, [4] Department of Critical Care Medicine, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai, China, [5] Department of Statistics, University of California, Riverside, Riverside, CA, United States, [6] Key Laboratory for Food Microbial Technology of Zhejiang Province, Zhejiang Gongshang University, Hangzhou, China, [7] Key Laboratory of Cell Differentiation and Apoptosis of Chinese Ministry of Education, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [8] State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, Shanghai, China

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) is the causative agent for the coronavirus disease 2019 (COVID-19) pandemic and there is an urgent need to understand the cellular response to SARS-CoV-2 infection. Beclin 1 is an essential scaffold autophagy protein that forms two distinct subcomplexes with modulators Atg14 and UVRAG, responsible for autophagosome formation and maturation, respectively. In the present study, we found that SARS-CoV-2 infection triggers an incomplete autophagy response, elevated autophagosome formation but impaired autophagosome maturation, and declined autophagy by genetic knockout of essential autophagic genes reduces SARS-CoV-2 replication efficiency. By screening 26 viral proteins of SARS-CoV-2, we demonstrated that expression of ORF3a alone is sufficient to induce incomplete autophagy. Mechanistically, SARS-CoV-2 ORF3a interacts with autophagy regulator UVRAG to facilitate PI3KC3-C1 (Beclin-1-Vps34-Atg14) but selectively inhibit PI3KC3-C2 (Beclin-1-Vps34-UVRAG). Interestingly, although SARS-CoV ORF3a shares 72.7% amino acid identity with the SARS-CoV-2 ORF3a, the former had no effect on cellular autophagy response. Thus, our findings provide the mechanistic evidence of possible takeover of host autophagy machinery by ORF3a to facilitate SARS-CoV-2 replication and raise the possibility of targeting the autophagic pathway for the treatment of COVID-19.

Keywords: SARS-CoV-2, autophagy, ORF3a, UVRAG, COVID-19

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Edited by:
Xiaonan Dong,
The University of Texas Southwestern Medical Center, United States

Reviewed by:
Yuting Zhao,
University of Texas Southwestern Medical Center, United States
Luis Henrique Franco,
Federal University of Minas Gerais, Brazil

***Correspondence:**
Yucai Zhang
zyucai2018@163.com
Ping Li
ping-biology@outlook.com
Rong Zhang
rong_zhang@fudan.edu.cn
Qiming Liang
liangqiming@shsmu.edu.cn

† These authors have contributed equally to this work

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INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) is a novel coronavirus confirmed as the causative agent of coronavirus disease 2019 (COVID-19; Wu et al., 2020; Zhou et al., 2020). As of 27 May 2021, there have been more than 169 million confirmed cases of SARS-CoV-2 infection with more than 3.5 million deaths attributed to the virus in 235 countries and territories. The clinical presentation of symptoms of COVID-19 ranges from asymptomatic to acute respiratory distress syndrome. The majority of cases will be mild to moderate but individuals with underlying comorbidities are at higher risk for developing more serious complications including respiratory failure, shock, and multiorgan system dysfunction (Huang et al., 2020). While there continues to be unprecedented collaboration to facilitate the development of therapeutic neutralizing antibodies, vaccines, and small molecule drugs against COVID-19 (Florindo et al., 2020), only one FDA-approved drug [Veklury (Remdesivir)] is currently available and there is an urgent need to better understand how SARS-CoV-2 manipulates the host responses.

Severe Acute Respiratory Syndrome Coronavirus-2 is an enveloped, positive-sense, single-stranded RNA virus that engages human angiotensin-covering enzyme 2 (hACE2) to mediate host cell entry in the nasal passage, respiratory tract, and intestine (Hoffmann et al., 2020a). Infection with SARS-CoV-2 can lead to excessive production of pro-inflammatory cytokines and dysregulation of type I interferon response (Blanco-Melo et al., 2020; Hadjadj et al., 2020; Zhang et al., 2020). Quantitative proteomic analysis of lung epithelial (A549) cells infected with SARS-CoV-2 and peripheral blood mononuclear cell (PBMC) specimens of COVID-19 patients revealed perturbations of a broad range of cellular signaling pathways, including macroautophagy (Stukalov et al., 2021). Macroautophagy, hereafter referred to as autophagy, is a critical house-keeping process involving the formation of double-membrane autophagosomes that later fuse with lysosomes to degrade and recycle damaged organelles, unused proteins, and invading pathogens (Dikic and Elazar, 2018). In mammalian cells, this process is orchestrated by Beclin 1 (Levine et al., 2015), a scaffolding protein that regulates the lipid kinase Vps34 (PI3KC3) and interacts with several cofactors/adaptor proteins (Atg14 or UVRAG) to promote the formation of mutually exclusive Beclin 1-Vsp34 subcomplexes with distinct functions (Dikic and Elazar, 2018). The PI3KC3-C1 (Beclin 1-Vps34-Atg14) mainly positively regulates autophagy by promoting autophagosome formation while the PI3KC3-C2 (Beclin 1-Vps34-UVRAG) accelerates autophagosome maturation by promoting the fusion of autophagosomes with lysosomes to form autolysosomes (Levine et al., 2015). HOPS complexes and SNARE complexes are also involved in autophagosome maturation (Zhao and Zhang, 2019), and abrogation of either one of these three complexes will impair autophagosome maturation.

Autophagy is an important homeostatic mechanism for cell protection and is evolutionarily conserved from yeast to higher eukaryotes; however, a subset of viruses have evolved mechanisms to subvert and hijack the autophagic pathway to benefit their replication (Dong and Levine, 2013; Choi et al., 2018). For example, Zika virus utilizes NS4A and NS4B to inhibit the Akt-mTOR signaling pathway, leading to aberrant activation of autophagy and increased viral replication (Liang et al., 2016); Kaposi's sarcoma-associated herpesvirus vBcl2, vFLIP, and K7 proteins serve as anti-autophagy molecules to inhibit autophagosome formation or maturation via targeting Beclin 1, Atg3, and Rubicon, respectively (Liang et al., 2008a, 2013; Lee et al., 2009; Gao et al., 2016); and herpes simplex virus 1 encodes protein ICP34.5 which binds to Beclin 1 and inhibits its autophagy function (Orvedahl et al., 2007). Studies on other beta-coronaviruses show that these viruses modulate autophagy (Miller et al., 2020; Zhao et al., 2021) but it is unknown whether viral evasion of autophagy is important in SARS-CoV-2 infection.

In this study, we found that SARS-CoV-2 infection or ORF3a expression triggers incomplete autophagy, resulting in elevated autophagosome formation but blockage of autophagosome maturation. By yeast two-hybrid screening, we found that SARS-CoV-2 ORF3a targets autophagy protein UVRAG to modulate Beclin 1 complexes by disrupting PI3KC3-C2 (Beclin 1-Vps34-UVRAG) but facilitating PI3KC3-C1 (Beclin 1-Vps34-Atg14). Importantly, SARS-CoV ORF3a does not share the autophagy modulatory activity with SARS-CoV-2 ORF3a. Our study not only highlights the role of ORF3a in autophagy modulation during SARS-CoV-2 infection but also raises the possibility of targeting the cellular autophagy for blocking SARS-CoV-2 replication.

RESULTS

SARS-CoV-2 Infection Induces Incomplete Autophagy

Severe Acute Respiratory Syndrome Coronavirus-2 can infect multiple hACE2-expressing cell lines and in refractory cell lines after exogenous hACE2 expression; however, it is unclear if SARS-CoV-2 infection modulates cellular autophagy response. To evaluate autophagic activity upon SARS-CoV-2 infection, we measured microtubule-associated protein light chain 3 (LC3) conversion (LC3-I to lipidated LC3-II), which is correlated with the formation of autophagosomes and is widely used as a marker to monitor autophagy (Klionsky et al., 2016). However, LC3-II is degraded together with the contents of the autophagosome and increased LC3-II may reflect either increased autophagosome formation or, alternatively, decreased autolysosome degradation (i.e., due to impairment of fusion between autophagosomes and lysosomes). Thus, it is important to measure SQSTM1/p62 turnover to evaluate autophagic flux (Klionsky et al., 2016). In stable hACE2 expressing HeLa cells infected with SARS-CoV-2 strain SH01, we detected dramatic conversion of LC3-I to LC3-II in the course of SARS-CoV-2 infection (**Figure 1A**), leading to accumulation of lipidated LC3-II. In addition, SQSTM1/p62 increased during the course of SARS-CoV-2 infection (**Figure 1A**), which strengthens the interpretation that infection blocked autophagosome-lysosome fusion and infected cells cannot form autolysosomes that degrade or recycle their contents. Similar LC3-I to LC3-II conversion and

SQSTM1/p62 stability were also observed in infected Vero-E6 cells (**Figure 1B**), suggesting SARS-CoV-2 induces incomplete autophagy in infected cells.

SARS-CoV-2 ORF3a Expression Induces Incomplete Autophagy

Since SARS-CoV-2 infection triggers an incomplete autophagy response in multiple cell lines, we attempted to determine which viral components could modulate the cellular pathways that control this process. The RNA genome of SARS-CoV-2 encodes 28 viral proteins, including 16 non-structural proteins (nsp1 to nsp16), four structural proteins [glycoprotein spike (S), membrane (M), envelope (E), and nucleocapsid (N)], and 8 accessory proteins (ORF3a, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10). We screened each individual SARS-CoV-2 gene for LC3 conversion in HeLa cells and found that ORF3a expression resulted in a significant increase in the conversion of LC3-I to LC3-II (**Figure 1C**). However, other viral proteins had little or no effect (**Figure 1C**). Similar findings were recapitulated when ORF3a was expressed in HeLa cells in the presence and absence of bafilomycin A1 (**Figures 1D,E**), suggesting ORF3a itself can efficiently induce autophagosome accumulation. Similarly, ORF3a expression facilitated LC3-I to LC3-II conversion in Vero-E6 cells (**Figure 1F**). We also observed that SARS-CoV-2 ORF3a expression increased the SQSTM1/p62 level (**Figure 1C**), indicating the fusion step between autophagosome and lysosome (autophagosome maturation) is blocked. Indeed, starvation-mediated SQSTM1/p62 degradation was also abolished by ORF3a in HeLa cells (**Figure 1G**), indicating that ORF3a expression activates autophagy but suppressing autophagic degradation. Consistently, expression of ORF3a led to dramatically elevation of LC3 puncta per cell in HeLa cells with or without Bafilomycin A1 treatment (**Figures 1H,I**), confirming the efficient induction of autophagosome formation. In addition, we utilized tandem fluorescent-tagged LC3 (mRFP-GFP-LC3) to differentiate between autophagosomes and autolysosomes upon SARS-CoV-2 ORF3a expression. Since GFP is unstable in the acidic environment of lysosome, unmatured autophagosomes, and matured autolysosomes are labeled by yellow LC3 puncta ($GFP^{+}mRFP^{+}$) or red LC3 puncta ($GFP^{-}mRFP^{+}$), respectively (Kimura et al., 2007). Although SARS-CoV-2 ORF3a increased matured autolysosome (red puncta), it promoted unmatured autophagosome (yellow puncta) to a higher level (**Figures 1J,K**), indicating that ORF3a triggers autophagy but also inhibits the fusion between autophagosomes and lysosomes. Taken together, our findings suggest that ORF3a efficiently triggers incomplete autophagy in multiple cell lines, which phenocopies SARS-CoV-2 infection.

SARS-CoV-2 ORF3a Interacts With UVRAG to Reshape PI3KC3 Complexes

Next, we sought to determine the mechanism through which SARS-CoV-2 ORF3a induces incomplete autophagy. In recent months, four independent virus-host interactomes analyzed by network based-approach provided insights into viral pathogenesis (Gordon et al., 2020a,b; Li et al., 2021; Stukalov et al., 2021), but it remains unclear how SARS-CoV-2 interacts with the cellular autophagy machinery to trigger incomplete autophagy. Using yeast two-hybrid screening with SARS-CoV-2 ORF3a as a bait, we captured a novel protein-protein interaction with UVRAG, a key autophagy regulator that activates PI3KC3 complex to promote autophagosome maturation and suppresses the proliferation of human colon cancer cells (Liang et al., 2006, 2008b). SARS-CoV-2 ORF3a interacted with UVRAG in yeast (**Figure 2A**). Among Beclin 1 complex components, UVRAG showed the strongest binding affinity to ORF3a in HEK293T cells (**Figure 2B**). Detailed mapping suggested that ORF3a interacted with the N-terminal C2 and coiled coil domain (CCD) of UVRAG (**Figures 2C,D**). The CCD provides a platform for many important protein interactions and is required for UVRAG binding to Beclin 1 (Liang et al., 2006). ORF3a also bound to and co-localized with endogenous UVRAG in HeLa cells (**Figures 2E,F**), further confirming UVRAG is the potential host target for SARS-CoV-2 ORF3a. Beclin 1 is an essential autophagy protein that orchestrates the assembly of functionally distinct PI3KC3 multiprotein complexes that regulate autophagosome formation and maturation (Levine et al., 2015). The core complex consists of Beclin 1, Vps34, and Vps15 which interacts with key modulators Atg14 and UVRAG to form mutually exclusive Atg14- or UVRAG-containing PI3KC3 subcomplexes that positively regulates autophagy by promoting early-stage autophagosome formation and late-stage autophagosome maturation, respectively (Levine et al., 2015). Since ORF3a binds to UVRAG and triggers incomplete autophagy, we speculated that ORF3a expression may lead to aberrant redistribution of Atg14- or UVRAG-containing PI3KC3 complexes and disrupt the balance between autophagosome formation and maturation. To test our hypothesis, we purified the Beclin 1 complex, UVRAG complex, and Atg14 complex, respectively, in the presence and absence of SARS-CoV-2 ORF3a. In line with previous reports, Atg14 and UVRAG were not present in the same complex and Rubicon is only associated with PI3KC3-C2 in the absence of ORF3a (Matsunaga et al., 2009; Zhong et al., 2009; **Figures 2G–I**). In contrast, the presence of ORF3a significantly reduced the interaction between UVRAG and Beclin 1 or other Beclin 1 complex components, such as Vps34 and Rubicon, suggesting that PI3KC3-C2 formation is disrupted by SARS-CoV-2 ORF3a (**Figures 2G,H**). Furthermore, SARS-CoV-2 ORF3a expression enhanced the interaction between Atg14 and Beclin 1, suggesting dissociation between Beclin 1 and UVRAG facilitated PI3KC3-C1 assembly (**Figures 2G,I**). Taken together, these findings demonstrate that the ORF3a-UVRAG interaction renders UVRAG less competitive than Atg14 for Beclin 1 binding and shifts the balance between PI3KC3-C1 and PI3KC3-C2, resulting in more efficient autophagosome formation but inhibition of autophagosome maturation.

Autophagy Deficiency Decreases SARS-CoV-2 Replication

Since SARS-CoV-2 infection or ORF3a expression modulates cellular autophagy pathway, we next analyzed the effect of

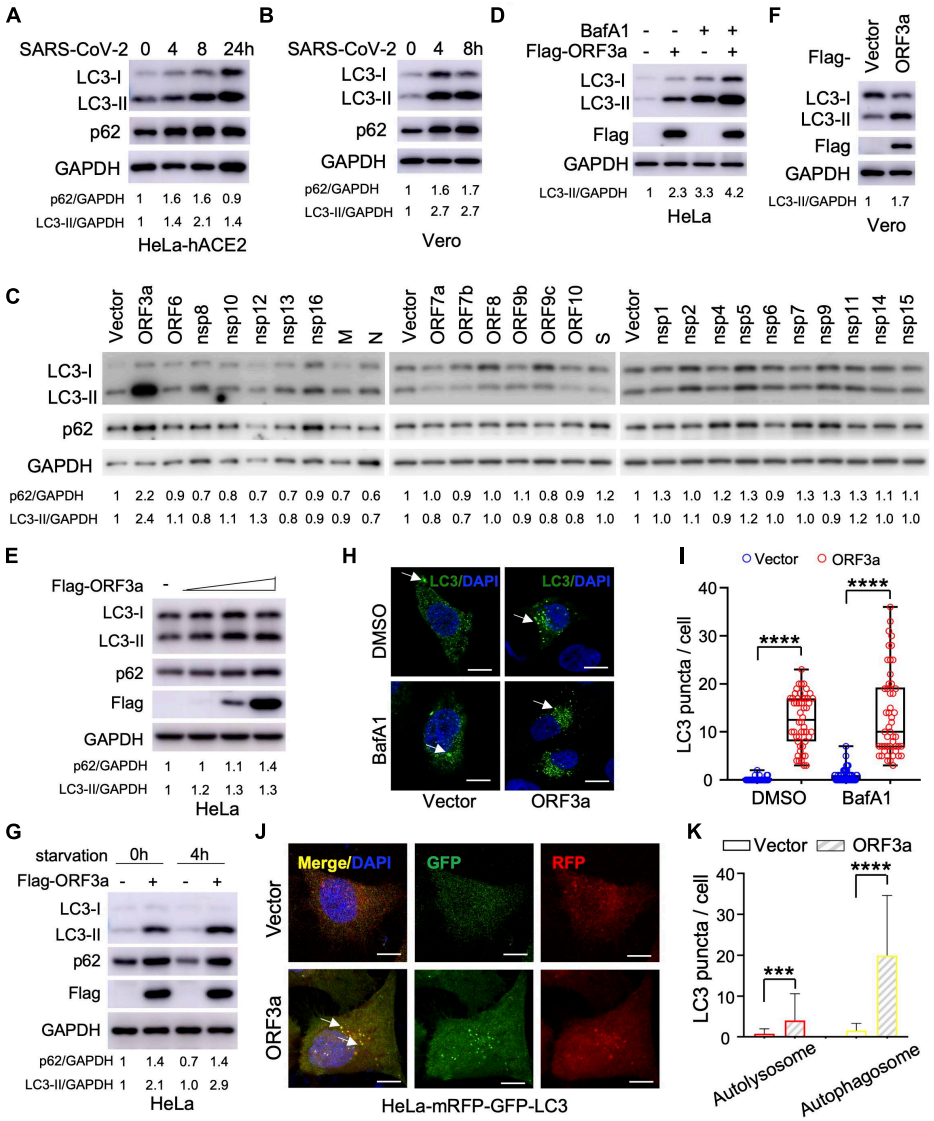

FIGURE 1 | Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) ORF3a triggers incomplete autophagy. **(A–B)** SARS-CoV-2 infection triggers incomplete autophagy. HeLa-hACE2 **(A)** or Vero-E6 **(B)** cells were infected with SARS-CoV-2 (MOI = 1) and cell lysates were collected at indicated time point post-infection for immunoblotting (IB) with indicated antibodies. **(C)** Screening of SARS-CoV-2-encoded proteins for LC3-I to LC3-II conversion in HeLa cells. **(D,E)** SARS-CoV-2 ORF3a induces incomplete autophagy. HeLa-vector and HeLa-ORF3a stable cells were treated with or without BafA1 (100 nM) for 4 h and the cell lysates were collected for IB with indicated antibodies **(D)**. HeLa cells were transfected with increasing amount ORF3a expressing plasmid and the cell lysates were collected for IB with indicated antibodies at 48 post-transfection **(E)**. **(F)** ORF3a expression modulates autophagy in Vero-E6 cells. **(G)** ORF3a blocks SQSTM1/p62 degradation upon starvation. HeLa-vector or HeLa-ORF3a stable cells were treated by serum starvation for 4 h and the cell lysates were collected for IB with indicated antibodies. **(H,I)** SARS-CoV-2 ORF3a induces LC3 puncta formation. HeLa-vector or HeLa-ORF3a cell line were treated with BafA1 (100 nM) and endogenous LC3 puncta were immunostained **(H)** and quantified **(I)**. Scale bar, 15 μm. Arrow: representative autophagosomes. **(J,K)** HeLa-mRFP-GFP-LC3 stable cells were transfected with ORF3a or vector control and transfected cells were fixed and the LC3 puncta were captured **(J,K)** quantified as indicated. Yellow puncta, autophagosomes; Red puncta, autolysosomes; Arrow, representative autophagosomes. p62/GAPDH or LC3-II/GAPDH levels were quantified by the band intensity in **(A–G)**. Similar results were obtained by three independent experiments. Mean ± SEM; $n = 50$; ***$p < 0.001$ and ****$p < 0.0001$ by Student's t test in panels **(I,K)**.

autophagy on SARS-CoV-2 replication using cell lines with genetic abrogation of autophagic essential genes. Atg3 and Atg5 are two indispensable proteins that mediate vesicle elongation during the autophagosome formation process and genetic knockout of Atg3 or Atg5 blocks LC3 conversion and SQSTM1/p62 degradation in MEFs (Dikic and Elazar, 2018; **Figure 3A**). Using MEF stable expressing hACE2 receptor, we investigated how abrogation of cellular autophagy response by Atg3 or Atg5 knockout affects SARS-CoV-2 replication. As shown in **Figures 3B–E**, the autophagy machinery is required for efficient SARS-CoV-2 replication, and infection of autophagy-deficient cells resulted a significant reduction in viral yield

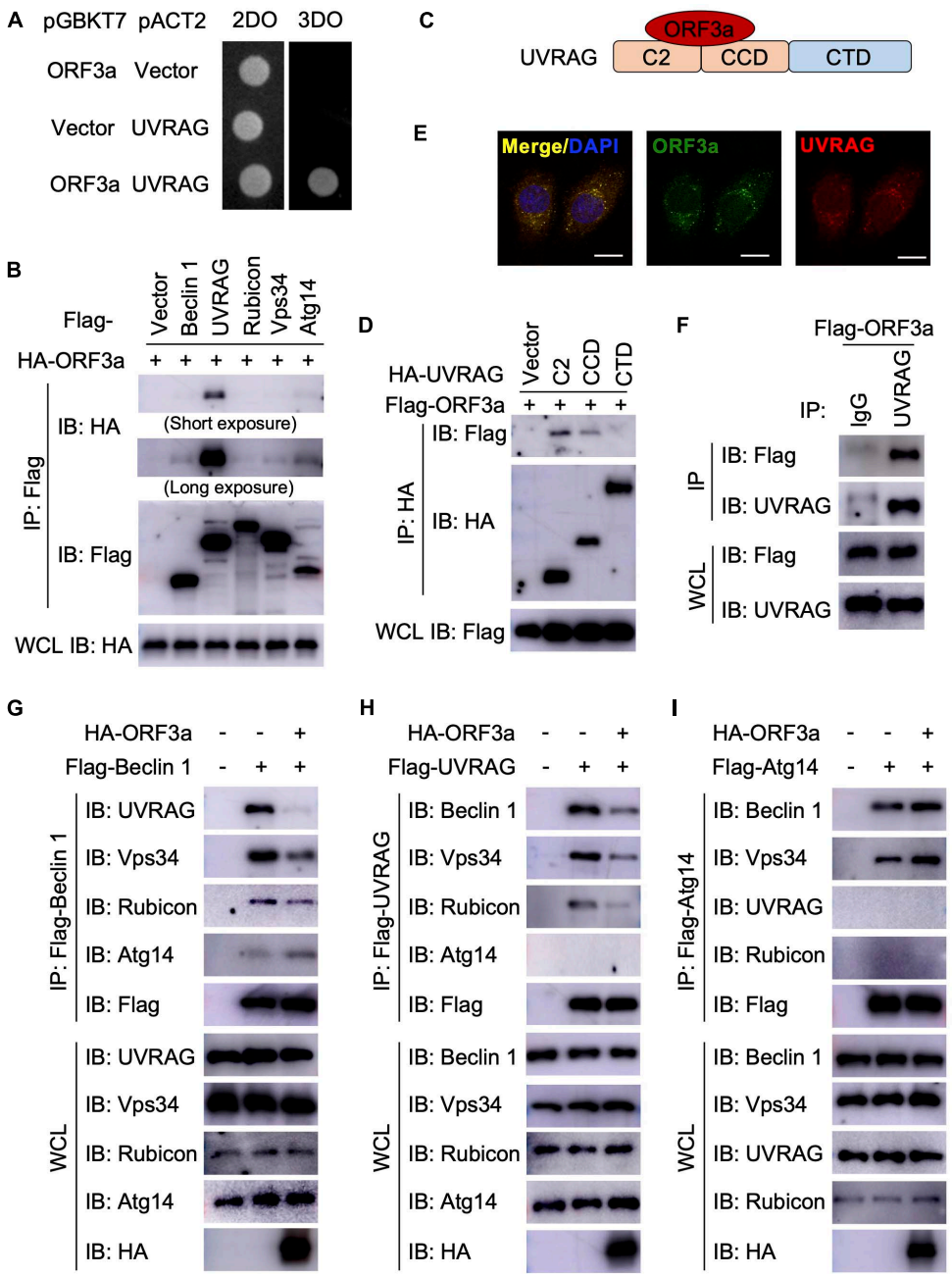

FIGURE 2 | Severe Acute Respiratory Syndrome Coronavirus-2 ORF3a targets UVRAG to modulate PI3KC3 complex formation. **(A)** SARS-CoV-2 ORF3a interacts with UVRAG in yeast two-hybrid system. **(B)** SARS-CoV-2 ORF3a binds to UVRAG in HEK293T cells. HEK293T cells were co-transfected with indicated plasmids and cell lysates were collected and subjected to immunoprecipitation (IP) and IB with indicated antibodies at 48 h post-transfection. **(C)** Schematic diagram of SARS-CoV-2 ORF3a-UVRAG interaction. **(D)** The C2 and CCD of UVRAG bind to SARS-CoV-2 ORF3a. **(E)** SARS-CoV-2 ORF3a co-localizes with endogenous UVRAG. HeLa-ORF3a stable cells were subjected to immunostaining with antibodies against Flag or UVRAG. Scale bar, 15 μm. **(F)** SARS-CoV-2 ORF3a binds to endogenous UVRAG in HEK293T cells. **(G–I)** SARS-CoV-2 ORF3a blocks PI3KC3-C2 but facilitates PI3KC3-C1. HEK293T cells were co-transfected with indicated plasmids and cell lysates were collected and subjected to IP and IB with indicated antibodies at 48h post-transfection.

compared to yield in control cells, as shown by immunoblot analyses of viral N protein expression (**Figures 3B,C**) and quantitative RT-PCR of viral transcripts (**Figures 3D,E**). To determine whether ORF3a affects SARS-CoV-2 replication, we infected Calu3-vector and Calu3-ORF3a stable cells with SARS-CoV-2 and evaluated virus replication. Compared to vector control, ORF3a expression significantly increased SARS-CoV-2 replication (**Figures 3F,G**). Collectively, these results demonstrated that cellular autophagy response is required for efficient SARS-CoV-2 replication.

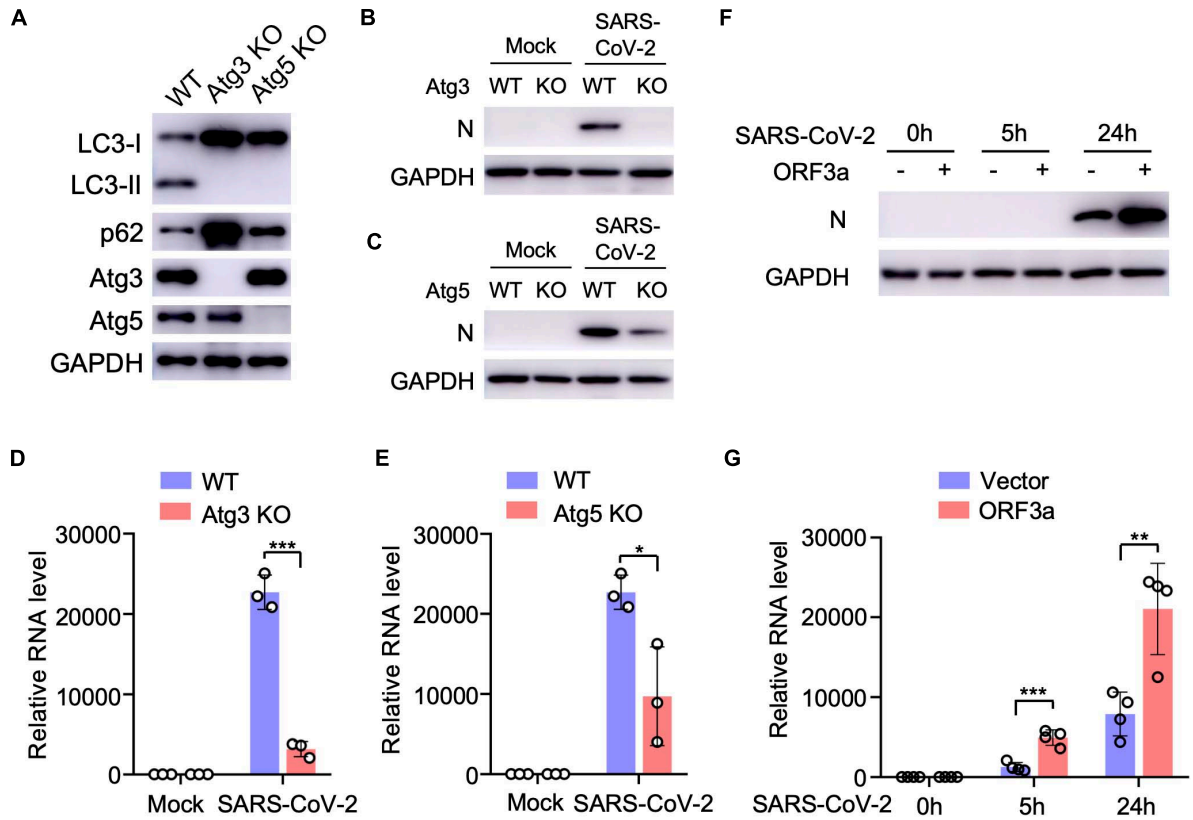

FIGURE 3 | Cellular autophagy is required for efficient SARS-CoV-2 replication. **(A)** Genetic abrogation of Atg3 or Atg5 blocks autophagy response in MEF-hACE2 cell lines. **(B–E)** Atg3 or Atg5 KO reduces SARS-CoV-2 replication efficiency in MEF-hACE2 stable cells. MEF-hACE2^{WT}, MEF-hACE2^{Atg3KO}, or MEF-hACE2^{Atg5KO} were infected with SARS-CoV-2 (MOI = 1) and virus replication was examined by IB of viral N protein **(B,C)** or quantitative RT-PCR of viral transcripts **(D,E)**. **(F,G)** ORF3a expression facilitates SARS-CoV-2 replication. Calu3-vector or Calu3-ORF3a stable cells were infected with SARS-CoV-2 (MOI = 1) and virus replication was examined by IB of viral N protein **(F)** or quantitative RT-PCR of viral transcripts **(G)**. Mean ± SEM; $n = 3$ **(D,E)** or $n = 4$ **(G)**; ns, not significant, $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ by Student's *t* test.

SARS-CoV ORF3a Does Not Interact With UVRAG and Has No Effect on Autophagy

The genome of SARS-CoV-2 is closely related to SARS-CoV, the first deadly coronavirus that caused the SARS epidemic in 2002–2003 (Zhong et al., 2003). Since ORF3a$^{SARS\text{-}CoV}$ shares 72.7% amino acid sequence identity to ORF3a$^{SARS\text{-}CoV\text{-}2}$ (**Figure 4A**), we sought to determine whether the ORF3a homolog from SARS-CoV could trigger similar incomplete autophagy. We generated a stable HeLa cell line that expresses ORF3a$^{SARS\text{-}CoV}$ and examined its effects on autophagy markers, including LC3-I to LC3-II conversion and LC3 puncta formation. Unlike ORF3a$^{SARS\text{-}CoV\text{-}2}$, ORF3a$^{SARS\text{-}CoV}$ expression led to neither dramatic increase in the amount of LC3-II nor accumulation of LC3 puncta (**Figures 4B–E**), indicating ORF3a$^{SARS\text{-}CoV}$ cannot efficiently induce the formation of autophagosomes or suppress autophagosome maturation. Furthermore, ORF3a$^{SARS\text{-}CoV}$ did not interact with endogenous UVRAG (**Figure 4F**) and could not modulate the formation of PI3KC3-C1 or PI3KC3-C2 (**Figures 4F–H**). These results show that ORF3a-mediated modulation of autophagy response is a unique signature of SARS-CoV-2 infection and may play an important role in controlling viral replication and pathogenesis.

DISCUSSION

Many coronaviruses trigger autophagy in infected cells (Brest et al., 2020; Mijaljica and Klionsky, 2020), however, how SARS-CoV-2 modulates cellular autophagy and whether autophagy affects SARS-CoV-2 replication remain elusive. In this study, we found that ORF3a triggers incomplete cellular autophagy to generate double-membrane autophagosome vesicles, which are required for efficient SARS-CoV-2 replication. Mechanistically, SARS-CoV-2 ORF3a interacts with UVRAG to positively regulate PI3KC3-C1 but suppress the PI3KC3-C2, thus elevating autophagosome formation and blocking autophagosome maturation (**Figure 5**). Knockout of Beclin 1 or Vps15 fully blocked SARS-CoV-2 replication in Huh7.5.1 cells (Wang et al., 2021), supporting the role of autophagosome formation is required for SARS-CoV-2 replication. Since ORF3a silenced UVRAG activity on autophagy by inhibiting PI3KC3-C2, knockout of UVRAG had little effect on SARS-CoV-2 replication

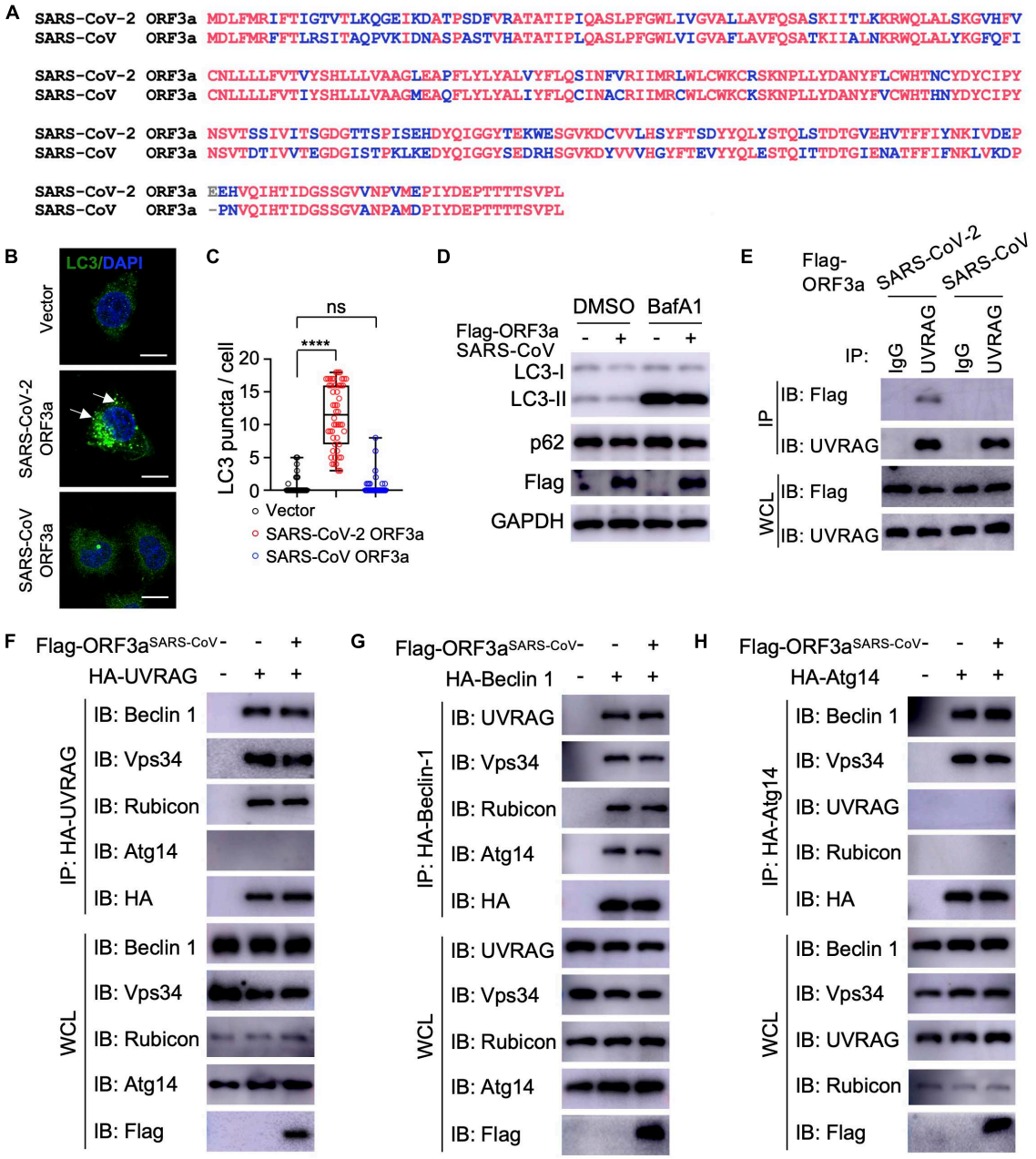

FIGURE 4 | SARS-CoV ORF3a cannot induce autophagy. **(A)** Sequence alignment between SARS-CoV-2 ORF3a and SARS-CoV ORF3a. **(B–D)** SARS-CoV ORF3a does not affect autophagy. HeLa-vector, HeLa-ORF3a$^{SARS\text{-}CoV\text{-}2}$, or HeLa-ORF3a$^{SARS\text{-}CoV}$ cell lines were treated with BafA1 (100 nM) and endogenous LC3 puncta were immunostained **(B)** and quantified **(C)**. Scale bar, 15 μm. Arrow: representative autophagosomes. Mean ± SEM; $n = 50$; *ns* and $^{****}p < 0.0001$ by one-way ANOVA and Bonferroni's *post hoc* test. HeLa-vector or HeLa-ORF3a$^{SARS\text{-}CoV}$ cell lines were treated with BafA1 (100 nM) for 4 h and the cell lysates were collected for IB with indicated antibodies **(D)**. **(E)** ORF3a$^{SARS\text{-}CoV\text{-}2}$ but not ORF3a$^{SARS\text{-}CoV}$ interacts with endogenous UVRAG. HEK293T cells were transfected with Flag-ORF3a$^{SARS\text{-}CoV}$ or Flag-ORF3a$^{SARS\text{-}CoV\text{-}2}$ and cell lysates were collected and subjected to IP and IB with indicated antibodies at 48h post-transfection. **(F–H)** SARS-CoV ORF3a does not affect the formation of UVRAG complex **(F)**, Beclin 1 complex **(G)**, or Atg14 complex **(H)**. HEK293T cells were co-transfected with indicated plasmids and cell lysates were collected and subjected to IP and IB with indicated antibodies at 48 h post-transfection.

(Wang et al., 2021). Recent study showed that SARS-CoV-2 ORF3a blocks autophagosome maturation by blocking HOPS-mediated SNARE complex assembly (Miao et al., 2021; Zhang et al., 2021), suggesting ORF3a may target multiple protein complexes required for autophagosome-lysosome fusion to facilitate SARS-CoV-2 replication. Given autophagy's role in the innate antiviral immune response to restrict infecting pathogens, it is not surprising that viruses are in a constant arms race to remodel the autophagic membranes for their own benefit during replication (Choi et al., 2018). SARS-CoV-2

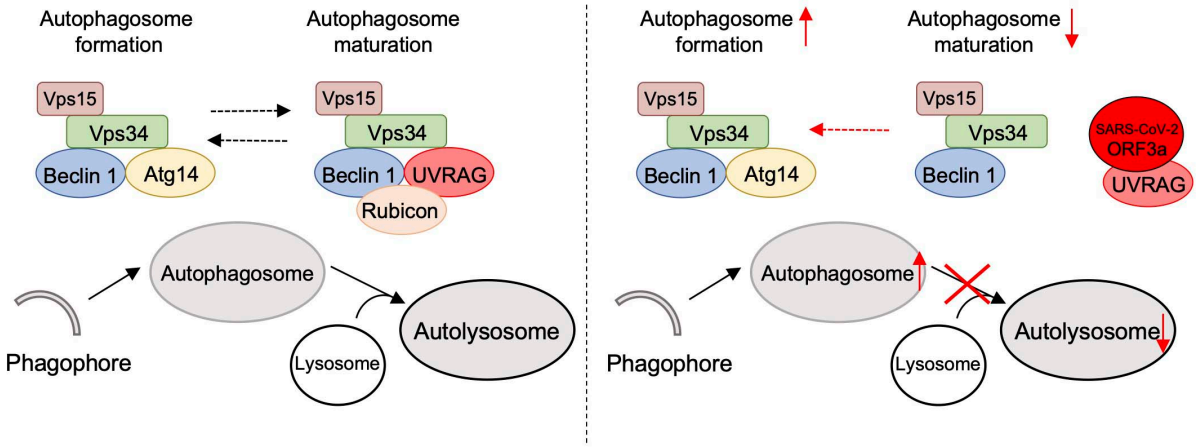

FIGURE 5 | Schematic diagram of SARS-CoV-2 ORF3a-mediated incomplete autophagy. SARS-CoV-2 ORF3a interacts with UVRAG to positively regulate PI3KC3-C1 but suppress the PI3KC3-C2, leading to elevated autophagosome formation but blockage of autophagosome maturation.

ORF3a hijacks the autophagy machinery to generate double-membrane autophagosome vesicles to facilitate viral replication but arrests the autophagosomes prior to lysosome fusion to avoid succumbing to lysosomal degradation. Interestingly, although ORF3a$^{SARS\text{-}CoV}$ shares 72.7% amino acid identity with the ORF3a$^{SARS\text{-}CoV\text{-}2}$, the former had no effect on the formation of double-membrane autophagosome vesicles. Coronaviruses are known to rely on the formation of convoluted autophagosome-like double membrane vesicles for optimal replication (Brest et al., 2020; Wolff et al., 2020), and ORF3a-mediated autophagosome formation may be one source of these double-membrane replication organelles. Although SARS-CoV ORF3a has no impact on cellular autophagy, nsp6 of SARS-CoV has been shown to limit autophagosome expansion (Cottam et al., 2014). Actually, nsp6 homologs from multiple coronaviruses, such as avian coronavirus, mouse hepatitis virus, SARS-CoV, and SARS-CoV-2, affect autophagosome membrane expansion, resulting in small autophagosome (Cottam et al., 2014). Besides nsp6 and ORF3a, ORF7a, and M of SARS-CoV-2 also inhibit autophagy at different levels, while within SARS-CoV-2-encoded proteins, ORF3a shows the most potent capability on autophagy modulation (Miao et al., 2021; Zhang et al., 2021). Manipulation of cellular autophagy pathway is a common signature of coronaviruses and ORF3a is a potential target to limit SARS-CoV-2 replication.

Veklury (Remdesivir) has just been approved for the treatment of SARS-CoV-2 infection and several preclinical investigations repurposing several FDA-approved drugs are underway. Since elevated autophagy facilitates coronaviruses replication efficiency (Brest et al., 2020), the drugs, such as rapamycin (Stukalov et al., 2021), that enhance autophagy should be avoided throughout the course of treatment. Antimalarial drugs chloroquine (CQ) and hydroxychloroquine are lysosomotropic agents that inhibit the pH-dependent replication steps in several viruses and many publications have urged the use of CQ as a potential treatment for COVID-19 based on *in vitro* considerations (Savarino et al., 2003; Colson et al., 2020; Devaux et al., 2020; Gao et al., 2020; Maisonnasse et al., 2020). However, there are still discrepancies and concerns on the sensitivity and therapeutic range of CQ as its effectiveness on limiting SARS-CoV-2 replication did not extend to TMPRSS2-expressing human lung cells (Hoffmann et al., 2020b), suggesting the cell specific responses may exist. In addition, CQ-mediated inhibition of cellular autophagy response may also contribute to its effects on SARS-CoV-2 (Klionsky et al., 2016). In summary, our work highlights the mechanism of how SARS-CoV-2 co-opts the autophagy pathway to enhance its own replication and spread, and raises the possibility of targeting the autophagic pathway for the treatment of COVID-19.

MATERIALS AND METHODS

Viruses, Plasmids, and Cell Culture

Severe Acute Respiratory Syndrome Coronavirus-2 strain SH01 (GenBank: MT121215.1) was described previously (Zhu et al., 2021). SARS-CoV-2 stocks were propagated in Vero-E6 cells and the titer of SARS-CoV-2 stocks were determined by standard plaque assay on Vero-E6 cells as described previously (Liang et al., 2014). Experiments related to SARS-CoV-2 infection are performed in BSL-3 laboratory in Fudan University.

Severe Acute Respiratory Syndrome Coronavirus-2 genes in pLVX-3xFlag-MCS-P2A-tagRFP (puro) have been described previously (Li et al., 2021). ORF3a$^{SARS\text{-}CoV\text{-}2}$ were subcloned into pEF-MCS-3xHA, pCDH (Hygro), or pGBKT7 vectors. The ORF3a$^{SARS\text{-}CoV}$ template was kindly provided by Dr. Peihui Wang (Shandong University, China) and cloned into pLVX vector. The constructs encoding Beclin-1, Atg14, Vsp34, UVRAG, and Rubicon have been previously described (Liang et al., 2013). All constructs were sequenced using an ABI PRISM 377 automatic DNA sequencer to verify 100% correspondence with the original sequence.

HEK293T (ATCC, #CRL-11268), HeLa (ATCC, #CCL-2), A549 (ATCC, #CCL-185), and MEF (wild-type, Atg3 KO, or Atg5 KO, gift from Dr. Qing Zhong at Shanghai Jiao Tong University

School of Medicine, China) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) containing 4 mM glutamine and 10% FBS. Vero-E6 cells were cultured in DMEM with 5% FBS and 4 mM glutamine. Calu-3 cells were cultured in DMEM with 20% FBS, 2 mM glutamine, 10 mM HEPES, and MEM Non-Essential Amino Acids. Transient transfections were performed with Lipofectamine 3000 (Thermo Fisher Scientific, #3000015). HeLa stable cell lines expressing SARS-CoV-2 proteins were generated using a standard lentivirus infection and selection protocol with puromycin (2 μg/ml) or hygromycin (200 μg/ml) for the expression of SARS-CoV-2 genes (Liang et al., 2016). Due to the low expression levels of nsp3 or E in HeLa, we excluded these two viral proteins in autophagy phenotype screening.

Detection of Autophagy

For the LC3 conversion assay, SARS-CoV-2-infected or ORF3a expressing cells were washed with cold PBS, lysed with 1% Triton X-100, and then subjected to immunoblot analysis (15% SDS-PAGE) with antibodies against LC3 (Cell Signaling, #3868) or SQSTM1/p62 (Cell Signaling, #39749). LC3-I is about 16 kD and lipidated LC3 (LC3-II) is about 14 kD. p62/GAPDH or LC3-II/GAPDH levels were quantified by the band intensity from immunoblotting. SARS-CoV-2-infected or ORF3a expressing cells were fixed and endogenous LC3 was detected by confocal microscope. Briefly, cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with Triton X-100 (0.2%) for 10 min, blocked with 1% BSA for 30 min, and immunostained with a primary antibody against LC3 (Cell Signaling, #3868) overnight at 4°C, followed by a fluorescent secondary antibody for 2 h at room temperature. Nuclei were stained by DAPI (Thermo Fisher Scientific, #62248) and images of cells were collected with Leica TCS SP8 confocal microscope. To quantitate LC3-positive autophagosomes per cell, cells from five random fields (>50 cells) were counted. The number of LC3 puncta per cells was counted manually in three independent experiments. Similar results were obtained by three independent experiments.

Immunoprecipitation and Immunoblot Analysis

For co-immunoprecipitation, 2×10^6 HEK293T cells were transfected with 20 μg of plasmid at a confluency of 90% with Lipofectamine 3000 (Thermo Fisher Scientific, #3000015). The cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in a whole cell lysis buffer (WCL) containing [50 mM *Tris*·HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol, protease inhibitor cocktail (Roche)] for 20 min on ice at 48 h post-transfection. The cell lysates were then centrifuged at 15,000 rpm for 15 min and the clear supernatants were subjected to immunoprecipitation with anti-Flag M2 agarose resin (Sigma, #F2426) or anti-HA agarose resin (Sigma, #E6779) following the manufacturer's instruction. After 4h incubation at 4°C, the beads were washed three times with WCL and twice with PBS, and then boiled with the 2 × loading buffer for 10 min. The immunoprecipitants were applied to standard immunoblotting analyses with indicated specific antibodies.

For immunoblotting, cell lysates were collected in WCL and separated by SDS-PAGE and transferred to PVDF membrane (Bio-Rad) by semi-dry transfer at 25 V for 30 min. All membranes were blocked in 5% milk in PBST and probed overnight with indicated antibodies in 5% BSA at 4°C. Primary antibodies included: mouse Flag (Sigma, #F1804), rat Flag-HRP (Biolegend, #637311), mouse HA (BioLegend, #901515), rabbit SQSTM1/p62 (Cell Signaling, #39749), rabbit LC3 (Cell Signaling, #3868), rabbit Beclin-1 (Cell Signaling, #3495), rabbit UVRAG (Cell Signaling, #13115), rabbit Atg14 (Cell Signaling, #96752), rabbit Rubicon (Cell Signaling, #8465), rabbit Vps34 (Cell Signaling, #4263), rabbit Atg3 (Cell Signaling, #3415), rabbit Atg5 (Cell Signaling, #12994), mouse SARS-CoV-2 N (GeneTex, #GTX635689), and mouse GAPDH (Santa Cruz, #365062). Appropriate HRP-conjugated secondary antibodies were incubated on membranes in 5% milk and bands were developed with ECL reagent (Thermo Scientific) and imaged on a Fuji LAS-4000 imager.

Yeast Two-Hybrid Assay

Yeast two-hybrid interaction assay was performed as previously described (Liang et al., 2018; Li et al., 2021). Briefly, Y2HGold Yeast Strain (Clontech, #630498) that contains four integrated reporter genes under the control of three distinct Gal4-responsive promoters are used to detect two-hybrid interactions. pGBKT7 (DNA binding domain) and pACT2 (activation domain) constructs were simultaneously transformed using Yeastmaker^{TM} Yeast Transformation System 2 (Clontech, #63039) following the manufactory manual. Transformed yeast cells were washed by sterile water one time and resuspended in sterile water and spotted onto -2DO plates (SD/-Leu/-Trp dropout medium) to assess transformation efficiency and onto -3DO plates (SD/-Ade/-Leu/-Trp selection medium) to evaluate the potential interactions. Plates were incubated for 4–6 days at 30°C for the positive clone growth. All constructs were tested for autoactivating properties to confirm no autonomous activation on the reporter genes. All the positive interactions were confirmed by at least three technical replicates.

RNA Extraction and Quantitative RT-PCR

Total RNA was isolated from cells with the RNeasy Mini Kit (Qiagen, #74106) and treated with RNase-free DNase according to the manufacturer's protocol. All gene transcripts were quantified by quantitative PCR using qScript^{TM} One-Step qRT-PCR Kit (Quanta Biosciences, #95057-050) on CFX96 real-time PCR system (Bio-Rad). Primer sequences for qPCR were as follow: SARS-CoV-2-N forward: GACCCCAAAATCAGC GAAAT, SARS-CoV-2-N reverse: TCTGGTTACTGCCAGTTG AATCTG; 18S forward: GTAACCCGTTGAACCCCATT, 18S reverse: CCATCCAATCGGTAGTAGCG.

Quantification and Statistical Analysis

All data were expressed as Mean ± SEM as indicated. Statistical significance across two groups was tested by Student's *t*-test;

one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test were used to determine statistically significant differences between multiple groups. *P*-values of less than 0.05 were considered significant.

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 authors.

AUTHOR CONTRIBUTIONS

QL, RZ, PL, and YCZ conceived of the research, designed the study, and wrote the manuscript. YQ, XW, CL, JL, ZL, and WW performed the experiments and analyzed the data. YKZ, YW, GH, and SR performed the SARS-CoV-2 infection experiments in BSL-3 Laboratory. MX helped with visual representation of data and edited the manuscript. All authors commented on the manuscript.

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Rubicon in Metabolic Diseases and Ageing

Satoshi Minami[1], *Shuhei Nakamura*[1,2,3]* *and Tamotsu Yoshimori*[1,2,4]*

[1]Department of Genetics, Graduate School of Medicine, Osaka University, Suita, Japan, [2]Department of Intracellular Membrane Dynamics, Graduate School of Frontier Biosciences, Osaka University, Suita, Japan, [3]Institute for Advanced Co-Creation Studies, Osaka University, Suita, Japan, [4]Integrated Frontier Research for Medical Science Division, Institute for Open and Transdisciplinary Research Initiatives, Osaka University, Suita, Japan

Autophagy is a conserved cellular degradation system that maintains intracellular homeostasis. Cytoplasmic components are engulfed into double-membrane vesicles called autophagosomes, which fuse with lysosomes, and resulting in the degradation of sequestered materials. Recently, a close association between autophagy and the pathogenesis of metabolic diseases and ageing has become apparent: autophagy is dysregulated during metabolic diseases and ageing; dysregulation of autophagy is intimately associated with the pathophysiology. Rubicon (Run domain Beclin-1 interacting and cysteine-rich containing protein) has been identified as a Beclin-1 associated protein. Notably, Rubicon is one of few negative regulators of autophagy whereas many autophagy-related genes are positive regulators of autophagy. Rubicon also has autophagy-independent functions including phagocytosis and endocytosis. In this mini-review, we focus on the various roles of Rubicon in different organs in the settings of metabolic diseases and ageing, and discuss its potential role as a promising therapeutic target.

Keywords: Rubicon, autophagy, LAP, metabolic disease, ageing, longevity

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***Correspondence:**
Shuhei Nakamura
shuhei.nakamura@fbs.osaka-u.ac.jp
Tamotsu Yoshimori
tamyoshi@fbs.osaka-u.ac.jp

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INTRODUCTION

The prevalence of metabolic diseases and age-related diseases is significantly increasing in the world (Christensen et al., 2009; Mozumdar and Liguori, 2011; von Ruesten et al., 2011; Ranasinghe et al., 2017; Foreman et al., 2018). Therefore, the development of effective treatment against these diseases is a pressing issue. Autophagy is a cellular degradation system that maintains intracellular homeostasis (Levine and Kroemer, 2008; Mizushima et al., 2008). Accumulating evidence supports that autophagy protects against metabolic diseases (Zhang et al., 2018) and age-related diseases (Hansen et al., 2018). In 2009, Rubicon (Run domain Beclin-1 interacting and cysteine-rich containing protein) has been discovered as a protein that negatively regulates autophagy (Matsunaga et al., 2009b; Zhong et al., 2009). Subsequent researches have clarified that Rubicon has various functions besides autophagy, and is intimately related to the pathogenesis of metabolic diseases and age-related diseases. In this mini-review, we first outline the diverse molecular functions of Rubicon. Next, we summarize the recent findings regarding the role of Rubicon in metabolic diseases and age-related diseases. Finally, we discuss current issues and propose future directions for the therapeutic application of Rubicon.

THE MOLECULAR FUNCTION OF RUBICON

Rubicon has been discovered as a negative regulator of autophagy in 2009 (Matsunaga et al., 2009b; Zhong et al., 2009). Besides autophagy, Rubicon has important roles in phagocytosis, endocytosis,

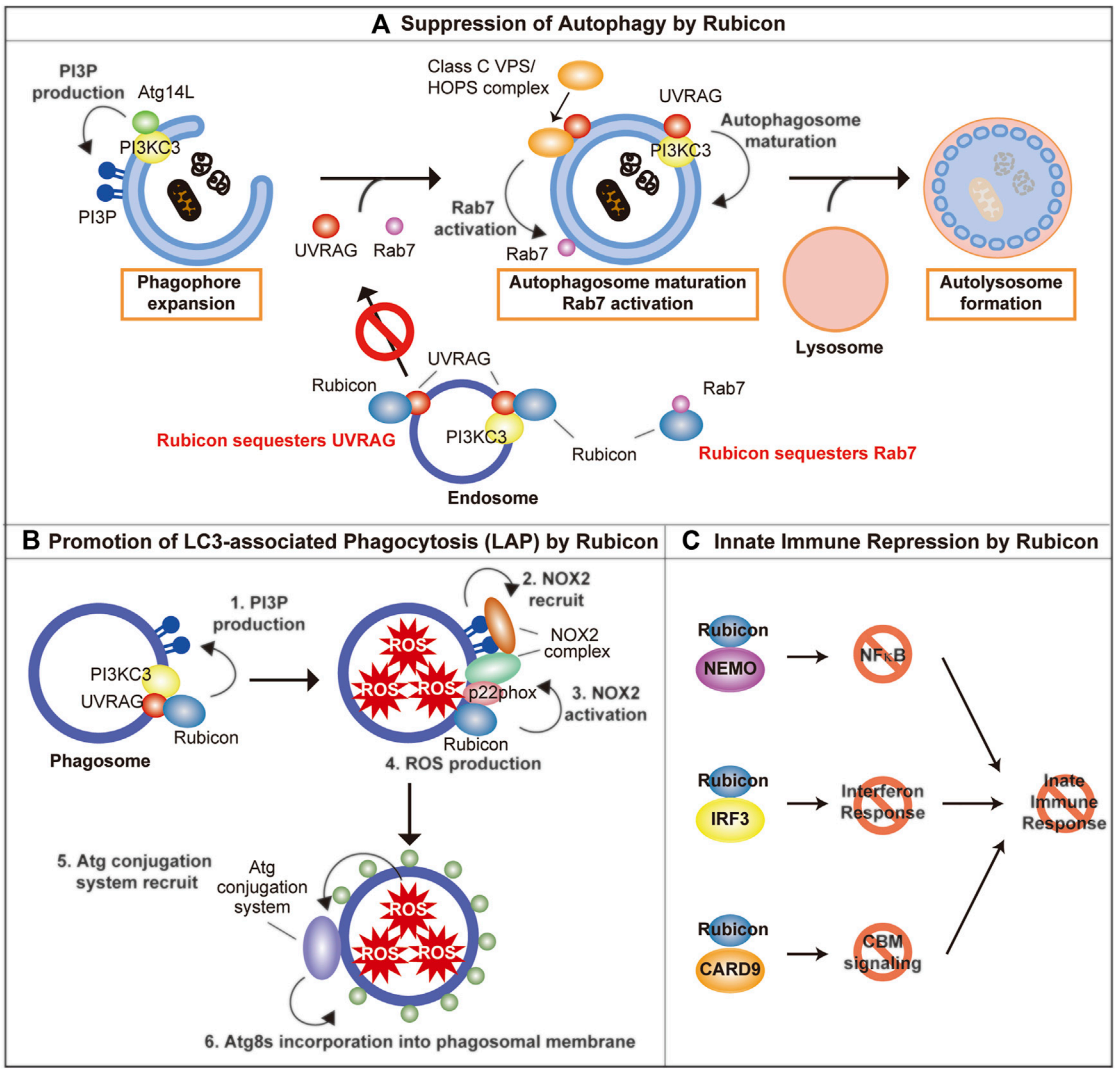

FIGURE 1 | The molecular function of Rubicon. **(A)** Rubicon suppresses autophagosome maturation via the sequestration of UVRAG, PI3KC3, and Rab7. **(B)** Rubicon promotes LAP *via* promotion of PI3P production and stabilization of NOX2 complex on phagosomes. **(C)** Rubicon inhibits innate immune response via the sequestration of NEMO, IRF3, and CARD9. PI3P, phosphatidylinositol-3-phosphate; PI3KC3, Class III phosphatidylinositol-3-kinase; NEMO, NF-κB essential modulator; IRF3, interferon regulatory factor 3; CARD9, caspase recruitment domain-containing protein 9.

microbial infection and innate immune response. In this session, we summarize the autophagy-dependent and–independent function of Rubicon (**Figure 1**).

Autophagy-Dependent Function of Rubicon

Class III phosphatidylinositol-3-kinase (PI3KC3), which consists of three core components, Vps34, p150, and Beclin-1 (Simonsen and Tooze, 2009; Sun et al., 2009), plays an important role for autophagosome biogenesis by catalyzing the formation of phosphatidylinositol-3-phosphate (PI3P) (Simonsen and Tooze, 2009). The core PI3KC3 complex interacts with mutually exclusive molecules Atg14L and UVRAG (Liang et al., 2006; Itakura et al., 2008; Sun et al., 2008). Atg14L contributes to phagophore expansion which leads to autophagosome formation, while UVRAG plays an important role in autophagosomal maturation (Liang et al., 2006). UVRAG is also reported to activate Rab7 by interacting with class C Vps/HOPS complex, which leads to promote the fusion between autophagosome, and lysosome (Liang et al., 2008).

Rubicon directly binds with UVRAG and Vps34 (one of PI3KC3 complex components), and suppresses their function, which impedes the maturation of autophagosome (Matsunaga et al., 2009b; Sun et al., 2011; Zambrano et al., 2019). In addition, Rubicon sequesters UVRAG from class C Vps/HOPS complex, which inhibits the activation of Rab7 (Sun et al., 2010). Rubicon also suppresses autophagic activity by directly interacting Rab7 via its RH domain (Bhargava et al., 2020). The function of Rubicon is regulated by its phosphorylation: The protein kinase HUNK-mediated phosphorylation of Rubicon inhibits its function and promotes autophagy (Zambrano et al., 2019).

Intriguingly, knockdown of Rubicon also promotes autophagosome formation (Matsunaga et al., 2009b), suggesting that the regulation of autophagosome maturation by Rubicon could be the rate limiting step for autophagic activity (Matsunaga et al., 2009a). Future research will be needed to elucidate the molecular mechanism regarding the regulation of autophagosomal formation by Rubicon.

Autophagy-Independent Function of Rubicon

LAP and LANDO

Recently, several components of autophagic machinery are closely associated with vesicular trafficking, including phagocytosis and endocytosis (Galluzzi and Green, 2019). Atg8s are reported to be incorporated into various degradative single-membrane vesicles like phagosome and endosome, and Atg8s help the smooth degradation of these vesicles in lysosomes. The incorporation of Atg8s into phagosomes and endosomes are called LC3-associated phagocytosis (LAP) (Romao and Münz, 2014) and LC3-associated endocytosis (LANDO) (Heckmann et al., 2019), respectively. Interestingly, the formation of LAP and LANDO is dependent on ATG8s-conjugation system, however, independent on autophagy-initiation machinery (Nieto-Torres et al., 2021). LAP assists efficient degradation of phagocytosed pathogens and cellular debris via acidification and maturation of phagosome as well as NOX2-dependent ROS production within phagosomes (Martinez et al., 2011). Therefore, LAP deficiency exacerbates infection via insufficient degradation of pathogens, and closely correlates with excessive activation of immune cells via insufficient degradation of cellular debris, and which lead to autoimmune diseases (Martinez et al., 2016; Heckmann et al., 2017). Furthermore, LAP in macrophage induces immune tolerance under the tumor environment via promoting polarization toward M2 phenotype (Cunha et al., 2018). LANDO is reported to be closely associated with endocytic receptor recycling (Heckmann et al., 2019). Interestingly, whereas Rubicon is a negative regulator of autophagy, Rubicon is reported to be an indispensable molecule of LAP and LANDO (Martinez et al., 2015; Martinez et al., 2016; Heckmann et al., 2019). The molecular mechanism of Rubicon to regulate LAP is following: 1) Rubicon recruits PI3KC3 to phagosomes and promotes PI3P production on phagosomes. 2) PI3P mediates the recruitment of NOX2 complex on phagosomes. 3) Rubicon stabilizes NOX2 complex by interacting with p22phox, one of the constituent molecules in NOX2 complex, thereby promotes ROS production within phagosomes. 4) ROS production within phagosomes is hypothesized to recruit ATG8s by unstabilizing the phagosomal membrane (Boyle and Randow, 2015; Martinez et al., 2015).

What is the significance of the role of Rubicon that promotes LAP and LANDO while suppresses autophagy? There is a limit to the lysosomal degradation capacity, and lysosomal overload could lead to lysosomal membrane permeabilization, which causes the release of lysosomal enzymes into the cytosol and activates the cell death pathway. Therefore, it is tempting to speculate that Rubicon might balance the activity of autophagy, phagocytosis and endocytosis, which are independent lysosomal degradation pathways, and in order to avoid lysosomal overload.

Microbial Infection and Innate Immune Response

Independent of autophagy, LAP and LANDO, Rubicon is reported to be associated with the exacerbation of microbial infection by suppressing the innate immune response. After viral infection, the expression of Rubicon is increased and Rubicon suppresses interferon response by directly binding to NF-κB essential modulator (NEMO) (Wan et al., 2017; Fang et al., 2019) or interferon regulatory factor 3 (IRF3) (Kim et al., 2017). Rubicon also inhibits cytokine production by binding caspase recruitment domain-containing protein 9 (CARD9). Rubicon-CARD9 complexes disassemble the CBM signaling complex, which consists of CARD9, BCL10, and MALT1, leading to the termination of pattern recognition receptors (PRRs)-induced cytokine production (Yang et al., 2012). As a result, Rubicon permits the intracellular proliferation of virus and fungus, which leads to the aggravation of microbial infection (Yang et al., 2012; Wan et al., 2017). We outlined the autophagy-dependent and–independent role of Rubicon. Whereas Rubicon has various roles, Rubicon exerts its roles properly by changing its binding partners in response to various environmental stimulations. On the other hand, the regulation of these various roles of Rubicon remains largely unknown and future detailed studies are desired.

METABOLIC DISEASES AND RUBICON

Whereas autophagy has been reported to be protective against various metabolic disease, recent studies have elucidated that autophagy is dysregulated in these settings (reviewed by (Zhang et al., 2018)). In 2016, Tanaka et al. have first elucidated a close relationship between Rubicon and autophagic dysregulation in metabolic disease (Tanaka et al., 2016). In this report, the protein level of Rubicon was increased concomitant with dysregulation of autophagy in the livers of human nonalcoholic fatty liver disease (NAFLD) patients, high fat diet-fed mice, and in the culture hepatocytes treated with saturated fatty acids. Furthermore, they clarified that Rubicon exacerbates hepatic steatosis in high fat diet-fed mice via suppression of autophagy by using hepatocyte-specific Rubicon knockout mice. NAFLD is a major risk factor for hepatocellular carcinoma (HCC), which is increasing worldwide (Huang et al., 2021). Therefore, targeting Rubicon could be a promising approach for therapeutic application in NAFLD, although further analyses are needed whether Rubicon suppression could prevent the development of HCC in NAFLD patients. The precise mechanism that Rubicon suppression ameliorates NAFLD remains unclear. Selective degradation of endoplasmic reticulum (ER-phagy) or lipid droplet (lipophagy) might ameliorate NAFLD as Rubicon suppression relieved ER stress and lipid droplet accumulation in hepatocytes treated with saturated fatty acids (Tanaka et al.,

2016). On the other hand, Rubicon suppression might ameliorate NAFLD via selective degradation of nuclear receptor co-repressor 1 (NCoR1), repressor of PPARα, which is recently identified as a selective substrate of autophagy (Saito et al., 2019).

Besides NAFLD, it is reported that there is a close relationship between Rubicon and myocardial infarction (Li et al., 2020; Nah et al., 2020). Nah et al. reported that the expression of Rubicon is up-regulated concomitant with autophagosome accumulation and autophagic cell death (autosis) after myocardial infarction, whereas the suppression of Rubicon ameliorates autophagosome accumulation, and autosis (Nah et al., 2020). On the other hand, Li et al. identified that Rubicon binds to caspase recruitment domain-containing protein 9 (CARD9) during myocardial infarction. They further elucidated that CARD9 deletion exacerbates myocardial infarction via autophagic dysregulation, indicating that autophagic activity is maintained during myocardial infarction via sequestration of Rubicon by CARD9 (Li et al., 2020).

Regarding diabetes mellitus, Aoyama et al. verified the role of Rubicon in pancreatic β cells using pancreatic β cell-specific Rubicon knockout mice, however, Rubicon deletion did not affect glucose homeostasis under a normal diet, and high fat diet (Aoyama et al., 2020). As for diabetic kidney disease, increased Rubicon expression concomitant with autophagic dysregulation was recognized in glomerular podocytes. Notably, upregulation of Rubicon was epidermal growth factor receptor (EGFR) signaling-dependent, and deletion of EGFR in glomerular podocytes ameliorated diabetic kidney disease via Rubicon suppression-dependent autophagic activation (Li et al., 2021). On the other hand, kidney proximal tubules-specific knockout of Rubicon have little influence on the streptozotocin-induced diabetic kidney disease mice model. In addition, kidney proximal tubules-specific Rubicon knockout mice exhibited obesity, hyperlipidemia and NAFLD via the increased exocytosis of fatty acids from kidney proximal tubules (Matsuda et al., 2020). As above, the role of Rubicon in diabetic kidney disease is different for each kidney constituent cells.

AGEING AND RUBICON

Accumulating evidence over the past decade indicates that autophagy has a key role in life span regulation (Hansen et al., 2018; Leidal et al., 2018; Nakamura and Yoshimori, 2018; Wong et al., 2020; Kaushik et al., 2021): autophagy is activated and required for the lifespan extension in various longevity paradigms, whereas autophagic activity is disturbed during the ageing process. On the other hand, the mechanism that autophagic activity decreases during ageing had for a long time been unclear and it became the biggest mystery to be solved in this study field. In 2019, we have elucidated that increment of Rubicon is the leading cause of autophagic disturbance during ageing. Importantly, the increased expression of Rubicon concomitant with autophagic disturbance is highly conserved among species including *Caenorhabditis elegans* (*C. elegans*), *Drosophila* and mammals, and suppression of Rubicon in *C. elegans* promotes longevity via autophagic activation (Nakamura et al., 2019). We also clarified that deletion of Rubicon suppressed organ fibrosis during natural ageing in mice. Furthermore, suppression of Rubicon ameliorated age-related disease, including PolyQ disease model in *Drosophila* and Parkinson's disease model in mice. The following report has indicated that age-related macular degeneration, one of the representative age-related diseases, could be improved by Rubicon suppression-dependent autophagic activation in retinal pigment epithelial cells (Ando et al., 2021).

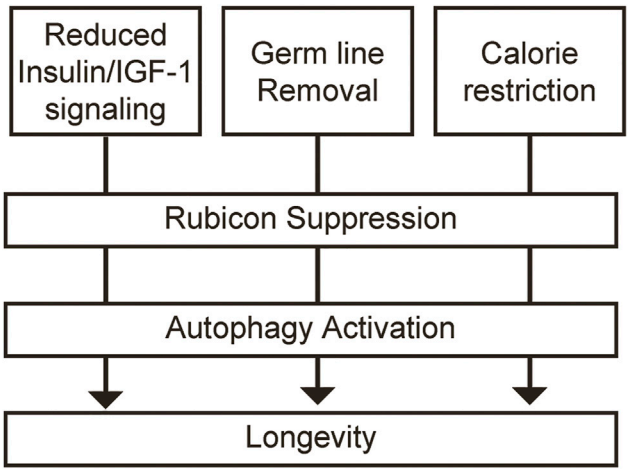

FIGURE 2 | Rubicon repression leads to the longevity *via* activation of autophagy in multiple longevity paradigms. Multiple longevity paradigms, including reduction of insulin/IGF-1 signaling, germ line removal and calorie restriction decrease the expression of Rubicon at the transcriptional level, and leading to the longevity *via* activation of autophagy.

Previous reports have indicated that genetic activation of autophagy contributes to lifespan extension in mammals (Pyo et al., 2013; Fernández et al., 2018), however, these studies have forcibly promoted the expression or function of the specific autophagy-related molecules. In contrast, we have for the first time elucidated that lifespan extension could be achieved by the intervention on the molecules that cause physiological autophagic dysregulation during ageing, indicating this study is clinically relevant. Furthermore, this study elucidated that the physical activity was increased by knocking down Rubicon in *C. elegans* and *Drosophila*. Although further analysis using mammals is needed, these results are clinically important because not only lifespan extension but also healthspan extension and sarcopenia prevention are important points in geroscience.

Furthermore, this study elucidated that various longevity paradigms, including *daf-2* mutant (reduction of insulin/IGF-1 signaling), *glp-1* mutant (germ line removal) and *eat-2* mutant (calorie restriction) in *C. elegans*, indicate the decreased expression of *Rubicon* at the transcriptional level, and leading to the longevity via activation of autophagy (**Figure 2**). Calorie restriction in mice also reduced Rubicon expression, although the precise mechanism remains unclear. Further studies are expected to elucidate the molecular mechanism that various longevity

TABLE 1 | Differential expression and role of Rubicon in various organs during ageing.

Organ	Age related disease	Rubicon expression by ageing	Ageing phenotype by Rubicon knockout
Liver	NAFLD	Increased	Improved
Kidney	Kidney fibrosis	Increased	Improved
Adipose tissue	Lipodystrophy Insulin resistance Ectopic fat accumulation	Decreased	Deteriorated

paradigms decrease Rubicon expression. In other words, we should clarify which signaling factor organisms sense and which transcriptional factor convert the signaling factor to increased expression of Rubicon during ageing process. If these questions could be answered, the development of therapeutic strategies against ageing that target autophagy could a reality.

We further investigated which organs of Rubicon are responsible for lifespan extension and clarified that specific knockdown of Rubicon in neuronal cells extended lifespan in *C. elegans* and *Drosophila*. How Rubicon in neuronal cells regulate the ageing process in the whole body? Neuronal cells are known to communicate with peripheral organs via neural circuit as well as humoral factor. Recently, accumulating evidence has elucidated that autophagy-related molecules have an important role in intercellular communication by promoting secretory autophagy (Ponpuak et al., 2015; Padmanabhan and Manjithaya, 2020) and/or exosomal secretion (Xu et al., 2018; Levine and Kroemer, 2019). Therefore, it is tempting to infer that the non-autonomous function of neuronal Rubicon regulates whole body ageing by intercommunicating with the peripheral organ. Future studies are needed to clarify the mechanism that neuronal Rubicon regulates whole body ageing.

Is Rubicon merely a bad guy in age-related disease? We answered the question in 2020 (Yamamuro et al., 2020). This study revealed that suppression of Rubicon in adipose tissue promoted excessive autophagic degradation of PPARγ coactivators, SRC-1 and TIF2, which led to lipodystrophy, insulin resistance, and ectopic fat accumulation including liver steatosis. In other words, Rubicon suppresses excessive autophagic activation and maintains homeostasis in ageing adipose tissue. Interestingly, during ageing process, the expression of Rubicon was significantly suppressed in adipose tissue in contrast to liver and kidney, indicating regulation of Rubicon expression is different for each tissue during ageing. This report is clinically relevant because lipodystrophy, insulin resistance and ectopic fat accumulation, which are closely related to downregulation of Rubicon, are generally observed in the human normal ageing process, and progeria. During ageing, Rubicon is upregulated in the liver and kidney which exacerbate age-related disease including NAFLD and kidney fibrosis, while Rubicon is downregulated in adipose tissue which is also closely associated age-related phenotype including lipodystrophy, insulin resistance and ectopic fat accumulation (**Table 1**). Elucidating the comprehensive regulatory factor that modulates the tissue-different expression of Rubicon is a future exciting challenge, which could combat the metabolic change of the whole body during ageing.

A recent report has identified that Rubicon affects male fertility: Rubicon deficiency in Sertoli cells causes defective spermatogenesis via accelerated autophagic degradation of GATA4, which is an essential transcription factor for Sertoli cell function (Yamamuro et al., 2021). This report is interesting from the viewpoint of ageing: it has been previously known that there is a negative correlation between fertility and longevity, although the causal relationship has been unclear (Partridge et al., 2005; Kenyon, 2010). A previous report identified that ELT-5 and ELT-6, which are GATA homologue in *C. elegans* accumulate during ageing and knockdown of ELT-5 and ELT-6 extends lifespan (Budovskaya et al., 2008). Considering together, the regulation of autophagic GATA4 degradation by Rubicon might be one of the mechanisms that explain the negative correlation between fertility and longevity.

The autophagy-independent function of Rubicon has also been identified to contribute to the pathogenesis of age-related disease. Heckmann et al. reported that deficiency of Rubicon-dependent LANDO exacerbates the pathogenesis of Alzheimer disease (Heckmann et al., 2019). Rubicon-dependent LAP is reported to be protective for various age-related disease including age-related macular degeneration (Kim et al., 2013; Muniz-Feliciano et al., 2017). Therefore, we should pay attention to whether the role of Rubicon is autophagy-dependent or not when we consider the function of Rubicon in age-related disease.

FUTURE PERSPECTIVES

Whereas Rubicon inhibition could be a promising therapeutic strategy, several concerns exist. Rubicon inhibition in the whole body might have side effects including lipodystrophy and infertility due to excessive autophagy, and exacerbation of autoimmune diseases, infectious diseases and neurodegenerative disease due to LAP and LANDO inhibition. To overcome these side effects, several issues should be resolved.

First, the regulatory mechanism of Rubicon during ageing should be clarified. Interestingly, there is a close relationship between Rubicon expression and the occurrence of age-related disease: Rubicon expression increases in the liver and kidney during ageing, which leads to the exacerbation of NAFLD, and kidney fibrosis. On the other hand, Rubicon expression decreases in adipose tissue during ageing, which leads to the exacerbation of

lipodystrophy, and ectopic fat accumulation (**Table 1**). Therefore, the identification of the comprehensive regulatory factor of Rubicon during ageing is the inevitable important issue that would enable the development of anti-ageing drug without side effects.

Second, it is critical to identify the region in the brain responsible for lifespan extension by Rubicon suppression. As noted above, Rubicon inhibition in the whole brain might have side effects including Alzheimer disease. Therefore, the site-specific inhibition approach is important to avoid side effects. Recent reports have elucidated that the hypothalamus or neural stem cells regulate ageing (Satoh et al., 2017; Zhang et al., 2017; Kim and Choe, 2019). It is tempting to speculate that Rubicon in these specific cells is essential for general ageing.

Finally, considering the relationship between Rubicon and diseases, human evidence is completely lacking at present. Future studies should accumulate human evidence.

CONCLUSION

Rubicon is a cell biologically interesting molecule that plays specific functions: Rubicon is one of few negative regulators of autophagy whereas Rubicon is indispensable for LAP and LANDO. There remain many questions unanswered. What is the regulator of Rubicon during ageing? How Rubicon exerts its various functions properly in response to various situations? Future studies to answer these questions will provide clues to a novel treatment for metabolic diseases and age-related diseases.

AUTHOR CONTRIBUTIONS

SM wrote the article. SN and TY edited/revised the article, and all authors approved the final version of the article.

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REVIEW
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Current Status of Autophagy Enhancers in Metabolic Disorders and Other Diseases

*Kihyoun Park and Myung-Shik Lee**

Severance Biomedical Science Institute/Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea and Soonchunhyang Institute of Medi-bio Science, Soonchunhyang University, Cheonan, Korea

Autophagy is pivotal in the maintenance of organelle function and intracellular nutrient balance. Besides the role of autophagy in the homeostasis and physiology of the individual tissues and whole organism *in vivo*, dysregulated autophagy has been incriminated in the pathogenesis of a variety of diseases including metabolic diseases, neurodegenerative diseases, cardiovascular diseases, inflammatory or immunological disorders, cancer and aging. Search for autophagy modulators has been widely conducted to amend dysregulation of autophagy or pharmacologically modulate autophagy in those diseases. Current data support the view that autophagy modulation could be a new modality for treatment of metabolic syndrome associated with lipid overload, human-type diabetes characterized by deposition of islet amyloid or other diseases including neurodegenerative diseases, infection and cardiovascular diseases. While clinically available bona fide autophagy modulators have not been developed yet, it is expected that on-going investigation will lead to the development of authentic autophagy modulators that can be safely administered to patients in the near future and will open a new horizon for treatment of incurable or difficult diseases.

Keywords: autophagy, modulator, metabolic diseases, lysosome, mitochondria, endoplasmic reticulum

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Zvulun Elazar,
Weizmann Institute of Science, Israel

Reviewed by:
Tobias Eisenberg,
University of Graz, Austria
Rohit Anthony Sinha,
Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGI), India
Ravi Manjithaya,
Jawaharlal Nehru Centre for Advanced Scientific Research, India

****Correspondence:***
Myung-Shik Lee
mslee0923@yuhs.ac
mslee0923@sch.ac.kr

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INTRODUCTION

Autophagy, derived from Greek words meaning "self-devouring," is a cellular process of degradation of cell's own material including organelles, proteins or cellular fluid in lysosome acting as the effector organelle of autophagic degradation (Mizushima and Komatsu, 2011; Agudo-Canalejo et al., 2021). Three major types of autophagy such as macroautophagy, microautophagy and chaperone-mediated autophagy have been described (Klionsky and Emr, 2000). Among them, macroautophagy (henceforth referred to as autophagy) is characterized by autophagosome which is encircled by double membrane assembled by subcellular membrane rearrangement sequestering organelles, target substrates and cytoplasm. After autophagosome fusion to lysosome, autophagolysosomes are formed in which sequestered material is decomposed or lysed by lysosomal enzymes (Klionsky and Emr, 2000). The main purpose of autophagy is quality control of organelles or proteins and protection of intracellular homeostasis or nutritional balance during energy deficiency. Thus, when nutrients are deficient, autophagy is initiated to mobilize intracellular nutrients and to avoid harmful effects caused by deficiency of vital nutrients or elements. When organelles are damaged, stressed or dysfunctional, autophagy receptors are recruited and act as bridges linking cargos to LC3 family proteins to initiate autophagic process. Detailed molecular and cellular mechanisms of the progression of autophagy after the initiation steps have been extensively investigated, and the

Nobel Prize for Physiology or Medicine 2016 was bestowed on Yoshinori Ohsumi, as an accolade to his epoch-making discovery of the Atg conjugation system in autophagosome expansion.

Since autophagy is critical in the maintenance of cellular homeostasis and organelle function, autophagy affects diverse aspects of numerous physiological and pathological processes. Hence, dysregulated autophagy would lead to or be associated with a variety of diseases such as metabolic disorders including type 2 diabetes (T2D) or metabolic syndrome, neurodegenerative disorders including Alzheimer's disease, Parkinson's disease or Huntington's disease, immune/inflammatory disorders, infectious diseases and cancer, etc. In T2D or metabolic syndrome, insulin and its downstream mTOR are well-known inhibitors of autophagy, while glucagon, a counter-regulatory hormone of insulin, is one of the first known inducers of autophagy (Deter and de Duve, 1967; Pfeifer, 1978; Sarbassov et al., 2005a). Furthermore, endoplasmic reticulum (ER) and mitochondria that play crucial roles in β-cell survival or function, insulin sensitivity or resistance and metabolic inflammation or inflammasome activation (Ozcan et al., 2004; Petersen et al., 2004; Laybutt et al., 2007; Misawa et al., 2013), rely on autophagy for proper function. Thus, autophagy is expected to affect diverse aspects of the pathogenesis of T2D or metabolic syndrome.

Because autophagy is involved in such various biological processes and diseases, searches for autophagy modulators have been conducted to develop novel compounds with therapeutic effects against those diseases or aging (Zhang et al., 2007; Eisenberg et al., 2009; Rubinsztein et al., 2012; Shoji-Kawata et al., 2013). In this review, the current development of autophagy modulators for clinical application will be summarized with a focus on therapeutic application to metabolic disorders. Since numerous papers are being published regarding autophagy enhancers for metabolic disorders and other diseases such as neurodegenerative diseases or cardiac disease, apologies are presented to the authors of the work which inadvertently was not covered in this review.

ROLE OF AUTOPHAGY IN THE SYSTEMIC METABOLISM AND METABOLIC DISORDERS

The role of autophagy in the maintenance of systemic or global metabolic homeostasis and in the pathogenesis of metabolic disorders has been controversial. *In vivo* role of autophagy in metabolic disorders has been mostly studied using genetic or site-specific autophagy gene-knockout (KO) mouse models that showed diverse metabolic features depending on the site of genetic alteration (Kim and Lee, 2014). For example, mice with KO of *Atg7*, an essential autophagy gene in pancreatic β-cells producing insulin, showed structural and functional defects of pancreatic β-cells, resulting in glucose intolerance and susceptibility to diabetes in the presence of metabolic stress (Ebato et al., 2008; Jung et al., 2008; Quan et al., 2012a) (**Figure 1**). On the contrary, KO of *Atg7* in insulin target tissues such as skeletal muscle cells or hepatocytes led to the induction of fibroblast growth factor 21 (FGF21) as a "mitokine" due to mitochondrial stress and to the resistance to diet-induced obesity and insulin resistance (Kim K. H. et al., 2013), contrary to the expectation that autophagy deficiency associated with mitochondrial dysfunction in insulin target tissues would lead to insulin resistance. In contrast, increased hepatic accumulation of lipid droplet has also been reported in mice with hepatocyte-specific *Atg5* KO or *Atg7* knockdown (Singh et al., 2009a; Yang et al., 2010) (**Figure 1**).

In mice with adipose tissue-specific KO of *Atg7*, defective differentiation of white adipocytes and reduced fat mass were observed (Singh et al., 2009b; Zhang et al., 2009). In contrast, adipose-specific KO of autophagy genes such as *Atg3, Atg16L1* or *Beclin 1* during adult period led to mitochondrial dysfunction, hypertrophic adipocytes due to impaired lipolysis, adipocyte death or inflammation in adipose tissue and peripheral insulin resistance or impaired glucose intolerance (Cai et al., 2018; Son et al., 2020) (**Figure 1**). Mice with deletion of *Atg7* in agouti-related protein (AgRP) neurons producing orexigenic hormones such as AgRP and neuropeptide Y (NPY) showed reduced fat mass and decreased hyperphagic response (Kaushik et al., 2011), while proopiomelanocortin (POMC) neuron-specific *Atg7*-KO mice showed obese phenotype and aggravated glucose intolerance after high-fat diet (HFD) feeding imposing metabolic stress (Coupe et al., 2012; Kaushik et al., 2012; Quan et al., 2012b) (**Figure 1**). Mice with macrophage-specific *Atg7* KO showed aggravated inflammasome activation in adipose tissue and metabolic deterioration after HFD feeding, which was associated with aggravated mitochondrial dysfunction (Kang et al., 2016; Lee et al., 2016) (**Figure 1**).

While these genetic models showed diverse metabolic phenotypes depending on the location and severity of autophagy deficiency, metabolic effects of systemic autophagy insufficiency of physiologically relevant degree rather than tissue-specific autophagy gene KO have been sparsely investigated. We have reported that global *Atg7*-haploinsufficient mice showed compromised adaptation to metabolic stress and accelerated progression from obesity to diabetes, which was associated with aggravated liver steatosis and augmented inflammasome activation in adipose tissue (Lim et al., 2014). In a similar vein, *Atg4b*-KO mice with reduced autophagic competence, exhibited aggravated metabolic profile after HFD (Fernández et al., 2017). Mice that are defective in stimulus-induced autophagy due to knock-in mutations in BCL2 phosphorylation sites (T69A, S70A and S84A) (*Bcl2 AAA* mice) also showed impaired exercise-mediated protection against glucose intolerance after HFD (He et al., 2012). On the other hand, overexpression of *Atg5* improved metabolic profile of aged mice (Pyo et al., 2013). These results indicate that adequate systemic autophagy is necessary for host adaptation to metabolic stress without which metabolic deterioration might ensue. These results also suggest the possibility that systemic enhancement of autophagic activity may confer beneficial metabolic effects against metabolic stress by enhancing cellular and global adaptive changes to the stress. In contrast, mice with genetic hyperactivation of autophagy through transgenic expression of constitutively active *Becn1* (*Becn1*^{F121A}) (Rocchi et al., 2017) displayed improved insulin sensitivity due to

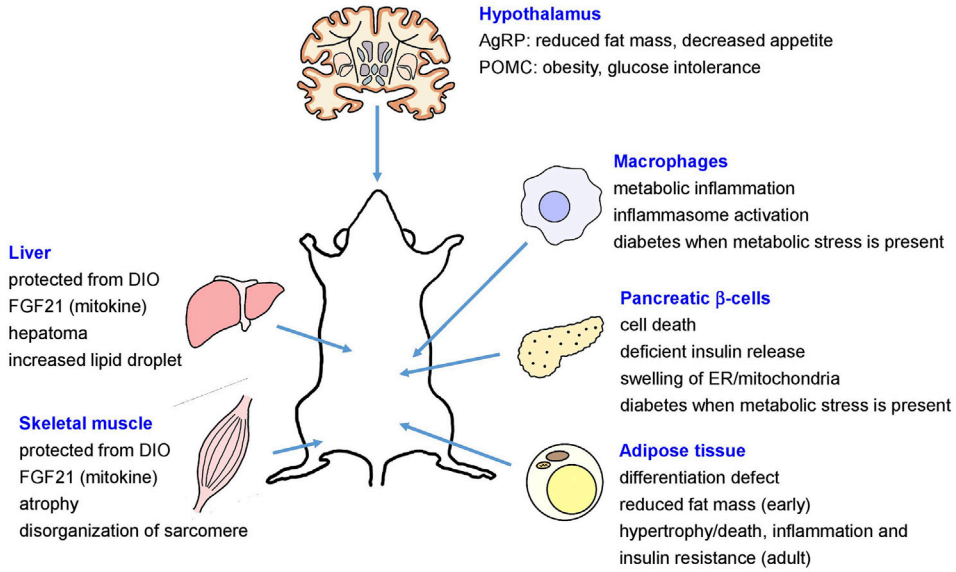

FIGURE 1 | Metabolic effects of site-specific KO of autophagy genes (AgRP, agouti-related protein; DIO, diet-induced obesity; ER, endoplasmic reticulum; FGF21, fibroblast growth factor 21; POMC, proopiomelanocortin).

attenuated endoplasmic reticulum (ER) stress in peripheral tissues but aggravated glucose intolerance due to compromised β-cell function when fed HFD (Yamamoto et al., 2018). Such results have been ascribed to excessive degradation of insulin secretory granules by hyperactive β-cell autophagy, termed "vesicophagy," and might suggest that intermittent rather than chronic activation of autophagy of an appropriate degree needs to be considered for treatment of T2D (Yamamoto et al., 2018).

While not an autophagy protein *per se*, effects of acyl-CoA binding protein (ACBP) involved in transport of acyl-CoA and lipid catabolism on autophagy and systemic metabolism has been studied. Upon autophagy induction, ACBP was released, and extracellular ABCP inhibited autophagy and promoted lipid anabolism or weight gain, which supports the view that systemic autophagy activation promotes lipid catabolism. On the other hand, intracellular ACBP stimulated autophagic flux, suggesting a feedback regulation of autophagy by ACBP, a lipid catabolic factor (Bravo-San Pedro et al., 2019).

AUTOPHAGY IN HUMAN DIABETES OR MURINE DIABETES MIMICKING HUMAN DIABETES

Most of the studies investigating the role of autophagy in T2D or metabolic disorders have been employing genetic mouse models, and studies of the relationship between autophagy and metabolism in humans are sparse. Technological limitation in the assay of autophagic flux in human tissues or samples could be one of the reasons impeding studies of the role of autophagy in the metabolic regulation in humans. Previous studies of tissue samples from patients with T2D have examined morphological features and number of autophagosomes employing electron microscopy. In pancreatic islets of T2D patients, accumulation of enlarged autophagic vacuoles and autophagosomes was observed which was decreased after treatment with metformin, suggesting impeded removal of autophagic substances in islets of T2D patients (Masini et al., 2009). Protective effects of Imatinib-induced autophagy on human islet cell apoptosis by cytokine combinations have also been reported (Marselli et al., 2013). In human metabolic liver diseases, genetic association between non-alcoholic fatty liver disease (NAFLD) and variants of *IRGM*, an autophagy gene activated by infection (Grégoire et al., 2011) has been observed (Lin et al., 2016), suggesting the role of decreased autophagy-mediated lipid clearance or lipophagy in the development of human NAFLD.

Although most studies on the relationship between autophagy and metabolic diseases have been conducted using genetic mouse models as mentioned above, murine models of metabolic diseases cannot exactly recapitulate human metabolic diseases. Specifically, pathophysiological features of human diabetes and murine one are different in several aspects. One of the most striking differences is amyloid deposition in pancreatic islets of >90% of T2D patients but not in those of murine diabetes, which is due to different amino acid sequences of amylin or islet amyloid polypeptide (IAPP). Human IAPP (hIAPP) is amyloidogenic, while murine IAPP (mIAPP) is non-amyloidogenic because proline-rich sequence in residues 20–29 of mIAPP inhibits β-sheet formation, a prerequisite for amyloidogenesis (Westermark et al., 2011). It is unknown why hIAPP acquired amyloidogenic propensity during evolution. Because insoluble or amyloid proteins are preferentially cleared by autophagic pathway or lysosomal degradation due to their large size in contrast to soluble or non-amyloid proteins that can be cleared by both proteasomal and autophagic pathways (Rubinsztein, 2006), autophagy may be more important in human diabetes than in murine diabetes.

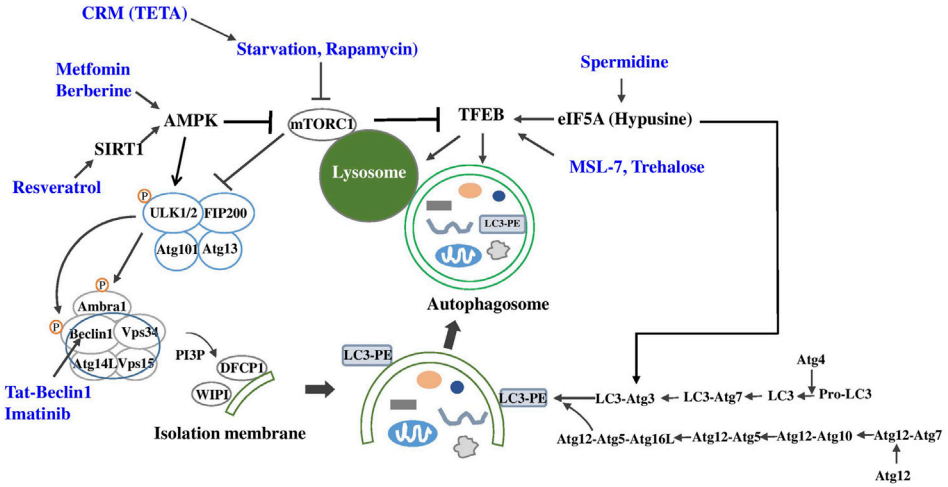

FIGURE 2 | Autophagy enhancers for metabolic diseases (blue) and their target molecules (black) in autophagy machinery (CRM, calorie restriction mimic; TETA, Triethylenetetramine dihydrochloride).

To study the role of autophagy in human-type diabetes characterized by islet amyloid accumulation, we have employed mice expressing *hIAPP* in pancreatic β-cells driven by rat insulin promoter (*hIAPP*$^{+}$ mice). While *hIAPP*$^{+}$ mice developed only mild hyperglycemia, *hIAPP*$^{+}$ mice crossed to β-cell-specific autophagy-KO mice (*hIAPP*$^{+}$*Atg7*$^{\Delta\beta\text{-cell}}$ mice) developed overt diabetes, accompanied by hIAPP oligomer and amyloid accumulation in pancreatic islets (Kim et al., 2014; Rivera et al., 2014). A study using *hIAPP* knock-in mice instead of *hIAPP*$^{+}$ mice also showed essentially similar results (Shigihara et al., 2014). These results demonstrate a critical role of β-cell autophagy in the clearance of hIAPP oligomer and in the prevention of islet amyloid deposition, which has implication in the understanding and treatment of human diabetes.

AUTOPHAGY ENHANCER IN METABOLIC DISORDERS

As the above results suggested potential therapeutic effects of autophagy modulation in T2D or metabolic syndrome and human-type diabetes, several autophagy enhancers have been developed or rediscovered from known drugs or chemicals against those diseases (**Table 1**).

Berberine

Berberine, a traditional Chinese medicine, has been reported to improve metabolic profile of experimental animals or human patients with metabolic syndrome, diabetes or NAFLD which has been attributed to activation of AMPK and suppression of reactive oxygen species or inflammatory changes (Ma et al., 2018) (**Figure 2**). Recent papers showed the role of autophagy activation in the metabolic improvement by Berberine which was dependent on SIRT1 activation (Sun et al., 2018; Zheng et al., 2021). Autophagy-independent effects such as FGF21 induction has also been reported to contribute to the metabolic improvement by Berberine (Sun et al., 2018).

Imatinib

Imatinib is a well-known anti-cancer drug that has a dramatic effect on chronic myelogenous leukemia (CML) and gastrointestinal stromal tumor by specifically inhibiting bcr-Abl and Kit kinases (Druker et al., 1996; Duensing et al., 2004). Imatinib has been reported to enhance autophagic activity of several cancer cells which may be related to the development of resistance against Imatinib. While conclusive mechanism of autophagy induction by Imatinib is still not clear, the mechanism of autophagy induction by Imatinib has been attributed to inhibition of Kit or Abl, increase of PARKIN-Beclin 1 interaction, induction of *Xiap, Atg5* or *Beclin 1*, and ER stress (Bellodi et al., 2009; Can et al., 2013; Etmer et al., 2007; Lin et al., 2015; Xie et al., 2017) (**Figure 2**). Imatinib can improve metabolic profile of experimental animals or patients with metabolic syndrome or diabetes (Breccia et al., 2005; Veneri et al., 2005; Han et al., 2009), which may be related to autophagy enhancement, while autophagy-independent mechanisms such as reduction of TNF-α release, ER stress or JNK activation might also contribute to the improved metabolic profile by Imatinib (Han et al., 2009). Furthermore, Imatinib could induce remission of diabetes of obese mice with insufficient autophagy, which was accompanied by autophagy induction *in vivo* and reduced insulin resistance (Lim et al., 2014). When Imatinib is considered as therapeutic agent inducing autophagic activity, potential adverse effects of Imatinib such as peripheral edema, cardiotoxicity and hematological side effects should be considered (Hartmann et al., 2009; Orphanos et al., 2009).

MSL-7

We recently identified and developed autophagy enhancer small molecules in a screening of a chemical library. We observed that MSL can enhance nuclear translocation of TFEB, a principal regulator of autophagy gene expression and lysosome biogenesis (Settembre et al., 2011) in an mTORC1-independent manner (Lim et al., 2018) (**Figure 2**). We chemically modified MSL to

enhance microsomal stability (MSL-7), and MSL-7 was able to exert beneficial metabolic effects on obese mouse models by enhancing the clearance of lipid in the liver tissue and ameliorating metabolic inflammation in adipose tissue (Lim et al., 2018). Attenuation of metabolic inflammation was due to amelioration of fatty acid-induced mitochondrial dysfunction and downregulation of fatty acid-induced inflammasome activation since mitochondrial events are important players in inflammasome activation (Misawa et al., 2013).

While these results showed improvement of metabolic profile of HFD-fed mice through enhanced clearance of lipid and reduced inflammasome activation, another potential metabolic effect of MSL-7 was studied because amyloidogenic hIAPP oligomer or islet amyloid (Westermark et al., 2011), is preferentially cleared by autophagy or lysosomal proteolysis rather than proteasomal degradation (Rubinsztein, 2006; Kim et al., 2014). Indeed, MSL-7 administration was able to improve glucose tolerance and β-cell function of HFD-fed *hIAPP*$^{+}$ mice expressing amyloidogenic *hIAPP* in β-cells, which was accompanied by reduction of hIAPP oligomer, islet amyloid or β-cell apoptosis and restoration of β-cell mass or insulinogenic index representing β-cell function (Kim et al., 2021b). These results suggest the possibility that autophagy enhancer may have more significant effects on human diabetes compared to its effects on murine diabetes because of additional effect of autophagy enhancer on β-cell viability and function.

Rapamycin

Rapamycin is a classical autophagy enhancer acting through inhibition of mTOR (Klionsky and Emr, 2000) (**Figure 2**). mTOR can associate with other factors forming two mTOR complexes, mTORC1 and mTORC2. mTORC1 complex consists of mTOR, Raptor (regulator-associated protein of mTOR), PRAS40 (proline-rich Akt substrate, 40 kDa), Deptor and mLst8 (mammalian lethal with SEC13 protein 8), while mTORC2 complex comprises mTOR, Rictor, mSin1 (mammalian stress-activated protein kinase-interacting protein 1), Protor1/2, Deptor and mLst8 (Ardestani et al., 2018). It is generally held that mTORC1 is sensitive to rapamycin, whereas mTORC2 is not. However, prolonged rapamycin treatment has been shown to inhibit mTORC2 (Sarbessov et al., 2006).

Rapamycin has been administered to several models of diabetes. Rapamycin has been shown to improve metabolic profile and β-cell function of Akita mice with a mutation in proinsulin gene leading to proinsulin misfolding and severe ER stress, and such improved β-cell function was attributed to autophagy enhancement (Bachar-Wikstrom et al., 2013). However, prolonged administration of Rapamycin has deleterious effects on β-cell mass which is due to abrogation of mTORC1 activity positively modulating β-cell mass (Barlow et al., 2013). Prolonged Rapamycin administration can also impair insulin sensitivity, which could be due to inhibition mTORC2, since mTORC2 is PDK2 regulating Akt473 phosphorylation by insulin (Sarbassov et al., 2005b).

The effect of Rapamycin on longevity has been a controversial topic. Rapamycin was shown to prolong lifespan of mice (Harrison et al., 2009), while the role of autophagy in this model is unclear. In contrast to the effect of Rapamycin on longevity, Rapamycin had no clear effect on aging-related features such as kyphosis, gait disturbance, motor incoordination, impaired hippocampal neurogenesis, decreased grip strength, glomerulosclerosis or decreased $CD4^{+}$ T cell number, suggesting that Rapamycin may prolong lifespan of mice independent of its effect on aging (Neff et al., 2013). In contrast, anti-aging effect or reversal of aging by Rapamycin such as reversal of reduced hematopoietic stem cell renewal, increased left cardiac ventricular mass and compromised myocardial performance or diastolic function in aged mice, have also been reported (Chen et al., 2009; Dai et al., 2014). In contrast to its effect on non-diabetic mice, Rapamycin shortened lifespan of diabetic *db/db* mice (Sataranatarajan et al., 2016).

Resveratrol

Resveratrol, which is a natural polyphenol in red wine and well-known activator of SIRT1, has been reported to improve metabolic profile and extend healthspan of HFD-fed mice, which was associated with increased biogenesis of mitochondria and enhanced activities of PGC-1α or AMPK (Baur et al., 2006) (**Figure 2**). Resveratrol can promote autophagic activity through SIRT1-dependent deacetylation of autophagy proteins or TFEB (Morselli et al., 2011; Zheng et al., 2021). However, resveratrol administration for 6 months did not have beneficial metabolic effects in human patients with T2D (Bo et al., 2016). In contrast, small but significant effects of resveratrol on blood glucose, lipid profile or bone mineral density of patients with T2D have recently been reported (Bo et al., 2018; Hoseini et al., 2019).

Rg2

Rg2, identified in a chemical screening using GFP-LC3 system, has been shown to improve glucose profile, insulin sensitivity and fatty liver change of mice fed HFD through autophagy activation (Fan et al., 2017). Rg2 also reduced body and fat weights of mice fed HFD; however, this effect on body weight was independent of autophagy.

Spermidine

Spermidine, a polyamine synthesized *in vivo* from ornithine, has been reported to induce autophagy through epigenetic regulation of autophagy genes. Spermidine inhibits EP300 (E1A-binding protein p300), a lysine acetyltransferase and endogenous inhibitor of autophagy (Pietrocola et al., 2015). Spermidine has been reported to improve metabolic profile and liver steatosis of HFD-fed mice through autophagy activation, which was accompanied by reduced metabolic inflammation, enhanced thermogenesis and improved gut barrier function (Ma et al., 2021). However, autophagy-independent effects such as antioxidant effect or nitric oxide (NO) production might also play a role in the metabolic improvement by spermidine (Madeo et al., 2018).

In addition to the effect of spermidine on metabolic diseases, spermidine may affect aging. Given that spermidine level decreases in multiple organs by aging (Nishimura et al., 2006), decreased autophagic activity in aging (Hansen et al., 2018) may

be causally related to spermidine insufficiency. Indeed, spermidine could extend longevity of *C. elegans* and *Drosophila* (Eisenberg et al., 2009). Furthermore, spermidine has also been reported to extend lifespan of aged mice by enhancing autophagy (Eisenberg et al., 2016). In a recent paper, spermidine has been shown to induce hypusination of eIF5A which is required for efficient translation of poly-proline proteins such as TFEB and ATG3, which led to the reversal of immune senescence of B lymphocytes (Zhang H. et al., 2019) and protection against premature brain aging or mitochondrial dysfunction (Liang et al., 2021) (**Figure 2**). The effect of spermidine on T cell autophagy has also been reported. Spermidine has been shown to enhance cytokine response of T cells from old vaccinees with suppressed autophagic activity by maintaining TFEB and autophagy (Alsaleh et al., 2020). It will be intriguing to investigate whether spermidine administration can reverse senescence or aging of other types of cells through similar mechanisms.

Trehalose

Trehalose is a glucose dimer acting as a chemical chaperone, and has been reported to enhance autophagy. The mechanism of autophagy induction by trehalose has been attributed to inhibition of solute carrier 2A (SLC2A), a glucose transporter called GLUT, by trehalose, leading to activation of 5′ AMP-activated protein kinase (AMPK) and autophagy (DeBosch et al., 2016). However, since SLC2A inhibition was observed at high concentrations of trehalose above 10 mM, it is still not clear whether inhibition of glucose transporter can entirely explain autophagy activation by trehalose. Trehalose has also been reported to activate transcription of autophagy genes or transcription factor EB (TFEB), a master regulator of autophagy gene expression and lysosome biogenesis through low-grade lysosomal stress in an mTOR-independent FOXO1-dependent manner (Sarkar et al., 2007a; Dehay et al., 2010; Castillo et al., 2013; Jeong et al., 2021) (**Figure 2**).

Regarding therapeutic potential, trehalose has been shown to improve glucose profile and insulin sensitivity of obese mice with autophagy insufficiency, which was accompanied by amelioration of fatty liver (Lim et al., 2014). The effect of trehalose has also been tested in mice expressing amyloidogenic human-type *IAPP* fed HFD. In these mice, administration of trehalose improved glucose profile and augmented insulinogenic index representing β-cell function. Accumulation of hIAPP oligomer and amyloid in pancreatic islets was also attenuated by trehalose (Kim et al., 2014). These results suggest possible therapeutic potential of autophagy enhancers in human-type diabetes.

Trehalose is degraded by intestinal trehalase, which may affect bioavailability of ingested trehalose. Lentztrehalose, a novel analog of trehalose with reduced susceptibility to trehalase, has been produced (Wada et al., 2015). Lactotrehalose, another trehalase-resistant, has been shown to ameliorate fatty liver after high-sucrose diet feeding (Zhang et al., 2020b). Contrary to these reports showing autophagy-enhancing effect of trehalose, a previous paper has questioned autophagy enhancing activity of trehalose (Kaizuka et al., 2016).

Triethylenetetramine Dihydrochloride

TETA, a Cu^{2+}-chelating agent that has been used for treatment of Wilson's disease, has been shown to improve metabolic profile of HFD-fed or *ob/ob* mice. Such metabolic effects were related not to chelation of Cu^{2+} but to stabilization and increased activity of spermidine N^1-acyltransferase (SAT1) that is also necessary for beneficial effects of spermidine on host metabolism (Castoldi et al., 2020) (**Figure 2**).

Other Autophagy Enhancers in Metabolic Diseases

Digoxin; ikarugamycin, a natural marine-derived product; and alexidin dihydrochloride, a synthetic compound, have been identified in a screening for TFEB agonists and have been reported to have beneficial metabolic effects on metabolic syndrome after HFD feeding and to extend lifespan of *C. elegans* (Wang et al., 2017). Dioscin, a natural steroid saponin, has been reported to improve metabolic profile and liver steatosis of obese mice through autophagy upregulation (Liu M. et al., 2015).

Known Drugs Against T2D or Other Metabolic Diseases

Some of the effects of drugs that are already being used in the clinic against T2D or other metabolic diseases have been ascribed to autophagy enhancement. Such drugs may work through both the originally proposed mechanisms and autophagy modulation, while the proportion of autophagy modulation in the pharmacological effects of the drug could be different from one drug to another.

Metformin

Metformin is a widely-used anti-diabetic medicine that has been recommended as the first-line drug against diabetes. While metformin has AMPK-independent mechanisms for improvement of metabolic profile (Kalender et al., 2010), most investigators concur with the opinion that metformin activates AMPK (Zhou et al., 2003). Then, metformin would be able to enhance autophagic activity since AMPK activation can upregulate autophagic activity through direct phosphorylation of ULK1 and Beclin 1, key molecules in the initiation of autophagy, or inhibition of mTORC1 (Egan et al., 2011; Howell et al., 2017; Kim J. et al., 2013; Kim et al., 2011) (**Figure 2**). Autophagy induction by AMPK activation is in line with the concept that autophagy is an adaptive process in response to nutrient deficiency and that AMPK is a sensor of intracellular energy balance. Hence, well-established AMPK activation by metformin suggests the possibility that improvement of metabolic profile by metformin might be related to autophagy induction through AMPK activation. Consistent with this concept, protection of pancreatic β-cells against lipoapoptosis by metformin was attributed to autophagy activation (Wu et al., 2013; Jiang et al., 2014). Metformin has also been shown to enhance disposal of accumulated autophagic vacuoles in β-cells (Masini et al., 2009), and to alleviate aging-

associated inflammation, fatty liver disease or diabetic kidney disease through autophagy induction (Song et al., 2015; Ren et al., 2019; Bharath et al., 2020). Thus, it is likely that anti-diabetic effect of metformin could be partly due to autophagy activation, in addition to autophagy-independent effects such as inhibition of mitochondrial complex I activity or alteration of gut microbiota (Owen et al., 2000; Shin et al., 2014).

GLP-1 Agonist

GLP-1 receptor agonists such as liraglutide have been reported to enhance autophagy level of insulinoma cells (Chen et al., 2013). While GLP-1 receptor agonists may upregulate autophagy in several tissues including the liver and thereby improve liver steatosis (Sharma et al., 2011; Noyan-Ashraf et al., 2013; Fang et al., 2020), the mechanism of autophagy induction by of GLP-1 receptor or other G-protein-coupled receptors (GPCR) is unclear (Wauson et al., 2014). In this regard, role of increased AMPK or cGMP and reduced mTOR or GSK3β signaling have been suggested (Candeias et al., 2018; Yang et al., 2018). Increases of autophagy by GLP-1 receptor agonists might also be related to the amelioration of ER stress (Yusta et al., 2006).

Additionally, GLP-1 agonists have been reported to have therapeutic effects on diabetic kidney disease (Yamada et al., 2021) through promotion of autophagy.

SGLT2 Inhibitor

Sodium-glucose transporter 2 (SGLT2) inhibitor is a novel class of anti-diabetic drug acting by increasing urinary loss of glucose and inducing a starvation-like state. SGLT2 inhibitors have been shown to enhance autophagic activity through AMPK activation (Hawley et al., 2016). SGLT2 inhibitors have also been reported to induce autophagy in diabetic heart and kidney tissues, which might be related to cardio- or reno-protective effects of SGLT2 inhibitors (Packer, 2020a; Packer, 2020b).

PPAR-γ Agonist

Peroxisome proliferator-activated receptor γ (PPAR-γ) is a transcriptional factor regulating expression of genes involved in adipogenesis and lipid uptake (Spiegelman, 1998). Rosiglitazone, a representative PPAR-γ agonist, has been reported to protect β-cells against lipoapoptosis, which was ascribed to the activation of autophagy through AMPK (Wu et al., 2013). On the other hand, inhibition of autophagy by rosiglitazone has also been reported (Ji et al., 2018; Li et al., 2018).

Pioglitazone, another PPAR-γ agonist, has also been reported to ameliorate fatty liver disease through autophagy activation (Hsiao et al., 2017).

PPAR-α Agonist

PPAR-α is a transcriptional factor which plays a crucial role in the expression of genes involved in fatty acid oxidation, lipid transport and ketosis (Torra et al., 2000). PPAR-α is induced by fasting (Kersten et al., 1999), suggesting its potential relationship with autophagy induction by fasting. Indeed, PPAR-α has been shown to bind promoters of several autophagy genes. Furthermore, GW7647, a PPAR-α agonist, has been reported to reverse feeding-induced autophagy suppression (Lee et al., 2014). Fenofibrate, a classical PPAR-α agonist, has been reported to alleviate HFD-induced kidney injury in a diabetic kidney disease model probably through autophagy activation (Sohn et al., 2017). While not directly related to diabetes or metabolic diseases, fenofibrate has also been reported to suppress cartilage degeneration *in vitro* through autophagy activation, and use of fenofibrate has been associated with improved clinical course of osteoarthritis in human patients (Nogueira-Recalde et al., 2019).

On the contrary, FXR agonist was reported to compete with PPAR-α for binding to promoters of autophagy genes and to suppress autophagy (Lee et al., 2014).

AUTOPHAGY MODULATORS IN OTHER DISEASES

Autophagy modulators have been developed for diseases other than metabolic disorders (**Table 2**).

Neurodegenerative Diseases

In the pathogenesis of many neurodegenerative diseases, accumulation of amyloidogenic, aggregate or misfolded proteins plays a critical role. Since such proteins are preferentially cleared by autophagy or lysosomal degradation pathway rather than proteasomal degradation pathway, potential relevance of autophagy modulators as future therapeutic agents against neurodegenerative diseases is immense (Rubinsztein, 2006). Defective autophagy or lysosomal dysfunction has been demonstrated to play a crucial pathogenic role in diverse neurodegenerative diseases such as Parkinson's disease or Alzheimer's disease (Nixon, 2007; Narendra et al., 2008; Bordi et al., 2016). In addition, aging, one of the most important risk factors for neurodegenerative diseases such as Alzheimer's disease (Thelen and Brown-Borg, 2020), diminishes autophagic activity in multiple tissues in a variety of non-mammalian and mammalian species (Sun et al., 2015; Hansen et al., 2018). For instance, the role of autophagy in aging or longevity is clearly seen in lower organisms such as *C. elegans* since knockdown of *Beclin 1* reduced lifespan of *C. elegans* (Melendez et al., 2003). In higher or vertebrate organisms, the role of autophagy in aging is less clear. However, a couple of papers suggested an important role of autophagy in aging or longevity of mammals. Overexpression of an autophagy gene has also been reported to prolong lifespan of mice and to improve metabolic profile of aged mice (Pyo et al., 2013). Furthermore, autophagic or mitophagic activity and lysosomal function decline with aging (Hütter et al., 2007; Salminen and Kaarniranta, 2009; Sun et al., 2015; Fernando et al., 2020), while the details of the changes could be different depending on the tissue and species. These results suggest that autophagy could be intimately related to aging of mammals as well.

Given the role of autophagy deficiency or lysosomal dysfunction in the pathogenesis of neurodegenerative disorders and the close relationship between autophagy or lysosomal function and aging, autophagy enhancers could have beneficial effects against diverse neurodegenerative disorders as discussed below. Further detailed discussion regarding the role of

TABLE 1 | Autophagy enhancers that have beneficial effects on T2D or metabolic syndrome.

Drugs or chemicals	Mechanism and effects	Molecular targets	References	Clinical trial (phase)
Berberine	improved metabolic profile	AMPK or SIRT1	Ma, et al., 2018; Sun et al., 2018; Zheng et al., 2021	1, 2
Imatinib	Improved metabolic syndrome or diabetes	Kit or Abl; Beclin 1	Breccia et al., 2005; Han et al., 2009; Veneri et al., 2005; Lim et al., 2014	—
MSL-7	Enhanced clearance of lipid in the liver and amelioration of metabolic inflammation	TFEB	Lim et al. (2018)	—
	Enhanced clearance of hIAPP oligomer and improved β-cell function of *hIAPP*$^{+}$ mice on HFD		Kim et al. (2021b)	—
Rapamycin	Improved metabolic profile and β-cell function of Akita mice	mTOR	Bachar-Wikstrom et al. (2013)	—
	Increased longevity of mice		Harrison et al. (2009)	2
Resveratrol	Improved metabolic profile and extended healthspan of HFD-fed mice	SIRT1	Baur et al. (2006)	2
	Improved metabolic profile of patients with T2D		Bo et al. (2016)	2
Rg2	Improved glucose profile, insulin sensitivity and fatty liver change of HFD-fed mice	—	Fan et al. (2017)	—
Spermidine	Improved metabolic profile and liver steatosis in HFD-fed mice	EP300 eIF5A	Ma et al. (2021)	—
	Extended lifespan of *C. elegans*, *Drosophila* and aged mice		Eisenberg et al. (2009)	—
	Induction of hypusination of eIF5A and enhanced translation of TFEB		Zhang et al. (2019b)	—
Trehalose	Improved glucose profile and insulin sensitivity of obese mice	TFEB; AMPK	Lim et al. (2014)	—
	Decreased accumulation of hIAPP oligomer and amyloid in pancreatic islets		Kim et al. (2014)	—
	Improved fatty liver		Zhang et al. (2020c)	—
Triethylene-tetramine dihydrochloride (TETA)	improved metabolic profile of HFD-fed or *ob/ob* mice	SAT1	Castoldi et al. (2020)	—
Digoxin	Beneficial metabolic effects on metabolic syndrome after HFD; extension of *C. elegans* lifespan	TFEB	Wang et al. (2017)	1
Dioscin	Improved metabolic profile and liver steatosis of obese mice	—	Liu et al. (2015a)	—
Metformin	Protection of pancreatic β-cells against lipoapoptosis	AMPK	Jiang et al., 2014; Wu et al., 2013	3, 4
	Alleviation of aging-associated inflammation, fatty liver disease or diabetic kidney disease		Bharath et al., 2020; Ren et al., 2019; Song et al., 2015	3
GLP-1 agonist	Improved liver steatosis	AMPK or cGMP	Fang et al., 2020; Noyan-Ashraf et al., 2013; Sharma et al., 2011	4
	Improved diabetic nephropathy		Yamada et al. (2021)	4
SGLT2 inhibitor	Cardio- and reno-protective effects	AMPK	Packer, 2020a, Packer, 2020b	1, 4
PPAR-γ agonist	Protected β-cells against lipoapoptosis	AMPK	Wu et al. (2013)	—
	Ameliorated fatty liver disease		Hsiao et al. (2017)	4
PPAR-α agonist	Alleviated HFD-induced kidney injury in a diabetic kidney disease model	Autophagy genes	Sohn et al. (2017)	—
	Improved clinical course of clinical osteoarthritis in human patients		Nogueira-Recaldea et al. (2019)	—

Abbreviations: HFD, high-fat diet; TFEB, transcription factor EB.

TABLE 2 | Autophagy enhancers that have beneficial effects on diseases other than metabolic disorders.

Drugs or chemicals	Mechanism and effects	Molecular targets	Target diseases	References	Clinical trial (phase)
AUTEN-67	Retarded disease progression	MTMR14	Huntington's disease or Alzheimer's disease	Billes et al., 2016; Papp et al., 2016	—
EN6	Accelerated clearing of TDP-43 aggregates	ATP6V1A	ALS	Chung et al. (2019)	—
ERRα Inverse Agonist	Enhancement of autophagosome fusion to lysosome	Autolyso-some	Parkinson's disease	Suresh et al. (2018)	—
HTT Linker	Ameliorated disease phenotype	LC3	Huntington's disease	Li et al. (2019)	—
ML246	Improved memory	Beclin 2	Alzheimer's disease	Rocchi et al., 2017; Kuramoto et al., 2016	—
ML-SA1	Protection of dopaminergic neurons	TFEB	ALS and Parkinson's disease	Tedeschi et al., 2019; Tsunemi et al., 2019	—
	Reduced apoptosis of photoreceptor cells		Retinal detachment	Yan et al. (2021)	—
Nilotinib	Degradation of α-synuclein and reversion of dopaminergic neuron loss	Beclin 1, Atg12	Parkinson disease	Hebron et al. (2013)	1,2
SMER	Increased clearance of mutant huntingtin or α-synuclein	—	Huntington's disease	Sarkar et al. (2007b)	—
	Attenuated neurodegeneration		Alzheimer's disease	Tian et al. (2011)	—
	Increased erythropoiesis		Diamond-Blackfan syndrome	Doulatov et al. (2017)	—
Spermidine	Improved cognition	PARKIN	Aging	Lazarou et al., 2015; Schroeder et al., 2021	—
	Improved cardiac function		Heart failure	Eisenberg et al. (2016)	—
Tat-beclin 1	Improved cognitive function	GAPR-1	Alzheimer's disease	Shoji-Kawata et al. (2013)	—
	Beneficial effects on cardiac function after TAC		Heart failure	Shirakabe et al. (2016)	—
	Reduced microbial replication and improved clinical outcome of infection		*Listeria* monocytogenes; Sindvis virus, chikungunya virus, West Nile virus, HIV	Shoji-Kawata et al. (2013)	—
Trehalose	Reduced Aβ deposition and improved cognitive deficit and learning disability	TFEB	Alzheimer's disease	Du et al. (2013)	1
	Reduced atherosclerotic plaque burden in *ApoE*-KO mice fed western diet		Atherosclerosis	Sergin et al. (2017)	2
	Attenuated inflammation of the brain		Mucopolysacchari-dosis IIIb	Lotfi et al. (2018)	—
UMI-77	Ameliorated neurological deficit	MCL-1	Alzheimer's disease	Cen et al. (2020)	—
Felodipine	Neuroprotection	—	Huntington's disease	Siddiqi et al. (2019)	—
Rilmenidine	Amelioration of neurological signs	—	Huntington's disease	Rose et al. (2010)	—
Lithium	Clearance of α-synuclein aggregate and mutant Huntingtin	Inositol monophos-phatase	Parkinson's disease; Huntington's disease	Sarkar et al. (2005)	1, 2
GLP-1 agonists	Improved motor function	mTOR	Parkinson disease	Zhang et al. (2020a)	—
	Attenuated fibrosis after aortic banding		Heart Diseases	Zheng et al. (2019)	—
PPAR-α agonist	Improved cognition	Autophagy genes	Alzheimer's disease	Luo et al. (2020)	1
Sildenafil	Improved hear failure	PGK1	Heart Diseases	Ranek et al. (2019)	4
Metformin	Ameliorated ultrastructural abnormalities	AMPK	Diabetic Heart Diseases	Xie et al. (2011)	
	Autophagy induction		Diabetic kidney disease	Kaushal et al. (2020)	4
Fenofibrate	Prevention of fibrosis in an animal model of T1D	Autophagy genes	Heart Diseases	Zhang et al. (2016)	—

(Continued on following page)

TABLE 2 | (*Continued*) Autophagy enhancers that have beneficial effects on diseases other than metabolic disorders.

Drugs or chemicals	Mechanism and effects	Molecular targets	Target diseases	References	Clinical trial (phase)
Artemisinin	Alleviation of atherosclerosis	AMPK	Atherosclerosis	Cao et al. (2019)	—
Fucoidan	Alleviation of atherosclerosis	TFEB	Atherosclerosis	Cheng et al. (2020)	—
Rapamycin	Ameliorated damage after coronary ligation	mTOR	Ischemic heart disease	Sciarretta et al. (2012)	—
Rapamycin	Suppressed photoreceptor degeneration or inflammation	mTOR	Retinitis	Okamoto et al. (2016)	—
Temsirolimus	Protected against sepsis-induced acute renal failure	mTOR	Kidney Diseases	Howell et al. (2013)	2
Urolithin A	Ameliorated I/R-induced injury	TFEB	Kidney Diseases	Wang et al. (2019b)	
Atrasentan	Amelioration of diabetic kidney disease and enhancement of Foxo1 expression	miR-21	Kidney Diseases	Wang et al. (2019a)	2
Klotho	Ameliorated FK506-induced injury	TFEB	Kidney Diseases	Lim et al. (2019)	—
A77 1726	Restriction of bacterial growth	AMPK	Infection (*Salmonella*)	Zhuang et al. (2020)	—
Flubendazole	Promoted clearance of intracellular bacteria	mTOR	Infection (HIV)	Chauhan et al. (2015)	—
BC18630	Reduced viral load and lung injury	TFEB	Infection (SARS-CoV-2)	Liu et al. (2021)	—
2-hydroxypropy l-β-cyclodextrin	Accelerated clearance of proteolipid aggregates	TFEB	Neuronal ceroid lipofuscinosis	Song et al. (2014)	—
Rosiglitazone	Attenuated post-operative fibrosis	Beclin 1	Glaucoma	Zhang et al. (2019a)	—
Artesunate (ART)	Ameliorated retinopathy	AMPK/SIRT1	Diabetic retinopathy	Li et al. (2021)	—
Apigenin	Improved clinical index and reduce intraocular oxidative damage	Nrf2	Age-related macular degeneration	Zhang et al. (2020b)	—
Latrepirdine	Improved cognition	mTOR	Alzheimer's disease	Steele and Gandy, (2013)	—
Calpastatin	Improved motor function and decrease tremor	Calpain	Huntington's disease	Menzies et al. (2015)	—
6-Bio	Improved motor coordination and locomotion	GSK3b	Parkinson's disease	Suresh et al. (2017)	—
Vitamin D	Inhibited viral replication	Beclin 1	HIV infection	Campbell and Spector, (2011)	2, 3
Acacetin	Reduced bacterial burden *in vivo*	TFEB	*Salmonella*	Ammanathan et al. (2020)	—

Abbreviations: ALS, amyotrophic lateral sclerosis; T1D, Type 1 diabetes; TAC, Transverse aortic constriction.

autophagy specifically in neurodegenerative diseases can be found in recent review articles (Rahman and Rhim, 2017; Park et al., 2020). Several autophagy enhancers against neurodegenerative diseases not covered in the text can be found in **Table 2**.

AUTEN-67

AUTEN-67 (Autophagy Enhancer-67), an MTMR14 inhibitor, has been reported to impede progression of the diseases in a *Drosophila* model of Huntington's disease and in a mouse model of Alzheimer's disease (Billes et al., 2016; Papp et al., 2016).

EN6

A small molecule autophagy activator (EN6) that covalently targets cysteine 277 of ATP6V1A subunit of lysosomal v-ATPase was identified in a screening using cysteine- and lysine-reactive ligands. EN6 decoupled v-ATPase from the Ragulator-Rag complex and induced mTORC1 inhibition, leading to accelerated clearing of TDP-43 aggregates through autophagy enhancement (Chung et al., 2019).

ERRα Inverse Agonist

It was reported that overexpression of ERRα inhibits autophagosome fusion to lysosome (Suresh et al., 2018). XCT 790, an inverse agonist of ERRα, was shown to exert neuroprotective effects in a preclinical model of Parkinson's disease through autophagy upregulation (Suresh et al., 2018).

HTT Linker

Recently, compounds linking target substrates to LC3 have been developed. An HTT-LC3 linker has been reported to ameliorate

Huntington's disease phenotype by tethering mutant Huntingtin to LC3 (Li et al., 2019). This compound belongs to the class of autophagosome tethering compounds (ATTECs).

ML246

ML246, a brain-permeable autophagy enhancer, has been shown to enhance clearance of Aβ, protect neuronal cells from Aβ-induced cell death, and improve memory of Alzheimer's disease mice, which was associated with a trend toward reduced brain amyloid accumulation (Rocchi et al., 2017). ML246 has also been shown to prevent cannabinoid tolerance by inhibiting Beclin 2 (BECN2) binding to GRASP1, a receptor for cannabinoid receptor 1 (CNR1) degradation (Kuramoto et al., 2016), which might be employed to enhance medical effects of cannabinoid such as analgesia.

ML-SA1

Transient receptor potential cation channel, mucolipin subfamily 1 (TRPML1) channel is a lysosomal Ca^{2+} exit channel that is important in activation of TFEB, a master regulator of autophagy gene expression and lysosomal biogenesis (Wang et al., 2015). ML-SA1, an agonist of TRPML channel (Shen et al., 2012), has been reported to protect dopaminergic neurons in cell models of amyotrophic lateral sclerosis (ALS) and Parkinson's disease by boosting autophagy or enhancing lysosomal Ca^{2+} exocytosis (Tedeschi et al., 2019; Tsunemi et al., 2019).

Nilotinib

As an Abl inhibitor related to aforementioned Imatinib, Nilotinib has been shown to induce degradation of α-synuclein and reverse loss of dopaminergic neurons through autophagy activation which was associated with increased levels of Beclin 1 and Atg12 (Hebron et al., 2013). Radotinib, a related compound, has also been reported to be neuroprotective in a preclinical Parkinson disease model (Lee et al., 2018). However, recent phase 2 clinical trials using Nilotinib showed mostly negative results (Simuni et al., 2021). A recent paper also reported no effect of Nilotinib on R6/2, an animal model of Huntington's disease (Kumar et al., 2021).

SMER

SMERs were initially selected by small-molecule screening in yeast and, their activity on mammalian autophagy was confirmed employing a cell system overexpressing A53T α-synuclein, an autophagy substrate. SMER10 (aminopyrimidone), SMER18 (vinylogous amide), SMER28 (bromo-substituted quinazoline) and their analogs increased autophagic clearance of mutant huntingtin or α-synuclein *in vitro* in an-mTOR-independent manner, and also attenuated neurodegeneration in *D. melanogaster* model of Huntington's disease (Sarkar et al., 2007b). Later studies showed that SMER28 can accelerate *in vitro* clearance of Aβ peptide or carboxy terminal fragment of amyloid precursor protein (APP-CTF) (Tian et al., 2011). Besides neurodegenerative disorders, SMER28 has been reported to increase erythropoiesis in cell or animal models of Diamond-Blackfan syndrome, a disorder of abnormal erythroid progenitor differentiation, through *Atg5* (Doulatov et al., 2017).

Spermidine

Aforementioned spermidine was recently reported to improve cognitive dysfunction in aged mice, which was associated with increased mitochondrial respiration and dependent on *Atg7* and *PINK1*, a kinase inducing mitophagy through recruitment of PARKIN onto dysfunctional mitochondria (Lazarou et al., 2015; Schroeder et al., 2021).

Tat-Beclin 1

HIV Nef-interacting domain of Beclin 1 attached to Tat transduction domain (Tat-beclin 1) could enhance autophagic flux by binding to GAPR-1, an autophagy inhibitor, and liberating Beclin 1 from Golgi complex (Shoji-Kawata et al., 2013) (**Figure 1**). Tat-beclin 1 has been reported to accelerate clearance of htt103Q aggregate or Aβ oligomer and to improve cognitive function of Alzheimer's disease model mice (Shoji-Kawata et al., 2013). In contrast, a high dose of Tat-beclin 1 has been reported to induce autosis, a special form of cell death characterized by high autophagic activity, inhibition by Na^+/K^+ ATPase inhibitors and unique morphological features including focal rupture of plasma membrane, electron-dense swollen mitochondria and ballooning of perinuclear space (Liu et al., 2013). Furthermore, Tat-Beclin 1 peptide has been shown to aggravate hypoxic injury of the rat brain, which might be related to the inhibition of autophagosome-lysosome fusion due to elevated expression of Rubicon (Nah et al., 2020).

Trehalose

Trehalose, a disaccharide with autophagy enhancing activity as discussed above, was also shown to exert effects on neurodegeneration and aging. Trehalose could prolong total and reproductive life span, retard decrease in pharyngeal pumping due to aging, enhance thermotolerance and suppress polyQ aggregation in *C. elegans*, while the role of autophagy in trehalose effects was not directly demonstrated in this paper (Honda et al., 2010). Trehalose also reduced Aβ deposition and improved cognitive deficit and learning disability in a mouse model of Alzheimer's disease (Du et al., 2013).

UMI-77

In a screening using an FDA-approved drug library, UMI-77, a BH3-mimetic for MCL-1, was identified as an inducer of mitophagy without effects on apoptosis. MCL-1 was suggested to be a mitophagy receptor, and UMI-77 ameliorated neurological deficit in a mouse model of Alzheimer's disease (Cen et al., 2020).

Other Previously Known Chemicals or Drugs

In several screening for autophagy modulators, some known chemicals or drugs have been identified and shown to enhance clearance of aggregate or amyloid proteins associated with neurodegeneration. For instance, GFP-LC3-based screening followed by assay of $FYVE\text{-}RFP^+$ vesicle identified eight known chemicals inducing autophagic degradation of long-lived proteins in an mTOR-independent manner (Fluspirilene, trifluoperazine,

pimozide, nicardipine, penitrem A, niguldipine, loperamide and amiodarone). Those chemicals also reduced accumulation of polyQ (Zhang et al., 2007).

Another autophagy enhancer screening using EGFP-LC3 vesicles identified verapamil, valproate and clonidine as autophagy enhancers acting through decreased $(Ca^{2+})_i$ or intracellular inositol 1,4,5-trisphosphate (IP_3) content and calpain inhibition (Williams et al., 2008). Such chemicals have been reported to delay Huntington's disease-like phenotypes in *Drosophila* or zebrafish models. A similar screening of autophagy enhancer using GFP-RFP-LC3 system and a chemical library identified flubendazole, an anti-helminthic agent, and bromhexine as autophagy enhancers. Flubendazole led to disrupted dynamic microtubule, followed by displacement and inhibition of mTOR. Flubendazole and bromhexine were also reported to reduce accumulation of total and hyperphosphorylated Tau (Chauhan et al., 2015). Felodipine, an anti-hypertensive drug acting on L-type Ca^{2+} channel similar to verapamil, has also been reported to have autophagy-enhancing and neuroprotective effects in animal models of Huntington's disease (Siddiqi et al., 2019). Rilmenidine, another anti-hypertensive drug, has been reported to enhance autophagy and to ameliorate neurological signs in an animal model of Huntington's disease (Rose et al., 2010).

Lithium, a well-known drug for bipolar disease, has been reported to induce autophagy through inhibition of inositol monophosphatase and depletion of free inositol or IP_3. Lithium has been shown to induce clearance of α-synuclein aggregate or mutant Huntingtin (Sarkar et al., 2005). A recent paper showed the effect of lithium in the clearance of Tau aggregate (Uddin et al., 2021).

GLP-1 agonists, aforementioned anti-diabetic drugs with potential autophagy enhancing activity, have also been reported to confer therapeutic effects on Parkinson disease animal models (Zhang L. et al., 2020).

PPAR-α agonist such as gemfibrozil or Wy14643 has been reported to ameliorate Alzheimer's disease-related phenotype in a murine model through autophagy induction (Luo et al., 2020).

Heart Diseases

Autophagy is crucial in the development of the heart and in the maintenance of cardiac function (Gatica et al., 2021). Dysregulated autophagy has been reported to be involved in the pathogenesis of several cardiovascular diseases.

As discussed above, spermidine induced autophagy through epigenetic regulation of autophagy genes (Pietrocola et al., 2015), and could extend lifespan of experimental animals (Eisenberg et al., 2016). Spermidine has also been reported to improve cardiac function in age- or hypertension-associated heart failure models (Eisenberg et al., 2016).

Sildenafil, an inhibitor of phosphodiesterase type-5, can activate protein kinase G1 (PGK1) that has been shown to phosphorylate TSC2 and induce autophagy through mTORC1 inhibition (Ranek et al., 2019). Sildenafil has been reported to reverse autophagy inhibition and heart failure after cardiac pressure overload in a phosphorylation-silencing *Tsc2* mutant-knockin mouse model (Ranek et al., 2019).

Everolimus, a relatively selective inhibitor of mTORC1, also had similar effects on failing heart in the same mice.

Metformin, a well-known anti-diabetic drug, has been reported to enhance cardiac autophagic activity and ameliorates cardiac ultrastructural abnormalities associated with diabetes through AMPK activation in an animal model of diabetic cardiomyopathy (Xie et al., 2011).

GLP-1 agonists have also been reported to have therapeutic effects on myocardial fibrosis after aortic banding through autophagy enhancement (Zheng et al., 2019).

Fenofibrate, a classical PPAR-α agonist, has been reported to prevent cardiac fibrosis in an animal model of type 1 diabetes (T1D), accompanied by enhanced autophagic activity *in vivo* (Zhang et al., 2016).

Tat-Beclin 1, aforementioned autophagy enhancer liberating beclin 1 from Golgi complex, was shown to confer beneficial effects on heart failure after transverse aortic constriction (TAC) (Shirakabe et al., 2016), which is consistent with a previous report that cardiac autophagy plays a protective role in ischemic cardiac disease of mice with diet-induced obesity (Sciarretta et al., 2012). On the other hand, Tat-Beclin 1 peptide has been shown to aggravate cardiac ischemia-reperfusion (I/R)-induced cardiac injury and autosis, which was accompanied by increased expression of Rubicon inhibiting multiple steps of autophagy including autophagosome-lysosome fusion (Nah et al., 2020).

Another example showing deleterious effect of autophagy on cardiac tissue has been published. A paper reported that dimethyl α-ketoglutarate inhibited mal-adaptive cardiac autophagy and suppressed TAC-induced heart failure in a pressure-overload-induced cardiomyopathy model after conversion to cytosolic acetyl CoA acting on EP300, an acetyltransferase (Mariño et al., 2014).

In addition to heart failure, atherosclerosis is another heart disease that is closely related to autophagy, particularly that of macrophages (Liao et al., 2012). Trehalose, an aforementioned autophagy enhancer, could reduce atherosclerotic plaque burden in *ApoE*-KO mice fed western diet by enhancing autophagy (Sergin et al., 2017).

Spermidine, an autophagy enhancer discussed above, has also been reported to attenuate atherosclerosis and to inhibit necrotic core formation through autophagy activation (Michiels et al., 2016).

Artemisinin is an endoperoxide sesquiterpene lactone with multiple beneficial effects such as anti-malarial, anti-inflammation and anti-oxidant effects. Artemisinin has also been reported to attenuate atherosclerosis in HFD-fed *ApoE*-KO mice by enhancing autophagic activity of macrophages (Cao et al., 2019).

Fucoidan, a marine sulfated polysaccharide derived from brown seaweeds with anti-inflammatory activity, has also been reported to alleviate atherosclerosis in HFD-fed *ApoE*-KO mice by enhancing autophagic activity (Cheng et al., 2020).

In ischemic heart disease, Rapamycin, a classical mTOR inhibitor and autophagy enhancer, could ameliorate cardiac damage after coronary ligation in mice fed HFD by enhancing

autophagy and protecting against cardiomyocyte death (Sciarretta et al., 2012).

Further detailed discussion regarding the role of autophagy specifically in cardiovascular diseases can be found in recent review articles (Lampert and Gustafsson, 2018; Gatica et al., 2021)

Kidney Diseases

Kidney is a critical target organ of diabetic complication. Previous papers showed the role of altered autophagy in the development of several kidney diseases including diabetic nephropathy and kidney fibrosis (Liu W. J. et al., 2015; Nam et al., 2019; Zhao et al., 2019). Several autophagy enhancers have been administered to such diverse kidney disease models.

Temsirolimus, an autophagy enhancer belonging to mTORC1 inhibitor family, has been administered to septic kidney disease model with beneficial clinical effects accompanied by autophagy enhancement (Howell et al., 2013). A variety of authentic or potential AMPK activators such as metformin, ω-3 polyunsaturated fatty acids, quercetin, neferine, astragaloside IV, mangiferin, cinacalcet, berberine, progranulin have also been administered to diabetic kidney disease, cisplatin-induced nephropathy or I/R kidney injury, leading to clinical improvement and autophagy enhancement (Kaushal et al., 2020).

Aforementioned autophagy enhancers such as fenofibrate, pioglitazone and SGLT2 inhibitors have also been employed to treat diabetic kidney disease or I/R kidney injury with clinical improvement.

Urolithin A, the main metabolite in pomegranate juice, has been reported to ameliorate I/R-induced kidney injury through TFEB activation (Wang Y. et al., 2019).

Atrasentan, an antagonist of endothelin 1 receptor subtype A (ET_A), was able to ameliorate diabetic kidney disease, by downregulating miR-21 expression, enhancing Foxo1 expression and upregulating autophagy (Wang J. et al., 2019).

Klotho, an obligate co-receptor for fibroblast growth factor 23 (FGF23), has also been reported to ameliorate FK506-induced renal injury with impaired lysosomal function by inducing TFEB activation through inhibition of GSK3β phosphorylation (Lim et al., 2019).

Further detailed discussion regarding the role of autophagy specifically in the kidney diseases can be found in recent review articles (Tang et al., 2020; Zheng et al., 2020).

Infection

Autophagy is also important antimicrobial defense mechanism against diverse infectious agents such as *Mycobacterium tuberculosis, Salmonella or Streptococcus*. Autophagy can contain cytosolic bacteria escaping from endosome/vacuole and induce maturation of phagosome into phagolysosome (Gutierrez et al., 2004; Nakagawa et al., 2004; Wild et al., 2008). In the intestine, secretory autophagy from Paneth cells mediates release of lysozyme, a critical antimicrobial peptide, and is an important host response against bacterial infection, which is defective in patients with Crohn's disease (Bel et al., 2017).

In a paper studying the effect of autophagy enhancer on infection, Tat-beclin 1 reduced replication of *Listeria monocytogenes* and viruses such as *Sindbis* virus, chikungunya virus, West Nile virus and HIV *in vitro* and improved clinical outcome of such infections *in vivo* (Shoji-Kawata et al., 2013).

A77 1726, an active metabolite of anti-inflammatory drug leflunomide, has been reported to restrict *Salmonella* growth through AMPK activation and autophagy enhancement (Zhuang et al., 2020).

SMER 28 has also been reported to have capability to kill *Mycobacterium* (Floto et al., 2008).

Flubendazole, an autophagy enhancer identified in a screening using GFP-RFP-LC3 system and a chemical library, inhibited HIV transfer from dendritic cells to T cells and promoted clearance of intracellular bacteria (Chauhan et al., 2015).

Everolimus or rapamycin has also been reported to suppress productive infection of HIV (Cloherty et al., 2021).

Intriguingly, therapeutic effect of a novel TFEB activator against COVID-19 was reported in a preprint paper. In corona virus infection, TFEB was shown to be degraded through proteasomal degradation. BC18630 selected by an *in silico* screening of inhibitors against DCAF7, a putative E3 ligase mediating degradation of TFEB, was shown to reduce viral load and lung injury in a Syrian hamster model of SARS-CoV-2 infection (Liu et al., 2021). Modulators of autophagy, lysosome or TFEB could have therapeutic effects against COVID-19 pandemic caused by infection with SARS-CoV-2 that egresses host cells through lysosomal trafficking and disrupts lysosomal function (Ghosh et al., 2020).

In contrast to the protective role of autophagy against infectious diseases, some pathogens such as *H. pylori* or uropathogenic *E. coli* have been reported to hijack host autophagic machinery for their survival and growth (Huab et al., 2020). In this case, inhibition of autophagy may have a therapeutic effect.

Lysosomal Storage Diseases

LSDs are a group of rare diseases in which lysosomal function is primarily impaired due to genetic causes and characterized by accumulation of excessive substrates in lysosome, leading to lysosomal dysfunction and a variety of systemic manifestations including neurodegeneration (Darios and Stevanin, 2020). As lysosomal dysfunction and autophagy impairment are observed in most of the LSDs, enhancement of autophagic or lysosomal activity could be a new modality to treat such diseases.

2-hydroxypropyl-β-cyclodextrin, an excipient and cholesterol-extracting agent, has been shown to activate TFEB and accelerate clearance of proteolipid aggregates in cells from patients with neuronal ceroid lipofuscinosis, a LSD (Song et al., 2014).

Trehalose has also been reported to attenuate inflammation of the brain and retina and to improve vision by activating TFEB and autophagic activity in a mouse model of mucopolysaccharidosis IIIB (MPS IIIB), a LSD caused by mutation of α-*N*-acetylglucosaminidase (NAGLU) (Lotfi et al., 2018).

Ocular Diseases

The risk of eye diseases such as macular degeneration is exponentially increasing in advanced age (Luu and Palczewski,

2018), which might be related to dysregulated autophagy in aging (Kivinen, 2018).

Rosiglitazone has also been reported to attenuate post-operative fibrosis in an animal model of glaucoma, which was associated with autophagy enhancement (Zhang F. et al., 2019).

Artesunate (ART) is a semi-synthetic derivative of aforementioned artemisinin, has also been reported to ameliorate diabetic retinopathy through AMPK/SIRT1 activation (Li et al., 2021).

ML-SA1, aforementioned agonist of lysosomal TRPML1 Ca^{2+} channel, has been shown to protect retinal structure, reduce apoptosis of photoreceptor cells and improve vision-dependent behavior in a rat model of retinal detachment, which was accompanied by enhanced autophagy (Yan et al., 2021).

Apigenin, a well-known antioxidant and anti-inflammatory flavonoid, has been reported to improve clinical index and reduce intraocular oxidative damage in a mouse model of age-related macular degeneration (AMD) by upregulating autophagy and expression of *Nrf2*, a master regulator of anti-oxidative gene expression (Zhang et al., 2020c).

Effects of Rapamycin in an animal model of endotoxin-induced uveitis and retinitis model have also been studied, which demonstrated suppression photoreceptor degeneration and inflammation together with inhibition of NF-κB or mTOR and enhancement of autophagy (Okamoto et al., 2016).

AUTOPHAGY INHIBITOR

Inhibitors of autophagy have been developed mostly for application in cancer. Autophagy has been reported to inhibit or accelerate the development of cancer, depending on the stage of carcinogenesis as summarized in the review (White, 2012). Thus, autophagy deficiency could promote the initiation of cancer by producing reactive oxygen species and inducing chromosomal abnormality. In a similar vein, cell death due to autophagy hyperactivation or autophagic cell death has been reported to limit chromosomal instability during replicative crisis causing telomeric DNA damage (Nassour et al., 2019). Some autophagy genes act as tumor suppressor genes (Qu et al., 2003; Liang et al., 2006; Cianfanelli et al., 2015). At the later stage of cancer development, autophagy can accelerate the progression of cancer by supplying amino acids or energy that is necessary for the progression of cancer through tumor cell-autonomous or non-tumor cell-autonomous manner (Kimmel and White, 2017). Autophagy of host cells might play a role in the maintenance of serum arginine level that is necessary for tumor growth (Poillet-Perez et al., 2021).

Several autophagy inhibitors have been developed as potential anti-cancer drugs such as chloroquine or ULK1 inhibitors (Chude and Amaravadi, 2017; Martin et al., 2018), exploiting metabolic requirement of cancer cells. For instance, chloroquine in conjunction with leucine-free diet has been reported to suppress growth of melanoma that is resistant to mTORC1 inhibition and autophagy activation by leucine deprivation (Sheen et al., 2011). In contrast, the effect of ULK1/2 inhibition has been questioned since abrogation of ULK1/2-mediated autophagy could not block extracellular protein-dependent cancer cell growth (Palm et al., 2015). Further discussion regarding the role of autophagy in the pathogenesis of cancer or that of autophagy modulators in the cancer treatment is beyond the scope of this article, and the readers are encouraged to consult excellent review papers (White, 2012; Amaravadi et al., 2017; Levy et al., 2017; Miller and Thorburn, 2021).

NEW APPROACHES FOR AUTOPHAGIC OR LYSOSOMAL DEGRADATION OF SELECTIVE TARGETS

Recently, novel autophagic or lysosomal protein degradation techniques such as lysosome targeting chimera (LYTAC), autophagy-targeting chimera (AUTAC) or autophagy-tethering compound (ATTEC) have been developed (Ding et al., 2021). LYTAC comprises an antibody fused to mannose-6-phosphate (M6P) that can be recognized by cation-independent M6P receptor (CI-M6PR), a lysosomal trafficking receptor (Banik et al., 2020). Using this method, extracellular targets such as apolipoprotein E (APOE) or plasma membrane-bound targets such as epidermal growth factor receptor (EGFR) could be targeted to endosome or lysosome for degradation. AUTAC consists of warheads to selective targets, linker and guanine degradation tag inducing S-guanylation-mediated autophagic degradation (Takahashi et al., 2019). This technique may allow degradation of large molecules, protein aggregates, organelles such as mitochondria or bacteria which has not been possible with PROTAC (proteolysis-targeting chimera) utilizing ubiquitin E3 ligase-binding ligands (Ding et al., 2021). ATTEC employs compounds interacting with both mutant huntingtin (HTT) protein targets and LC3, directing target proteins to autophagic degradation without ubiquitination (Li et al., 2019). These novel approaches will provide outstanding tools for the development of next-generation modulators of autophagy or lysosomal degradation.

CONCLUSION AND FUTURE PERSPECTIVES

While the significance of autophagy in the physiological function and pathogenesis of diverse diseases or aging is becoming more and more clear, authentic pharmacological autophagy modulators are still not available in the clinics except drugs that have already been used but were found to have autophagy-modulating effects later. Pharmacological modulation of specific processes or molecules among a wide array of the related genes or proteins at the specific sites is still not possible, precluding the use of autophagy modulators in the current circumstances. Lack of reliable biomarkers and suitable assay systems for measurement of autophagic flux in humans is also a big hurdle. Nonetheless, as detailed molecular mechanisms of selective autophagy are being discovered, specific modulation of specialized aspects of autophagy possibly at the specific tissues or sites will not be a remote possibility, which will be of a paramount importance for clinical application of autophagy modulators.

However, some caution might be exercised in the use of autophagy enhancer for treatment of human diseases.

Lysosomal dysfunction is frequently observed in diverse tissues of patients with Alzheimer's disease, obesity, T2D and associated conditions or aging (Hütter et al., 2007; Liu W. J. et al., 2015; Bordi et al., 2016; Zhao et al., 2019; Fernando et al., 2020; Kim et al., 2021a). In such conditions, accumulation of autophagic intermediates due to lysosomal dysfunction may be further increased after administration of autophagy enhancers activating earlier steps of autophagy, which might lead to autophagic stress or autophagic cell death (Zheng et al., 2020). Thus, it might be necessary to manage lysosomal dysfunction together with or before administration of autophagy enhancers. Possibility of aggravation of pre-existing cancer or infection due to pathogens exploiting host autophagic machinery for their survival by autophagy enhancers should also be kept in mind. Therefore, maintaining the exquisite balance of timing, duration and the extent of autophagy modulation depending on the types and stages of the diseases is crucial.

AUTHOR CONTRIBUTIONS

MS-L conceived the paper. KP and MS-L wrote the manuscript. All authors approved the submitted version.

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Evaluation of Parkin in the Regulation of Myocardial Mitochondria-Associated Membranes and Cardiomyopathy During Endotoxemia

Matthew Kim[1†], *Azadeh Nikouee*[1†], *Yuxiao Sun*[2], *Qing-Jun Zhang*[3], *Zhi-Ping Liu*[3] *and Qun Sophia Zang*[1*]

[1]Department of Surgery, Burn & Shock Trauma Research Institute, Loyola University Chicago Stritch School of Medicine, Maywood, IL, United States, [2]Department of Surgery, University of Texas Southwestern Medical Center, Dallas, TX, United States, [3]Internal Medicine-Cardiology Division, University of Texas Southwestern Medical Center, Dallas, TX, United States

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Xiaonan Dong,
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Giuseppe Arena,
University of Luxembourg, Luxembourg

***Correspondence:**
Qun Sophia Zang
qzang@luc.edu

[†]These authors have contributed equally to this work

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Background: Mitochondrial deficiency is a known pathology in sepsis-induced organ failure. We previously found that mitochondria-associated membranes (MAMs), a subcellular domain supporting mitochondrial status, are impaired in the heart during endotoxemia, suggesting a mechanism of mitochondrial damage occurred in sepsis. Mitophagy pathway *via* E3 ubiquitin ligase Parkin and PTEN-induced kinase 1 (PINK1) controls mitochondrial quality. Studies described here examined the impact of Parkin on cardiac MAMs and endotoxemia-induced cardiomyopathy. Additionally, point mutation W403A in Parkin was previously identified as a constitutively active mutation *in vitro*. *In vivo* effects of forced expression of this mutation were evaluated in the endotoxemia model.

Methods: Mice of wild type (WT), Parkin-deficiency (*Park2*$^{-/-}$), and knock-in expression of Parkin W402A (human Parkin W403A) were given lipopolysaccharide (LPS) challenge. Cardiac function was evaluated by echocardiography. In the harvested heart tissue, MAM fractions were isolated by ultracentrifugation, and their amount and function were quantified. Ultrastructure of MAMs and mitochondria was examined by electron microscopy. Mitochondrial respiratory activities were measured by enzyme assays. Myocardial inflammation was estimated by levels of pro-inflammatory cytokine IL-6. Myocardial mitophagy was assessed by levels of mitophagy factors associated with mitochondria and degrees of mitochondria-lysosome co-localization. Parkin activation, signified by phosphorylation on serine 65 of Parkin, was also evaluated.

Results: Compared with WT, *Park2*$^{-/-}$ mice showed more severely impaired cardiac MAMs during endotoxemia, characterized by disrupted structure, reduced quantity, and weakened transporting function. Endotoxemia-induced cardiomyopathy was intensified in *Park2*$^{-/-}$ mice, shown by worsened cardiac contractility and higher production of IL-6. Mitochondria from the *Park2*$^{-/-}$ hearts were more deteriorated, indicated by losses in both structural integrity and respiration function. Unexpectedly, mice carrying Parkin W402A showed similar levels of cardiomyopathy and mitochondrial damage when compared with

their WT counterparts. Further, Parkin W402A mutation neither enhanced mitophagy nor increased Parkin activation in myocardium under the challenge of endotoxemia.

Conclusion: our results suggest that Parkin/PINK1 mitophagy participates in the regulation of cardiac MAMs during endotoxemia. Point mutation W402A (human W403A) in Parkin is not sufficient to alleviate cardiomyopathy induced by endotoxemia *in vivo*.

Keywords: mitophagy, mitochondria, parkin, cardiac dysfunction, inflammation, sepsis, endotoxemia

INTRODUCTION

Sepsis is currently a leading cause of fatality in critical care units (Singer et al., 2016). Despite improvements in antibiotic therapies and critical care techniques (Levy et al., 2010), reported incidences of sepsis are still rising (Iwashyna et al., 2010). Thus, to understand the molecular mechanisms of sepsis pathogenesis and to explore new therapeutic interventions for this critical condition are in urgent need.

Cardiomyopathy is a critical component of multi-organ failure occurring in severe sepsis, and it often serves as a main predictor of poor outcomes (Blanco et al., 2008; Zanotti-Cavazzoni and Hollenberg, 2009; Walley, 2018). Previously, research from others and our laboratory demonstrated that impairments in mitochondria is a major drive inducing cardiac failure in sepsis, likely due to deficiency in energy production, elevation in oxidative stress, and overproduction of mitochondria-derived danger-associated molecular patterns (DAMPs) (Zang et al., 2007; Zang et al., 2012a; Zang et al., 2012b; Yao et al., 2015). The quality and quantity of mitochondria are tightly regulated by a multi-step process that includes mitochondrial biogenesis, mitochondrial fusion-fission dynamics, and mitophagy-mediated recycling. Interestingly, studies in recent years revealed that mitochondria-associated membranes (MAMs), the regions of close physical connection between mitochondrial outer membrane and membranes of endoplasmic reticulum (ER), are an important subcellular domain providing additional control to ensure mitochondrial properties (Mannella et al., 1998; Axe et al., 2008; Hayashi-Nishino et al., 2009; Patergnani et al., 2011; Raturi and Simmen, 2013; Giorgi et al., 2015). Proper mitochondrial physiology is dependent on the specific communication between mitochondria and ER for transporting Ca^{2+} (Patergnani et al., 2011) and lipids (Vance, 2014). Further, MAMs also function as a signaling hub, filled with dynamically translocated molecules that are involved in important cellular events of protein sorting, ER stress, apoptosis, inflammation, and autophagy (Poston et al., 2013; van Vliet et al., 2014). Currently, abnormalities in MAMs have been detected in disease models that involve mitochondrial dysfunction as a major pathogenesis component, such as in neurodegenerative diseases, diabetes, obesity, and infectious diseases (Bach et al., 2003; Ishikawa et al., 2009; Zorzano et al., 2009; Area-Gomez et al., 2012; De Strooper and Scorrano, 2012; Park et al., 2013). However, whether MAMs play a significant role in sepsis-induced cardiomyopathy has not been well understood. We recently found that endotoxemia caused MAM impairments in the heart tissue, suggesting a signaling mechanism underlying the deficiencies of mitochondria in septic hearts (Sun et al., 2021). Future investigations to address how aberration in MAMs occurs and its related pathological consequences during sepsis are important to identify novel therapeutic targets.

We previously found that the promoting autophagy *via* autophagy initiation factor Beclin-1 limited inflammation, governed mitochondrial quality control, reduced mitochondria-derived DAMPs, and thus, improved cardiac function during endotoxemia (Sun et al., 2018a). We also obtained results suggesting that Beclin-1 removed dysfunctional mitochondria by selectively activating an adaptive mitophagy *via* PTEN-induced kinase 1 (PINK1) and E3 ubiquitin ligase Parkin. Our finding is also in consistency with previous reports implicating the adaptive feature of PINK1-Parkin mitophagy in cardiac performance (Kubli et al., 2013; Piquereau et al., 2013). While disrupted Parkin expression was shown to cause insufficient mitophagy, resulting in an accumulation of damaged mitochondria and eventually caused cardiac failure (Kubli et al., 2013; Piquereau et al., 2013; Dorn, 2016), activation of Parkin was expected to provide, at least partial, cardiac protection. Recent studies using *in vitro* structure-guided mutagenesis and evaluation in cultured cells successfully selected several activating mutant forms of Parkin, within which mutant W403A held the highest promise of developing potential Parkin-targeted therapeutic strategies (Tang et al., 2017; Yi et al., 2019). In the investigation presented in this report, by using genetically engineered mouse strains with knockout expression of Parkin and knock-in expression of Parkin W403A, we evaluated the functional significance of Parkin in the regulation of myocardial MAMs, mitochondria, and cardiomyopathy in the model of endotoxemia. We also examined whether introducing mutant W403A Parkin may deliver any beneficial cardiac protection during endotoxemia.

MATERIALS AND METHODS

Experimental Animals

Mouse strains that carry Parkin knockout (*Park2*$^{-/-}$) and knock-in of Parkin W402A mutation (Parkin W402A KI) (W403A in human Parkin) were obtained from the Jackson Laboratory (Bar Harbor, ME) (stock numbers 006582 and 029317). According to the vender's

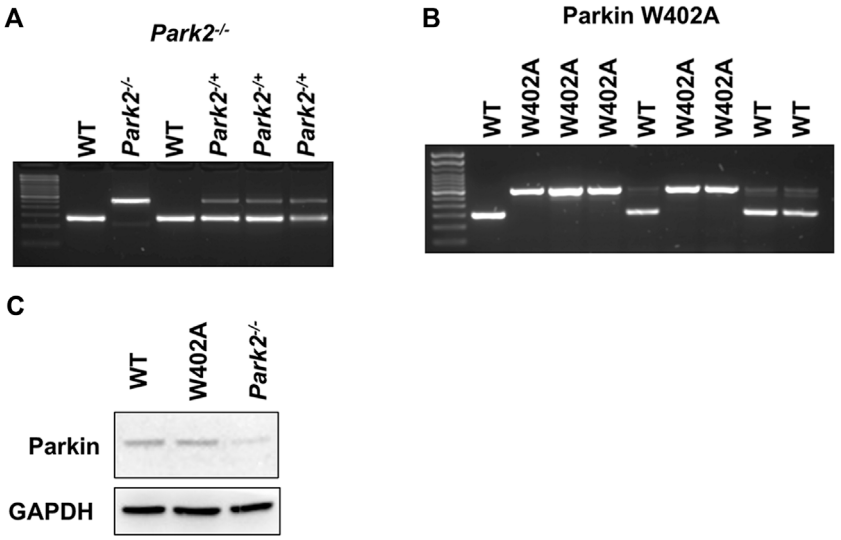

FIGURE 1 | Genotyping and the expression of Parkin in WT, *Park2*$^{-/-}$, and Parkin W402A mice. Pups of strain *Park2*$^{-/-}$ **(A)** and Parkin W402A **(B)** breeders were subjected to genotyping PRC according to genotyping protocols provided by the vendor. Protein expression of Parkin was verified in the heart tissue of adult WT, *Park2*$^{-/-}$, and Parkin W402A mice by western blot **(C).** Images shown are representative of at least 5 independent experiments.

suggestion, wild type mice C57BL/6NJ (Jackson Laboratory, stock number 005304) were chosen as the appropriate control. All animals were conditioned in-house for 5–6 days after arrival with commercial diet and tap water available at will. To properly maintain the colonies, genotyping was performed in each individual animal according to the vender's protocols (protocol 22730 for *Park2*$^{-/-}$ and protocol 20141 for Parkin W402A KI). **Figure 1** provided example results of genotyping verification for the colony management

The care of animal work described in this study was reviewed by and conducted under the oversight of UTSW institutional animal care and use committee and conformed to the National Research Council's "Guide for the Care and Use of Laboratory Animals" when establishing animal research standards.

Endotoxemia Model

Male mice, 8–12 weeks old, were weighed to determine the amount of lipopolysaccharide (LPS) (MilliporeSigma, Burlington, MA; catalog number L3012) required to achieve indicated doses and administered intraperitoneally (i.p.) in a volume of 100 µl per mouse. Sterile endotoxin-free PBS was used as a vehicle control in sham groups.

Echocardiography

Transthoracic echocardiograms were recorded in sedated mice using Visualsonics Vevo 2,100 small animal echocardiography machine. Views were taken in planes that approximated the parasternal short-axis view and the apical long-axis view in humans.

Transmission Electron Microscopy

Hearts were retrograde perfused (buffer: 4% paraformaldehyde/1% glutaraldehyde/0.1 M Na Cacodylate, pH7.4). Small blocks of tissue from the midsection of the left ventricular wall were fixed (buffer: 2.5% glutaraldehyde/0.1 M Na Cacodylate, pH7.4). Sections (75–80 nm) were cut using a Leica Ultramicrotome and examined under TEM. Quantification of MAM areas was achieved by Image J software.

Preparation of Serum and Tissue Lysates

Freshly collected blood was immediately centrifuged at 3,000 g for 15 min at 4°C to isolate serum. The serum preparations were then allocated and stored at −80°C until analyzed. Tissues were harvested, washed in PBS, snap clamp frozen, and kept at −80°C. Tissue lysates were prepared using tissue protein extraction reagent (Thermo Fisher Scientific, Rockford, IL; catalog number 78510). Protein concentrations were quantified using detergent compatible Bradford assay kit (Thermo Fisher Scientific; catalog number 23246).

Preparation of MAM and Mitochondria Fractions

Heart tissues were harvested, washed in PBS, snap-clamp frozen, and kept at −80°C until used. Procedures for the isolation of MAMs and mitochondria were performed according to a previously established procedure (Sun et al., 2021). Briefly, tissue pieces of one mouse heart were homogenized in 1 ml IB_{heart} buffer (220 mM mannitol, 70 mM sucrose, 10 mM HEPES, and 1 mM EGTA, pH7.4) using a Potter-Elvehjem PTFE pestle and glass homogenizer (MilliporeSigma; catalog number P7734), which was driven by a stirrer motor with electronic speed controller (Cole-Palmer, Vermon Hills; catalog number EW-04369-10) by 40 strokes at a speed of 1,500 rpm followed by another 40 strokes at 800 rpm. Crude mitochondrial fractions were then obtained by differential centrifugation in the following two steps. First, the homogenized heart lysates were subjected to twice-repeated centrifugation at 740 g for 5 min to remove unbroken cells and nuclei. Second, the supernatant mixtures were centrifuged at 9,000 g for

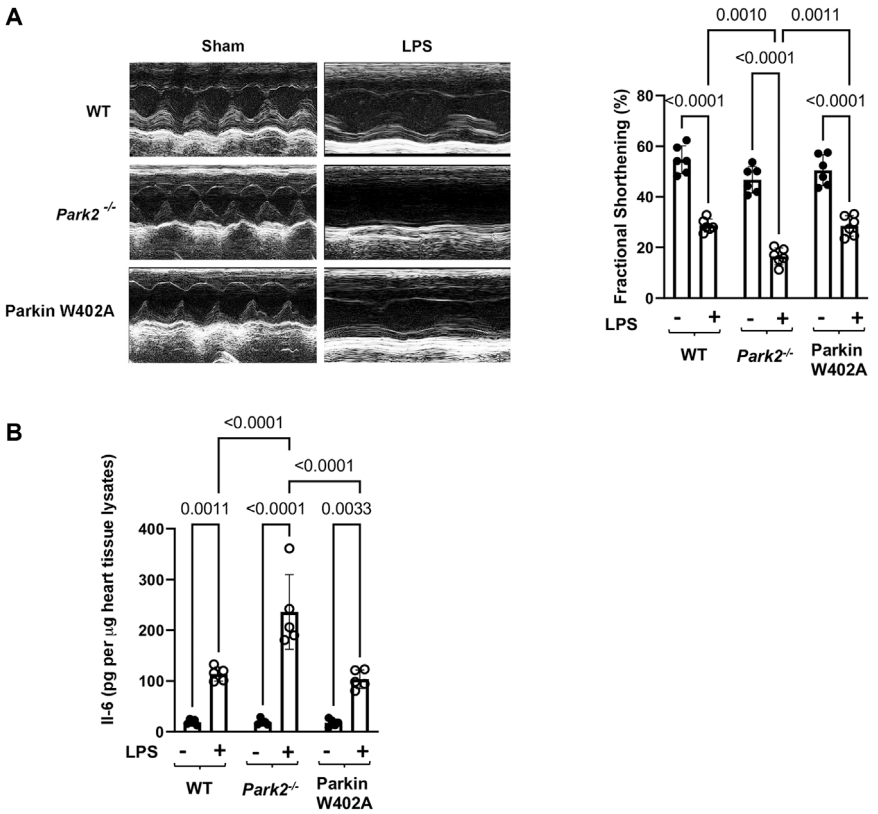

FIGURE 2 | Cardiac outcomes in LPS challenged WT, *Park2*$^{-/-}$, and Parkin W402A mice. Mice were given 5 mg/kg LPS or PBS (in shams) *via* i.p... At 18 h post LPS challenge, echocardiography was applied to measure cardiac function **(A).** The heart tissue was then harvested, and IL-6 levels were quantified in the tissue lysates by ELISA **(B).** All values are presented as means ± SEM. *p* values with less than 0.05 showing significant differences, are labeled (n = 5).

10 min to collect pellets. These pellets were then resuspended in freshly prepared mitochondria-resuspension buffer (MRB; 250 mM mannitol, 5 mM HEPES, and 0.5 mM EGTA, pH7.4) and subjected to twice-repeated centrifugation at 10,000 g for 10 min to collect crude mitochondria. The crude mitochondria pellets were then resuspended in MRB at the ratio of 0.5 ml MRB per heart and subjected to ultracentrifugation (Sorvall MX 120 Plus Micro-Ultracentrifuge with rotor S50-ST; Thermo Scientific; catalog number 50135645) to isolate MAMs and pure mitochondria (PM) by the following three steps. First, in each 7 ml ultracentrifuge tube, 6 ml of freshly made percoll medium [225 mM mannitol, 25 mM HEPES (pH7.4), 1 mM EGTA, and 30% percoll (v/v)] was layered with 0.5 ml of crude mitochondria resuspension and 0.5 ml of MRB, from the bottom to the top, and centrifuged at 95,000 g for 30 min. Fractions of mitochondria, dense bands located approximately at the bottom, and MAMs, diffused white bands located above the mitochondria, were collected. Second, the collected bands of mitochondria and MAMs were diluted 10 times with MRB and further centrifuged at 6,300 g for 10 min. Third, mixtures of MAMs bands and mitochondria bands were centrifuged at 100,000 g for 1 h. For fractions of MAMs, the pellets were collected and stored at −80°C until used. For fractions of mitochondria, the pellets were collected and resuspended with MRB again, followed by another two washes by centrifugation at 6,300 g for 10 min, and the PM pellets were then collected and stored at -80°C until used. All chemicals were purchased from MilliporeSigma.

Quantification of Mitochondrial Phospholipids

Levels of phospholipids in mitochondria were measured with a phospholipid assay kit (MilliporeSigma; catalog number MAK122) as described previously (Sun et al., 2021). Briefly, fractions of mitochondria were diluted to 1–2.5 μg protein per assay using the assay buffer provided. Each reaction mix was set up by adding a prepared sample or standard to phospholipid D that degrades phospholipids to release choline. The amount of choline was determined with choline oxidase and an H_2O_2 specific dye. A colorimetric reading at wavelength 570 nm was proportional to the phospholipid concentration in the sample. Results were calculated according to the standard curve and normalized by protein amount per sample, and measurements were performed in triplicates.

Mitochondrial Respiratory Complex I, II, and III Enzyme Assays

The activities of mitochondrial complexes were measured using enzyme assay kits according to manufacturer's

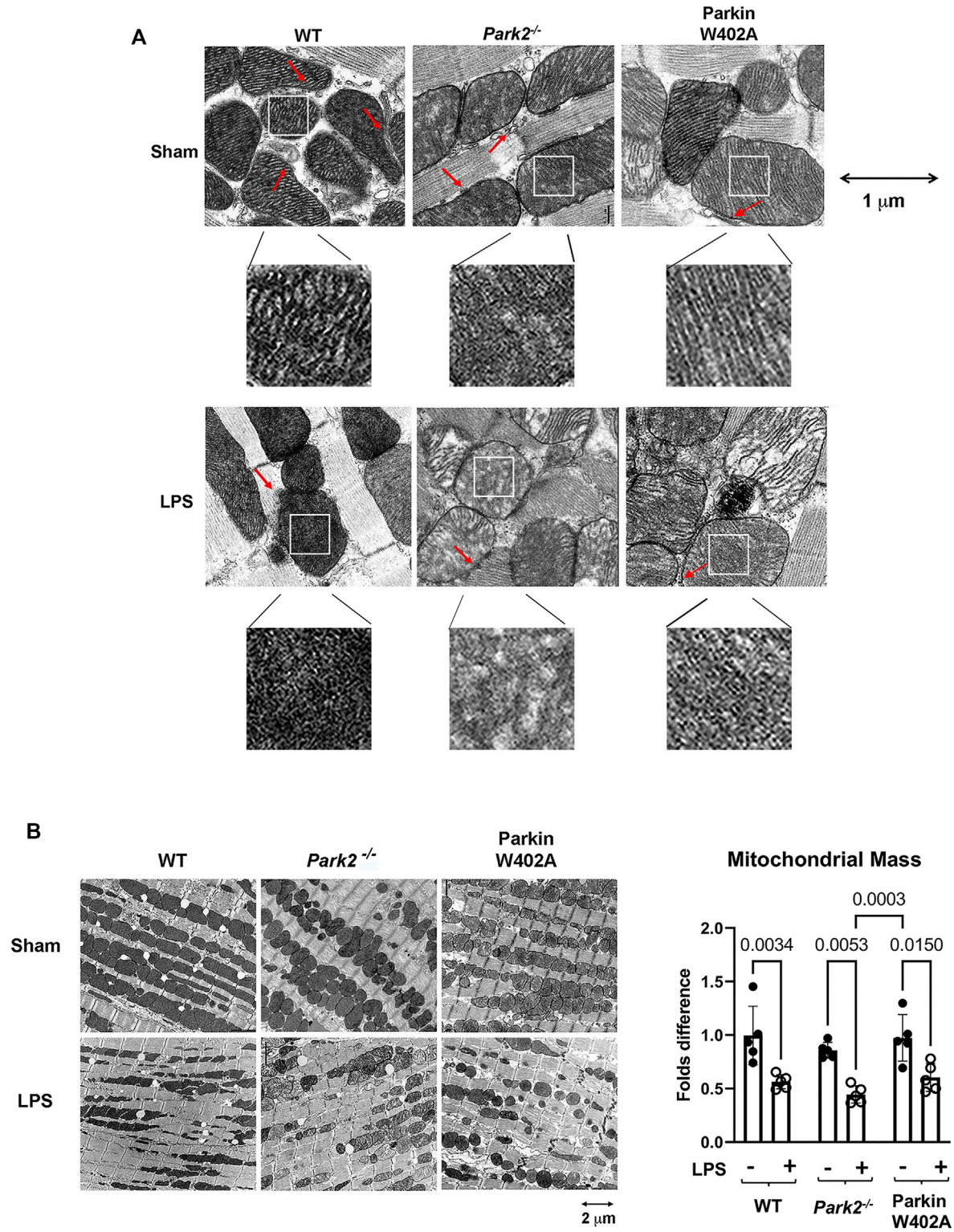

FIGURE 3 | Ultrastructure of cardiac mitochondria in WT and *Park2*$^{-/-}$ mice pre- and post-LPS challenge. Mice were given 5 mg/kg LPS or PBS (in shams) *via* i.p., and heart tissues were harvested 18 h post LPS challenge. Ultrastructure of mitochondria in myocardium was observed under TEM. **(A).** Images show the details of individual mitochondria in myocardium at the indicated magnification. Each white square-circled area was enlarged 9-time for viewing the inside of mitochondria. Red arrows indicate the locations of MAMs. **(B).** Images show the numbers and sizes of mitochondria in myocardium at the indicated magnification. Numbers of intact mitochondria in 10 × 10 μm^2 were counted, and >5 areas per sample were examined. Results were expressed as folds compared with that in the group of WT shams. In A and B, images are representative of at least 5 animals per group. **(C).** Enzymatic activities of mitochondrial respiratory complexes I and II/III were quantified in the mitochondrial fractions isolated from the heart tissue harvested at 18-h post LPS challenge. All values are presented as means ± SEM. *p* values with less than 0.05, showing significant differences, are labeled (n = 5).

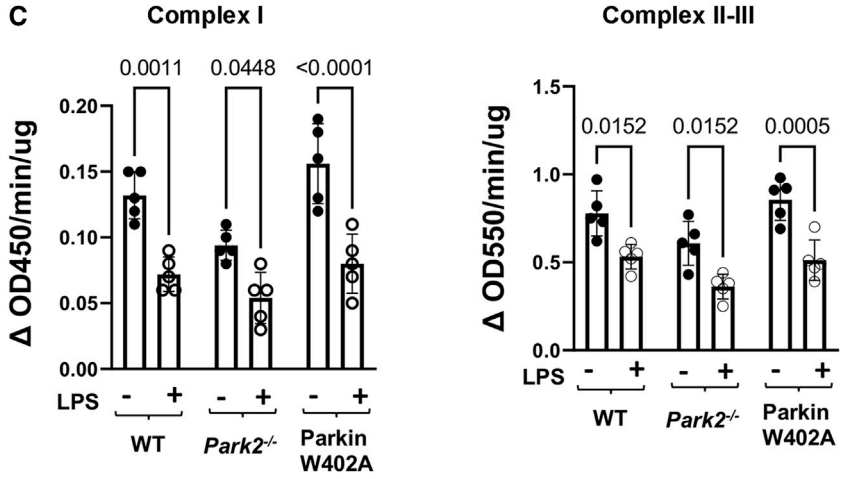

FIGURE 3 | (continued).

protocols (Abcam, Cambridge, MA; catalog numbers ab109721 and ab109905). Freshly isolated mitochondrial pellets were resuspended in PBS supplemented with 10% detergent provided in the kits. Protein concentrations of these mitochondrial lysates were estimated and 25 μg (for complex I) or 100 μg (for complex II + III) mitochondrial protein was used per reaction. Enzyme activities were measured spectrophotometrically in triplicate and expressed as changes of absorbance per minute per mg of protein.

Measurements of Cytokines by Enzyme-Linked Immunosorbent Assay

Cytokine levels in serum or in total tissue lysates were measured using Bio-Plex Mouse Cytokine Panel A 6-Plex (Bio-Rad, Hercules, CA; catalog number M6000007NY) according to vendor's instructions. Results were normalized by volume of serum samples or by protein amount in tissue lysates.

Western blots

Prepared SDS-PAGE protein samples were loaded and run on 20–4% tris-glycine stain free ready gels (Bio-Rad) and transferred to PVDF membranes. Stain free total protein measurement Membranes were blocked with 5% nonfat milk-PBS at room temperature for 1 h and subsequently probed with antibodies against Parkin (Santa Cruz Biotechnology, Santa Cruz, CA, catalog number sc-30130), phospho-Parkin (Ser65), LC3II (Cell Signaling, Danvers, MA; catalog number 368665 and 4,108), cytochrome C, FALC4, VDAC1, and PEN2 (Abcam, Cambridge, MA; catalog numbers ab110325, ab155282, ab14734, and ab18189), PINK1 (Novus Biologicals, Littleton, CO, catalog number BC100-494), and GAPDH (Millipore, Billerica, MA, catalog number MAB374). The membranes were then rinsed and incubated with corresponding horseradish peroxidase-conjugated secondary antibody (Rockland Immunochemicals, Pottstown, PA; catalog numbers 170-6515 and 170-6516). Antibody dilutions and incubation time were according to manufacturer's instructions. At the end, membranes were rinsed, and bound antibodies were detected by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific; catalog number 34077). Densitometry analysis was performed using Bio-Rad ChemiDoc MP Imaging System.

Immunostaining

Fresh heart tissues were fixed in 4% paraformaldehyde, transferred to 18–10% sucrose in PBS, and embedded in OCT. Samples were sectioned at 8 μm, air-dried and stored at −80°C until used. Frozen slides were then thawed, permeabilized, blocked with 3% donkey serum (Jackson ImmunoResearch catalog number 017-000-121) in PBS, and subjected to staining by a rat monoclonal anti-Lamp1 (Santa Cruz Biotechnology, Santa Cruz, CA, catalog number sc-1992; 1:300) or a rabbit monoclonal anti-Mfn2 (Cell Signaling, Danvers, MA, catalog number 9482; 1:100 at room temperature, 1 h). Upon completion of secondary antibody incubation with Alexa Fluor 448 conjugated donkey anti-rat IgG (Thermo Scientific; catalog number A-21208; 1:300) or Alexa Fluor 647 conjugated donkey anti-rabbit IgG (Thermo Scientific; catalog number A-31573; 1:300), the slides were washed, sealed with DAPI/antifade mounting solution (Thermo Fisher Scientific, Rockford, IL; catalog number 36931), and examined. Images were acquired with a LSM 510 confocal microscope equipped with an Axio Observer Z1 motorized inverted microscope and Zen software (Carl Zeiss Microscopy) and analyzed offline with Imaris software (version 9.5, Bitplane).

Statistical Analysis

All data were expressed as mean ± SEM of at least 3 independent experiments using 4-6 animals/group. Data were analyzed by GraphPad using two-way ANOVA test for comparisons of multiple groups. Differences were considered statistically significant as $p \leq 0.05$.

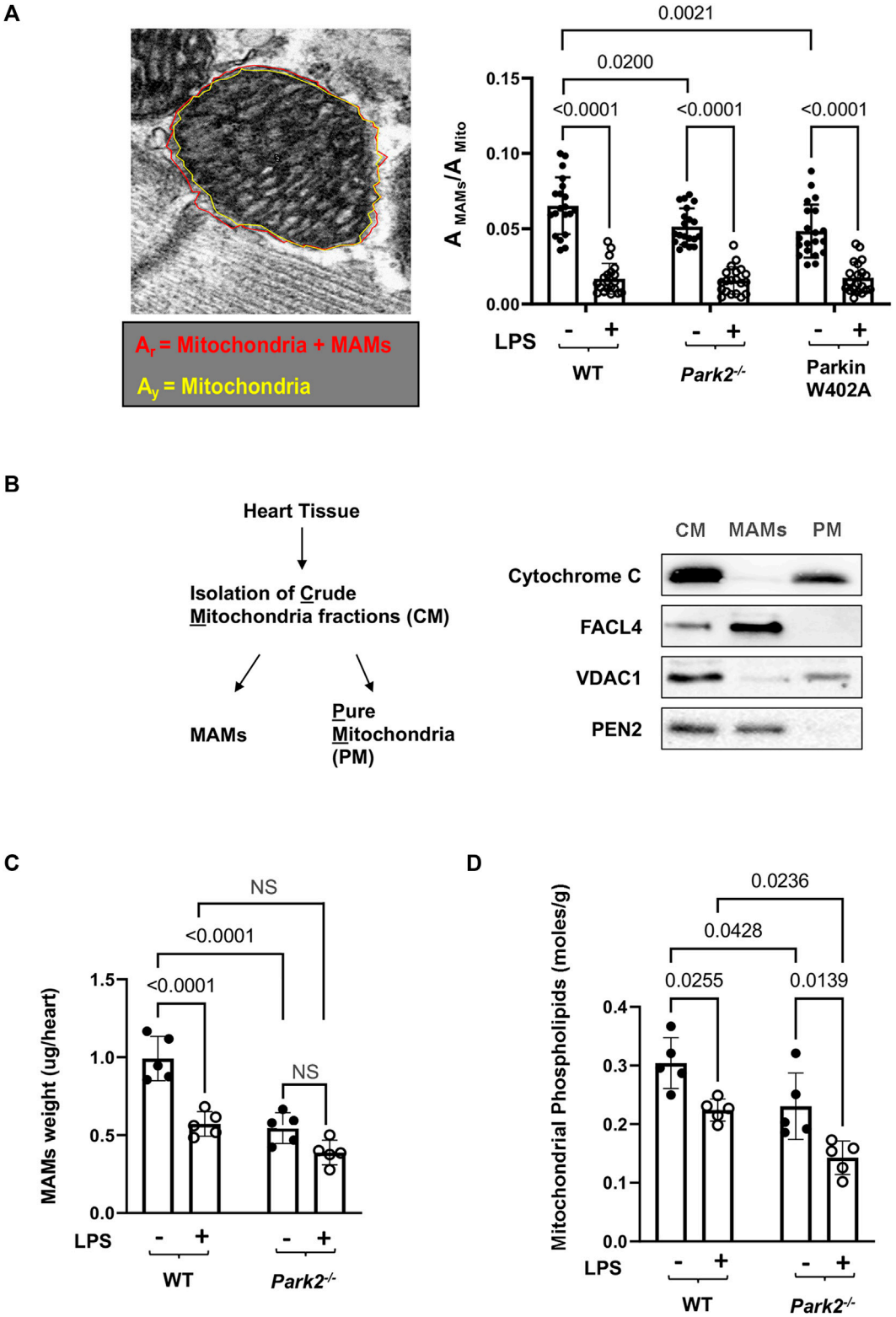

FIGURE 4 | LPS-induced reductions in the total mass and function of cardiac MAMs in WT and *Park2*$^{-/-}$ mice. Mice were given 5 mg/kg LPS or PBS (in shams) *via* i.p., and the heart tissues were harvested 18 h post LPS challenge. **(A).** Quantification of MAM areas was achieved by Image J software based on the images obtained from TEM. The area of mitochondria together with surrounding MAMs, circled in red (A_r), and the area of mitochondria only, circled in yellow (A_y, A_{Mito}), were quantified using the polygon selection tool in Image J. The difference between A_r and A_y is calculated as the area of MAMs, A_{MAMs}. More than 20 mitochondria per sample

(*Continued*)

FIGURE 4 | were examined and ratios of A_{MAMs}/A_{Mito} were calculated. **(B)**. Validation of the extraction procedure of crude mitochondria (CM), pure mitochondria (PM), and MAMs by ultracentrifugation according to the protocol described in the section of *Methods*. Successful isolation of CM, PM, and MAMs from the heart tissue of WT mice was demonstrated by western blots detecting markers of proteins located in mitochondria, cytochrome C and VDAC1, and proteins reside between mitochondrial outer membrane and ER, FACL4 and PEN2. **(C).** Mass of MAMs was calculated as the total amount of protein in MAM fraction isolated per heart, and the value was normalized by the heart tissue weight. **(D)**. Levels of phospholipids in mitochondrial fractions were measured and results were normalized by the amount of protein. All values are means ± SEM. *p* values with less than 0.05, showing significant differences, are labeled (n = 5).

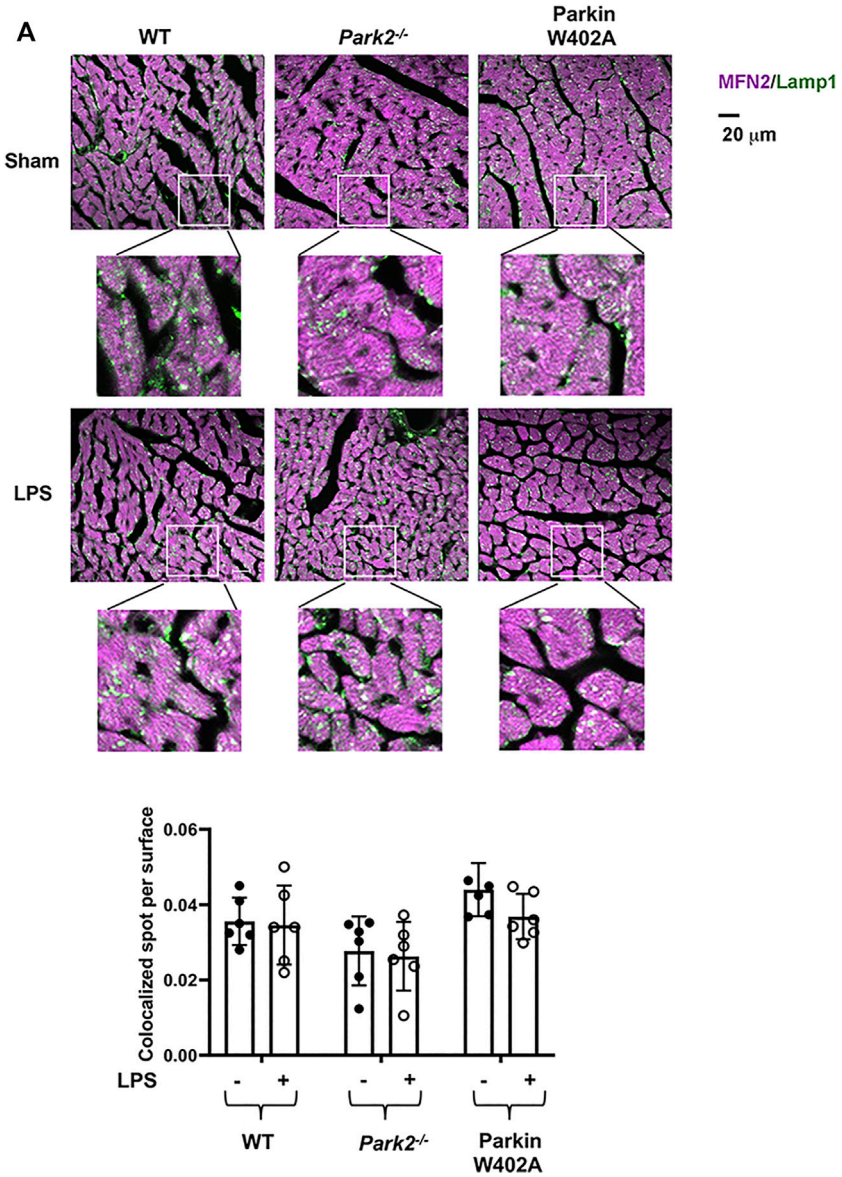

FIGURE 5 | Introducing Parkin W402A mutation is unable to promote cardiac mitophagy during endotoxemia. Mice were given 5 mg/kg LPS or PBS (in shams) *via* i.p., and hearts were harvested 18 h post LPS challenge **(A)**. Heart tissue sections were co-stained with antibodies against lysosomal marker Lamp1 (green) and mitochondrial marker Mfn2 (purple). Colors in white and pale green are resulted from co-localization of the two markers. Each white square circled area was enlarged 9 times for viewing the co-localization status. Additionally, co-localization areas were quantified using Imaris cell imaging software, in which co-localization of Lamp 1 and Mfn2 was determined when the distance between these two signals was ≤0.15 μm. The numbers of co-localization areas were normalized to the cross-sectional area of the Mfn2 fluorescence to permit between-groups statistical comparisons. **(B)** Levels of LC3II, Parkin, PINK1, and VDAC1 in the mitochondrial fractions isolated from the heart tissue were evaluated by western blot. The intensity of signals was quantified by densitometry analysis. **(C)** Levels of Parkin and phosphorylated Parkin (serine 65) were detected in heart tissue lysates by western Blot using antibodies against Parkin and Parkin p-ser65. All values are presented as means ± SEM. *p* values with less than 0.05, showing significant differences, are labeled (n = 5).

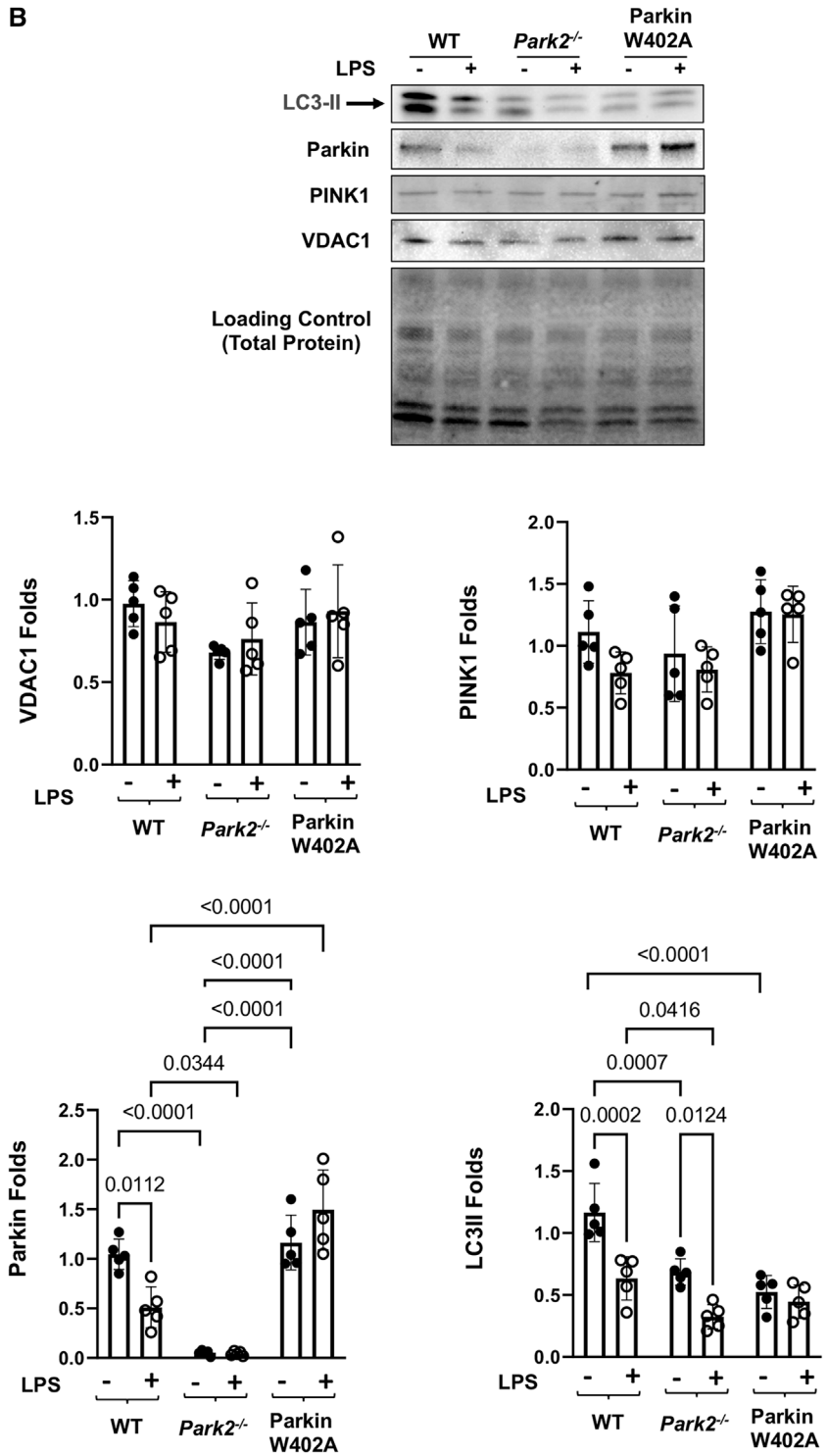

FIGURE 5 | (continued)

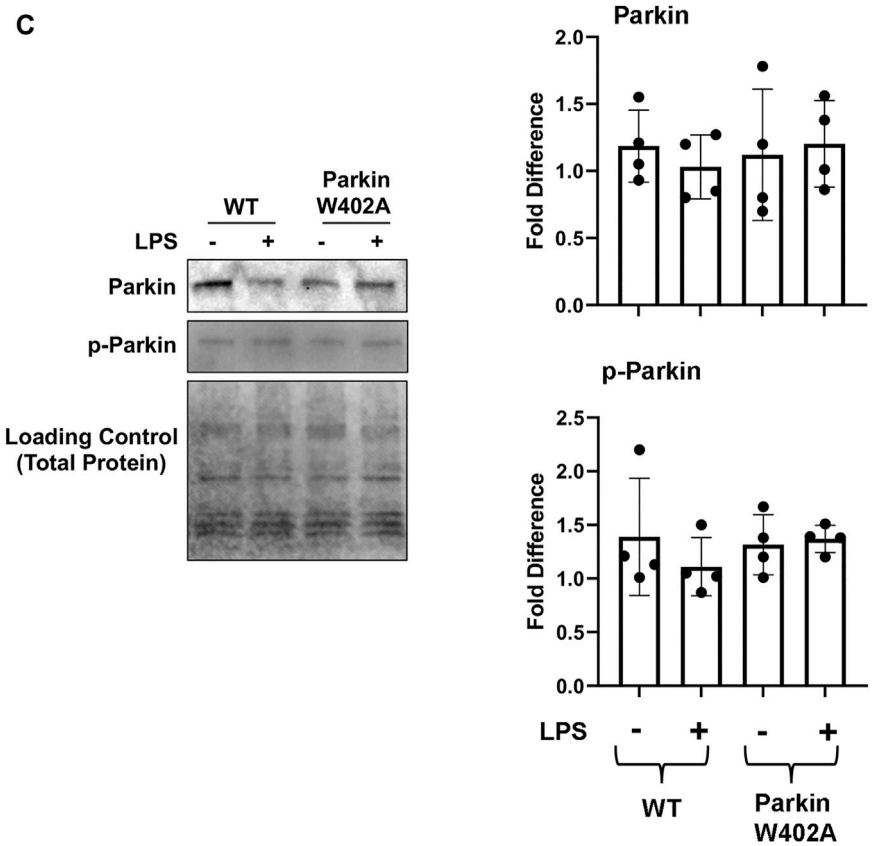

FIGURE 5 | (continued)

RESULTS

Cardiomyopathy in Mouse Strains of WT, *Park2*$^{-/-}$, and Parkin W402A During Endotoxemia

Previous studies in animal models with Parkin deficiency showed that the Parkin-mediated mitophagy is essential for cardiac performance and mitochondrial physiology in myocardium (Kubli et al., 2013; Piquereau et al., 2013; Sun et al., 2018a). On the other hand, *in vitro* analysis revealed that point mutation W403A in Parkin resulted in a constitutive activation, and this mutation was shown to enhance Parkin activity and promote PINK1-Parkin mitophagy (Tang et al., 2017; Yi et al., 2019).

Therefore, we speculated that a knock-in expression of this active mutant Parkin might provide protection in cardiac disease models *in vivo*. We previously established a mouse model of survival endotoxemia, in which cardiomyopathy is induced by a toxic dose of LPS (Sun et al., 2018a; Sun et al., 2018b). In this report, experiments were designed to evaluate the effects of disrupted *parkin* expression and knock-in, forced expression of Parkin mutant W402A (human W403A) on cardiomyopathy during endotoxemia. Mice strains carrying *parkin* knockout, *Park2*$^{-/-}$, and Parkin W402A are commercially available. These mice are viable with no apparent abnormalities at baseline when observed up to 12 months (Simpkins et al., 2005; Kubli et al., 2013). Proper genotyping of the mouse strains was performed according to the vendor's protocols, as described in the section of *Materials and Methods* (**Figure 1**).

WT, *Park2*$^{-/-}$, and Parkin W402A mice was given LPS challenge at 5 mg/kg and subjected to the assessment of cardiac performance by echocardiography at 18 h post-challenge. As the results summarized in **Figure 2A**, we found that LPS caused significant decreases in cardiac contractility of all three strains. The fractional shortening was reduced about 40–50% by LPS in the WT and Parkin W402A strains, whereases LPS induced the worst cardiac performance in *Park2*$^{-/-}$ mice–a more than 60% reduction in contractility. This observation of cardiac dysfunction in *Park2*$^{-/-}$ mice is consistent with the literature (Kubli et al., 2013; Piquereau et al., 2013), likely due to the halted mitophagy that causes overaccumulation of deficient mitochondria in myocardium. However, the result obtained in Parkin W402A mice is unexpected since this mutant was suggested carrying Parkin activation in previous published *in vitro* studies (Tang et al., 2017; Yi et al., 2019).

Overwhelming inflammation is characteristic in sepsis, which is hypothesized as the main reason for causing multi-organ

failure. We previously showed that IL-6 is the most responsive cytokine to LPS challenge in the heart tissue (Sun et al., 2018b). To evaluate Parkin in myocardial inflammation, heart tissue from WT, *Park2*$^{-/-}$, and Parkin W402A mice was harvested 18 h post LPS challenge and levels of IL-6 in tissue lysates were compared. Results summarized in **Figure 2B** showed that LPS triggered an over 5-fold increase in IL-6 production in both WT and Parkin W402A mice, and the most dramatic stimulation of IL-6 by LPS was detected in the *Park2*$^{-/-}$ hearts with a ~ 12-fold increase. Thus, the data suggest Parkin as a critical control over the stimulation of cardiac inflammation during endotoxemia. However, like its effect on cardiac function, Parkin mutant W402A provided little protection again LPS-induced inflammatory response in the heart tissue.

Status of Myocardial Mitochondria in Mouse Strains of WT, *Park2*$^{-/-}$, and Parkin W402A During Endotoxemia

Mitochondrial damage is a known mechanism of sepsis pathology, and it is closely associated with sepsis severity. In this study, we compared LPS-induced changes in cardiac mitochondrial structure and function in WT, *Park2*$^{-/-}$, and Parkin W402A mice. Following given LPS challenge at 5 mg/kg, and the left ventricular wall of hearts was harvested at 18 h post challenge and applied to the assessment by transmission electron microscopy (TEM). As shown in **Figure 3A**, we observed that LPS triggered a significant loss of mitochondrial outer membrane integrity and disruption of inner membrane cristae structures in all three strains. There was no evident difference detected between the WT and Parkin W402A mice. When the heart tissue slides were examined in a larger field under TEM (**Figure 3B**), numbers of mitochondria in a fixed area of myocardium were counted and statistical analysis was applied. Data indicated that LPS stimulated a trend of reduction in the mass of total mitochondria, which was also associated with an accumulation of small-sized dysfunctional mitochondria in all three mouse strains. Quantification of the intact mitochondria showed that LPS challenge reduced the total number of mitochondria about 50% in WT and Parkin W402A mice and about 60% in *Park2*$^{-/-}$ mice.

To evaluate mitochondrial function, mitochondrial fractions isolated from the heart tissue harvested at the same time point were applied to the measurements of the enzymatic activities of mitochondrial respiratory complexes I and II/III. Summarized in **Figure 3C**, data indicate that LPs induced significant decreases in the enzymatic activities of mitochondrial respiratory complexes I-III in animals of all strains. In addition, this response in Parkin W402A mice was similar to that in the WT. These results showed that LPS stimulates mitochondrial damage, including structural abnormality and functional deficiencies, in the heart of WT, *Park2*$^{-/-}$, and Parkin W402A mice, among which responses in the strain of *Park2*$^{-/-}$ was the most severe. Data also demonstrated that introducing Parkin mutation W402A has little effect on this LPS-induced harmful response.

Parkin is Essential to Maintain the Structure and Function of Myocardial MAMs During Endotoxemia

MAMs are an essential structural entity to support mitochondrial health (Mannella et al., 1998; Axe et al., 2008; Hayashi-Nishino et al., 2009; Patergnani et al., 2011; Raturi and Simmen, 2013; Giorgi et al., 2015). Recently, our investigation revealed that endotoxemia causes impairments in cardiac MAMs (Sun et al., 2021). Since PINK1-Parkin mitophagy is a major quality control mechanism for mitochondria (Kubli et al., 2013; Piquereau et al., 2013; Sun et al., 2018b), we next examined whether myocardial MAMs are also subjected to regulation *via* the PINK1-Parkin signaling axis during endotoxemia.

To compare cardiac MAMs of WT with that of the *Park2*$^{-/-}$ counterparts, mice were given 5 mg/kg LPS, and the heart tissue was harvested 18 h post challenge. Since MAMs tightly surround mitochondria, the ultrastructure of MAMs can be examined together with mitochondria under TEM. In the images shown in **Figure 3A**, red arrows indicate the locations of MAMs. Observation of the TEM images suggested that LPS decreased the amount of MAMs and also caused fragmentation of MAMs, especially in *Park2*$^{-/-}$ mice. To obtain a more precise evaluation, we developed a method to quantify the areas of MAMs and mitochondria based on TEM images using Image J software, as described in **Figure 4A**. Statistical analysis of the ratios of MAMs to mitochondria clearly showed that LPS triggered a significant decrease in the amount of MAM in the heart tissue of all three mouse strains. In addition, compared with WT, both *Park2*$^{-/-}$ and Parkin W402A showed a decrease in MAMs at baseline.

Further, a biochemical approach was applied to compare the amount and function of MAMs in the heart tissue of WT and *Park2*$^{-/-}$ mice at baseline and following the challenge by LPS. The procedure of isolating MAMs fractions by ultracentrifugation was previously validated (Sun et al., 2021). As illustrated in **Figure 4B**, successful isolation of MAMs from the total tissue lysates and then subsequential crude mitochondria (CM) fraction was shown by specific markers of subcellular locations. Cytochrome C, an enzyme located in the mitochondrial intermembrane space, was exclusively located in purified mitochondria (PM) but not in MAMs. Mitochondrial outer membrane protein VADC1 was detected primarily in PM. Fatty acid CoA ligase 4 (FACL4), an enzyme enriched in MAMs to facilitate lipid metabolism, and PEN2, a subunit of gamma secretase complex located on the ER membranes, were detected mainly in MAMs but not in PM. As shown in **Figure 4C**, when the amount of MAM isolation in the heart was quantified based on heart tissue weight, we found that LPS triggered a significant, about a 40% drop in the quantity of MAMs in WT mice. Lacking Parkin expression in *Park2*$^{-/-}$ mice resulted in a near half decrease in the total amount of MAMs at the baseline and about 30% more loss in response to LPS. The result is consistent with the observation of MAM ultrastructure obtained under TEM shown in **Figure 3A**.

A main function of MAMs is to coordinate the synthesis and transport of phospholipids to other organelles such as mitochondria (Vance, 1990). Since mitochondria are unable to synthesize phospholipids *de novo*, the level of phospholipids inside mitochondria relies entirely on the supply from ER (Voeltz et al., 2002; Vance, 2015; Annunziata et al., 2018). Thus, quantification of mitochondrial phospholipids has been used as an indirect measurement of the transporting function of MAMs (Vance, 1990; Hedskog et al., 2013; Vance, 2015). By this approach, we measured levels of phospholipid accumulation in the mitochondrial fractions from the heart tissue. The result showed that knockout Parkin expression severely reduced phospholipids in mitochondria, suggesting an impaired transporting function of MAMs (**Figure 4D**).

W402A Point Mutation in Parkin is not Sufficient to Improve Mitophagy in the Heart During Endotoxemia

According to previously established protocol (Sun et al., 2018b), we determined whether introducing Parkin mutation W402A affected cardiac mitophagy in the endotoxemia model by examining mitochondria-lysosome association and the levels of mitophagy/autophagy factors located at mitochondria. As shown in **Figure 5A**, heart tissue slides were subjected to co-immunostaining with antibodies against mitochondrial protein mitofusin 2 (Mfn2) and lysosomal protein Lamp1. Co-localization of mitochondria and lysosomes, shown in white and pale green, indicate the occurrence of mitophagy. Quantification of Mfn2-Lamp1 co-localization signals showed that there were little LPS-initiated changes in mitochondria-lysosome association in all three mouse strains.

In the mitochondrial fractions isolated from the heart tissue, we examined mitophagy factors Parkin and PINK1, autophagy marker LC3II, and mitochondrial membrane marker protein VDAC by western blot (**Figure 5B**). Statistical analysis of the signal density showed that LPS caused a significant decrease in mitochondria-located Parkin in the WT but not in Parkin W402A mice. In fact, the level of mitochondria-associated Parkin was significantly higher in LPS-challenged Parkin W402A mice. However, these differences were not associated with any changes in levels of PINK1, suggesting that W402A mutation in Parkin did not promote PINK1 to mitochondria. Further, LPS caused decreases in LC3II in the mitochondria in WT and *Park2*$^{-/-}$ mice, suggesting a reduction in mitophagy by LPS, consistent with our previously published result (Sun et al., 2018b). In Parkin W402A mice, though not changed by LPS, mitochondrial LC3II level was significantly lower than that in the WT at baseline, suggesting mutation W402A in Parkin does not at all improve mitophagy *in vivo*. Since the same amount of mitochondrial protein was loaded on the western blot in this experimental setting, differences in VDAC were neither expected nor observed.

Phosphorylation of Parkin on serine 65 located at its N-terminal ubiquitin-like domain is shown to be essential for Parkin activation (McWilliams et al., 2018). In the heart tissue lysates, Parkin phosphorylation in Parkin W402A mice was compared with that in WT by western Blot using the antibody specific for Parkin serine 65 phosphorylation. The detection revealed no statistically significant difference between these two groups (**Figure 5C**). Together, these results suggest that W402A mutation in Parkin was not able to promote the Parkin-mediated mitophagy in myocardium under the pathological condition of endotoxemia.

DISCUSSION

This report summarized our investigation regarding the role of mitophagy factor Parkin in cardiomyopathy induced by endotoxemia. Using a mouse model of endotoxemia, cardiac responses in the mouse strains carrying knockout Parkin expression, *Park2*$^{-/-,}$ and a forced expression of active mutant Parkin W402A were compared with those in the WT counter parts. Data demonstrated that deficiency in Parkin expression exacerbated cardiac dysfunction and cytokine production in response to LPS challenge (**Figure 2**). Unexpectedly, little improvement of cardiac performance was obtained in Parkin W402A mice under the same condition. Furthermore, evaluation of mitochondrial status, including the ultrastructure, total population, and respiratory function, showed that LPS causes severe impairments in myocardial mitochondria, which response was worsened in *Park2*$^{-/-}$ mice (**Figure 3**). Since MAM property is essential for maintaining mitochondrial physiology in the heart (Sun et al., 2021), the relationship of Parkin and MAMs was examined in this model. Our results showed that disruption of Parkin expression reduced the total quantity and quantity of cardiac MAMs, either at baseline or under LPS challenge, suggesting that Parkin has a functional role in the regulation of MAMs (**Figure 4**). Additionally, our evaluation of Parkin W402A mice showed that cardiac mitophagy was not improved by this mutation *in vivo* (**Figure 5**). Together with published results from other groups, our data suggest that Parkin is essential for supporting proper mitochondrial status *via* Parkin-mediated mitophagy as well as maintaining MAM physiology in the heart under the shock stress of endotoxemia. Mutation W402 on Parkin was not found to associate with detectable benefits of enhancing mitophagy and improving cardiac function.

Research in the past decades has well recognized mitochondrial deficiency as a major signaling to incite functional failure in multiple organs during sepsis. However, the underlying causative mechanisms are still not well-established. MAMs are dynamic interaction domains between mitochondria and ER that sustain mitochondrial health (Patergnani et al., 2011; Vance, 2014), but their pathological role in sepsis is largely unknown. Our recent published investigation detected that endotoxemia caused impairments in myocardial MAMs (Sun et al., 2021). Data summarized in this report suggest that Parkin-dependent regulation of mitochondrial quality control is tightly associated with the properties of MAMs in response to the challenge by

endotoxemia (**Figure 4**). Our data clearly indicate that deficiency in Parkin expression severely impaired cardiac MAMs, shown by disruption in structural integrity, reduction in total mass, and malfunction of transporting phospholipids into mitochondria. This damage in MAMs occurred in parallel with worsened cardiomyopathy induced by endotoxemia, shown by a significant decrease in cardiac contractility, an increase in cytokine production (**Figure 2**), as well as an intensified adversity of mitochondria status (**Figure 3**). The newly obtained evidence supports the hypothesis that damage in MAMs is an important cellular component that mediates pathogenesis in mitochondria in septic hearts. Therefore, strategies that protect MAMs may potentially possess therapeutic effectiveness.

The results summarized in this report indicate a Parkin-dependent regulation of cardiac MAMs, suggesting that the formation of MAMs may demand an active machinery of Parkin/PINK1 mitophagy. In this regard, Parkin and its partner protein PINK1 were previously found to be located at MAMs, suggesting a plausible involvement of Parkin in regulating MAM properties (Michiorri et al., 2010; Choubey et al., 2014; Gelmetti et al., 2017). On the other hand, *in vitro* studies have linked the initiation of PINK1/Parkin mitophagy to a signal of phosphorylation on Mfn2 (Shiba-Fukushima et al., 2012; Chen and Dorn, 2013; Jin and Youle, 2013), which is by far the most important identified key regulatory factor of MAMs (Cipolat et al., 2004; de Brito and Scorrano, 2008). Additionally, MAMs are also known as a signaling hub harboring key molecules during protein sorting, ER stress, apoptosis, inflammation, and autophagy (Poston et al., 2013; van Vliet et al., 2014). Therefore, a proper maintenance of MAM status is likely a necessary component for starting mitophagy as well as regulating other cellular functions that depend on energy supply from the mitochondria. Though the potential signaling interactions between Parkin and molecules in MAMs is beyond the scope of this report, our ongoing investigation is set up to apply omics approaches to obtain a comprehensive signaling diagram in near future.

The upstream signaling regulation of MAMs may involve autophagy. Previously, we reported that insufficient, maladaptive autophagy occurs during cardiomyopathy in endotoxemia, and promoting autophagy *via* Beclin-1 attenuates mitochondrial damage and improves cardiac performance at the same condition (Sun et al., 2018a). Data also suggested that this Beclin-1-mediated protection on mitochondria is accomplished *via* a selective activation of Parkin/Pink1 mitophagy (Sun et al., 2018a), which target mitochondria with lost membrane potential and subsequently bring these dysfunctional mitochondria to autophagosomes for degradation (Narendra et al., 2008; Narendra et al., 2010). Our recent study further revealed that targeted activation of Beclin-1, either genetically or pharmacologically, is capable of protecting the structure and function of cardiac MAMs from endotoxemia challenge (Sun et al., 2021). Together, these investigations suggest that sepsis-induced myocardial mitochondrial damage is a result of MAM abnormality stimulated by inadequate autophagic responses.

Research from other and ours support the notion that PINK1/Parkin mitophagy is cardiac-protective, an adaptive response, during sepsis as well as other pathological conditions of heart failure (Sun et al., 2018b) (Kubli et al., 2013; Piquereau et al., 2013). A structure-guided mutagenesis screen and *in vitro* cell culture studies identified point mutation W403A in Parkin with enhanced Parkin activity and increased PINK1-Parkin mitophagy (Tang et al., 2017; Yi et al., 2019). We therefore designed *in vivo* experiments to evaluate the cardiac responses in mice carrying knock-in expression of Parkin W402A (human W403A). However, when challenged with LPS, introducing this mutant Parkin neither improved cardiac function nor protected myocardial mitochondria (**Figures 2**, **3**). It is also worthy to point out that our analysis using TEM did not detect any sign of increase in mitophagy, in which situation mitochondria would be engulfed by the double-membrane structure of autophagosomes and/or autolysosomes. Our further analysis of mitophagy and Parkin phosphorylation at serine 65, which represents its activation, did not detect any difference between the WT and Parkin W402A mice (**Figure 5**), confirming little impact provided by W402A on mitophagy or activation of Parkin. It remains possible that this mutation in Parkin may convey Parkin function in a tissue or cell type specific feature. Alternatively, the level of Parkin activation may need to be optimized to accomplish proper mitophagy. Nonetheless, current data from our investigation suggest that point mutation W402A (human W403A) in Parkin by itself is not sufficient to provide cardiac protection or promote mitophagy *in vivo* under the challenge by endotoxemia. Thus, the results suggest that point mutation W402 (human W403) in Parkin is unable to promote Parkin activation *in vivo*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Texas Southwestern Medical Center institutional animal care and use committee.

AUTHOR CONTRIBUTIONS

QSZ conceived the project, designed the study, and wrote the manuscript. MK, AN, YS, and Q-JZ conducted all the experiments. QSZ, MK, AN, YS, Q-JZ, and ZPL contributed to the data analysis and interpretation. All authors approved the final draft.

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REVIEW
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Degradative and Non-Degradative Roles of Autophagy Proteins in Metabolism and Metabolic Diseases

*Kenta Kuramoto and Congcong He**

Department of Cell and Developmental Biology, Feinberg School of Medicine, Northwestern University, Chicago, IL, United States

Autophagy is a stress-induced lysosomal degradation pathway regulated by evolutionarily conserved autophagy-related (ATG) genes. Recent research has revealed that autophagy plays an important role in the regulation of energy metabolism, development of metabolic tissues, and pathogenesis of metabolic disorders. Bulk and selective degradation by autophagy helps maintain protein homeostasis and physiological function of cells. Aside from classical degradative roles, ATG proteins also carry out non-classical secretory functions of metabolic tissues. In this review, we summarize recent progresses and unanswered questions on the mechanisms of autophagy and ATG proteins in metabolic regulation, with a focus on organelle and nutrient storage degradation, as well as vesicular and hormonal secretion. Such knowledge broadens our understanding on the cause, pathophysiology, and prevention of metabolic diseases including obesity and diabetes.

Keywords: autophagy-related gene, mitophagy, lipophagy, ER-phagy, adipose tissue, liver, beta cell (β cell), secretion

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Weizmann Institute of Science, Israel

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Du Feng,
Guangzhou Medical University, China
Paolo Grumati,
Telethon Institute of Genetics and Medicine (TIGEM), Italy

****Correspondence:***
Congcong He
congcong.he@northwestern.edu

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INTRODUCTION ON THE AUTOPHAGY PATHWAY

Autophagy is an evolutionally conserved intracellular degradation system, which degrades various types of cellular components by delivering cargos into the lysosome that contains degradative enzymes (Dikic and Elazar, 2018; Aman et al., 2021). Various cellular stress stimuli, including nutrient depletion, exercise and oxidative stress, induce autophagy. Autophagy plays a critical role in both supplying metabolites and eliminating damaged cellular compartments to maintain cellular homeostasis. Dysregulated autophagy is associated with the pathogenesis of a variety of metabolic disorders, such as obesity and diabetes (Rocchi and He, 2015). Based on differences in the mechanism of lysosomal cargo delivery, autophagy is categorized into three major types, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy involves the formation of double-membrane autophagosomes and requires the coordination and execution of more than 40 autophagy-related (ATG) genes. Based on cargo selectivity, macroautophagy can be non-selective bulk degradation of the cytosol (bulk autophagy), or selective degradation of cargos via specific cargo receptors (selective autophagy). Depending on cargo types, selective autophagy can be further classified into several subtypes, such as lipophagy (autophagy of lipid droplets), mitophagy (autophagy of mitochondria), and ER-phagy (autophagy of the endoplasmic reticulum/ER) (**Figure 1**). In comparison, microautophagy degrades target components by directly engulfing cargos into the lysosome, through the invagination of the lysosomal membrane in an ESCRT (endosomal sorting complexes required for transport) machinery-dependent manner. CMA, on the other hand, is the transport of a specific subset of

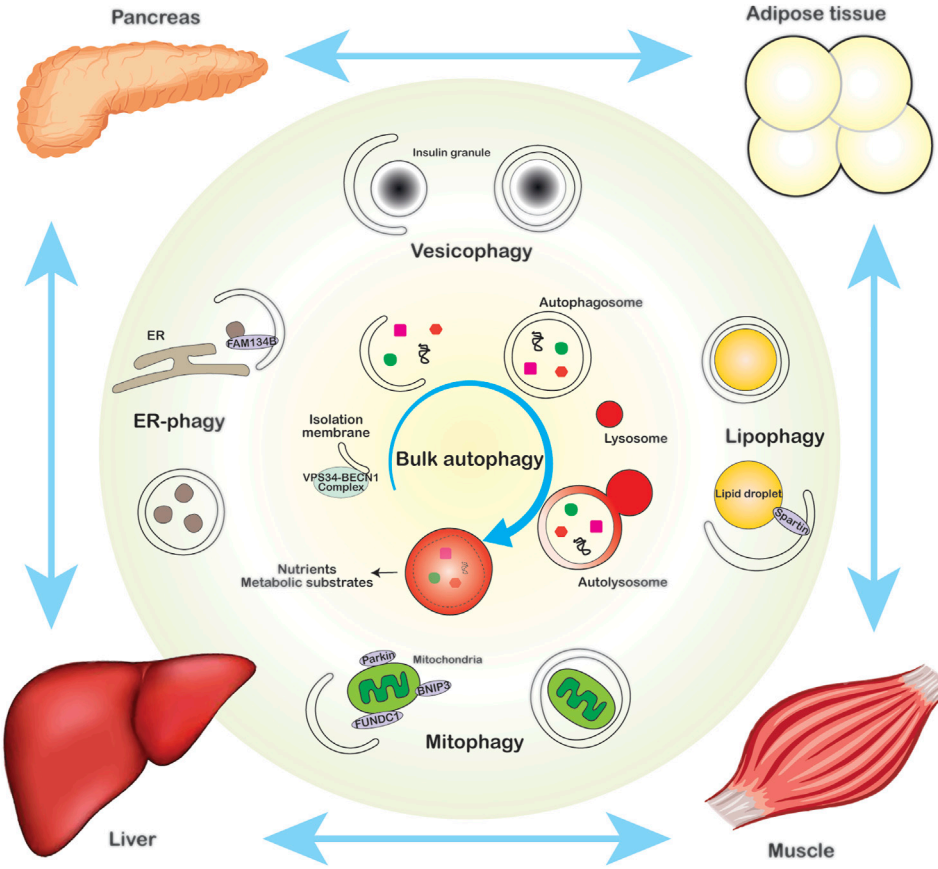

FIGURE 1 | The role of autophagy in intracellular and systemic energy homeostasis. Bulk autophagy (center) and organelle-specific selective autophagy, such as vesicophagy (top), ER-phagy (left), lipophagy (right) and mitophagy (bottom), play pivotal roles in maintaining intracellular energy homeostasis via supplying nutrients and removing defective organelles. Autophagy also regulates systemic metabolic homeostasis via the cross-talk among metabolic organs in a non-cell autonomous manner.

proteins via a lysosomal membrane receptor and transporter LAMP2A (lysosomal associated membrane protein-2 type A). The CMA substrates must possess a KFERQ-like pentapeptide sequence, which binds to heat shock-cognate chaperon 70 KDa (HSC70). The HSC70-substrate complex then interacts with LAMP2A, through which the target proteins are translocated into the lysosome. Based on existence of the predicted KFERQ-like targeting motif, approximately 30% of total cellular proteins are potentially degraded via CMA (Alfaro et al., 2018).

Macroautophagy is the main focus of this review. Various types of stress stimuli induce autophagy via multiple signaling cascades, including activation of AMP-activated protein kinase (AMPK) and inhibition of mammalian target of rapamycin (mTOR) signaling pathways. Both AMPK and mTOR can phosphorylate the autophagy-initiating Unc-51-like kinase 1 (ULK1/ATG1) complex, which is composed of ULK1/ATG1, ATG13, ATG101 and RB1-inducible coiled-coil protein 1 (RB1CC1/FIP200). While AMPK-mediated ULK1 phosphorylation activates ULK1 and autophagy, mTOR-mediated ULK1 phosphorylation inhibits the ULK1 function. Thus, the ULK1 kinase activity is activated under autophagy-inducing conditions. ULK1 further phosphorylates and activates the class III VPS34-BECN1 phosphatidylinositol 3-kinase (PI3KC3) complex, which produces phosphatidylinositol 3-phosphate (Ptdlns3P) on the isolation membrane, the nascent forming autophagosomal membrane. BECN1 is the mammalian homolog of yeast Atg6, and stabilizes and activates the VPS34 kinase complex. Ptdlns3P is important for isolation membrane growth through recruiting WD repeat domain phosphoinositide-interacting (WIPI/ATG18) proteins and ATG9-containing vesicles. Among all ATG proteins, ATG9 is the only integral membrane protein essential for autophagosome formation and functions as a lipid scramblase (Matoba et al., 2020; Ghanbarpour et al., 2021). At the steady state, ATG9 is localized at the trans-Golgi network and recycling endosomes, and upon autophagy induction, ATG9-containing vesicles are translocated to the autophagosome assembly site to drive the expansion of the isolation membrane. The transport of ATG9 is regulated by the late endosome-associated small GTPase RAB7.

On the isolation membrane, yeast Atg8, and its mammalian homologs including LC3 (microtubule-associated protein light chain 3) and GABARAPs (GABA Type A receptor-associated proteins), are conjugated to the lipid phosphatidylethanolamine (PE) through a multi-step ubiquitination-like conjugation

process regulated by ATG4, ATG7, ATG3 and the ATG12-ATG5-ATG16L1 complex. Isolation membrane-localized LC3 is essential for autophagosome formation, as well as for cargo recruitment via its binding to autophagy cargo receptors. Completed autophagosomes fuse with the lysosome and form autolysosomes, where autophagic substrates are degraded. Multiple protein complexes involved in membrane fusion participate in the autophagosome-lysosome fusion step, including the HOPS (homotypic fusion and protein sorting) complex, the SNARE complex, and RAB proteins.

REMOVAL OF DAMAGED OR EXCESS CELLULAR STRUCTURES

Overall Roles of Autophagy in Metabolic Regulation

Whole-body knockout (KO) of several essential ATG genes, including *Atg5*, *Becn1* and *Atg7*, leads to embryonic or postnatal lethality, suggesting that autophagy is essential for embryonic and postnatal development. Thus, studies on energy metabolism using whole-body ATG gene KO mice are rare and are based on KO mouse models with partial autophagy reduction, such as *Atg4b*-null mice and *Becn1* heterozygous mice. ATG4 family proteins function in LC3/GABARAP processing and delipidation, and are composed of four isoforms, ATG4A, ATG4B, ATG4C and ATG4D, among which ATG4B possesses the highest processing activity (Maruyama and Noda, 2018). *Atg4b*-null mice show partial autophagy inhibition and do not show embryonic lethality phenotypes. In response to high-fat diet feeding, *Atg4b*-null mice have hepatic steatosis, decreased energy expenditure, increased weight gain and disrupted blood glucose clearance, compared to wild-type mice (Fernández et al., 2017). Similarly, *Becn1* heterozygous mice also show partially decreased autophagy activity in metabolic organs, and systemic glucose intolerance and insulin intolerance upon high-fat diet feeding, even though their body weight is comparable to that of wild-type mice (Yamamoto et al., 2018). These studies suggest that systemic suppression of autophagy activity disrupts energy homeostasis and exacerbates metabolic dysfunctions.

On the other hand, activating autophagy has different metabolic effects among various metabolic organs, since the demands of autophagy activity in each metabolic organ are different. Ubiquitous *Atg5*-overexpressing transgenic mice show improved systemic glucose clearance, decreased fat and body mass upon aging, and extended lifespan compared to wild-type mice (Pyo et al., 2013). These beneficial effects are attributed to increased autophagy activity and antioxidant levels via Atg5 overexpression. In line with this, mutant mice defective in stress-induced autophagy but not basal autophagy, $BCL2^{AAA}$ mice, fail to show improvements in glucose metabolism induced by exercise (He et al., 2012), suggesting that whole-body autophagy activation during exercise is metabolically beneficial and essential for exercise-induced metabolic improvements. Furthermore, pharmacological activation of autophagy also shows overall beneficial effects on energy metabolism homeostasis. For example, spermidine and Rg2 activate bulk autophagy through inhibition of the histone acetyltransferase EP300, and activation of the AMPK-ULK1 signaling pathway, respectively (Pietrocola et al., 2015; Fan et al., 2017). Short-term *in vivo* administration of these autophagy inducers suppresses lipid accumulation in the white adipose tissue and liver, and limits body weight gain in response to high-fat diet treatment, resulting in improved glucose tolerance and insulin sensitivity (Fan et al., 2017; Fernández et al., 2017). These findings highlight that autophagy activation is important to maintain whole-body energy homeostasis. However, long-term genetic or pharmacological activation of autophagy may also negatively impact metabolism. Although hyperactivating autophagy by a constitutively active BECN1 mutant ($BECN1^{F121A}$) suppresses ER stress and improves systemic insulin sensitivity in response to high-fat diet feeding, it reduces insulin storage in pancreatic β cells and causes systemic glucose intolerance by excessive degradation of insulin granules, which can be reversed by treatment of autophagy inhibitors (Yamamoto et al., 2018). Consistently, in response to high-fat diet treatment, partial suppression of the VPS34 kinase by systemic heterozygous expression of a kinase-dead VPS34 mutant ($Vps34^{D761A/+}$), as well as VPS34 inhibitor treatment, suppresses hepatic steatosis and enhances glucose tolerance and insulin sensitivity, via suppression of metabolic substrate supplies to mitochondria, activation of AMPK signaling pathway, and increase of fatty acid β-oxidation (Bilanges et al., 2017). Thus, the roles of autophagy in energy metabolism are complex, and further investigations are necessary to discover tissue-specific mechanisms of ATG proteins in metabolic regulation.

Lipophagy

Lipid droplets are ubiquitous lipid-storing organelles, composed of a core of neutral lipids (such as triacylglycerol) surrounded by a phospholipid monolayer. Lipid droplets play a pivotal role in lipid energy metabolism, and also in gene expression by providing the ligands for peroxisome proliferator-activated receptors (PPARs). The canonical lipid droplet degradation pathway, lipolysis, occurs in the cytosol and is controlled by cytosolic lipases, including adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). Lipolysis stimuli, such as norepinephrine stimulation, induce phosphorylation of cytosolic lipases and lipid droplet-associated proteins (such as PLINs) through the PKA signaling pathway, resulting in increased lipolysis. In the past decade, growing evidence has revealed that macroautophagy also functions as a lipid droplet degradation pathway, known as lipophagy (Kounakis et al., 2019) (**Figure 1**). Through lipophagy, triacylglycerol and cholesterol esters stored in lipid droplets are delivered to the lysosomes for degradation and lipids are released for reuse. The direct evidence is the presence of accumulated lipid droplets within double-membrane autophagosomal structures. Pharmacological or genetic (knockdown of Atg5) inhibition of autophagy in cultured hepatocytes increases intracellular lipid contents. Consistently, liver-specific KO of Atg7 in mice increases hepatic lipid levels compared with those of control mice (Singh et al., 2009a), whereas liver-specific KO of Rubicon, an autophagy-inhibiting protein, ameliorates hepatic lipid accumulation in response to

high-fat diet challenge (Tanaka et al., 2016). Moreover, RAB proteins are also shown to participate in lipophagy (Schroeder et al., 2015; Li et al., 2016). However, although many efforts have been devoted to lipophagy research, the molecular mechanism of lipophagy is still largely unclear. Notably, lipophagy-specific receptors have not been identified until recently. Spartin, a protein mutated in the Troyer syndrome characterized by the degeneration of upper motor neurons (Patel et al., 2002), is identified as a lipophagy receptor (Chung et al., 2021). Spartin localizes to lipid droplets and mediates autophagy-dependent lipid droplet delivery to lysosomes through interacting with LC3A and LC3C, although a canonical LC3-interacting region (LIR) motif has not been determined. KO of Spartin, or expression of the Spartin dominant-negative mutant, leads to accumulation of lipid droplets in breast cancer cells and mouse motor cortex neurons. These findings support that the macroautophagy machinery regulates cellular lipid turnover. Furthermore, a positive interplay between lipophagy and lipolysis has been discovered. In brown adipose tissue, autophagosomes and autophagosome-associated LC3 act as scaffolds for cytosolic lipases, such as ATGL, to localize on lipid droplets, where lipophagy and cytosolic lipolysis complement each other in lipid droplet degradation (Martinez-Lopez et al., 2016). Future investigation is needed to further demonstrate the molecular mechanisms and the physiological functions of lipophagy.

In addition, CMA also facilitates lipid droplet degradation. Although it does not directly break down lipid droplets, CMA degrades the lipid droplet-associated coating proteins, PLIN2 and PLIN3, which contain the KFERQ targeting motif. CMA-mediated PLIN degradation enhances the translocation of both cytosolic lipases and macroautophagic proteins onto the lipid droplets for degradation (Kaushik and Cuervo, 2015; 2016).

Mitophagy

Mitochondria are the main site of energy production and the powerhouse of the cell, generating up to 95% of ATP in the cell. Under nutrient-rich conditions, mitochondria generate reactive oxygen species, which in turn induce mitochondria malfunction and lead to systemic energy disruption. Mitophagy is an important mitochondrial quality control pathway that selectively eliminates dysfunctional mitochondria (Palikaras et al., 2018) (**Figure 1**). Several mitophagy-mediated proteins are identified, including PTEN-induced kinase 1 (PINK1) and the cytosolic E3 ubiquitin ligase Parkin, and the mitochondrial outer-membrane proteins BNIP3 and FUNDC1. PINK1 accumulates specifically on the surface of damaged mitochondria by defective proteolytic cleavage due to the loss of mitochondrial membrane potential. Accumulated PINK1 recruits, phosphorylates and activates Parkin on the mitochondrial membrane, and active Parkin then polyubiquitinates outer mitochondrial membrane proteins. Ubiquitinated damaged mitochondria are recognized and bound by mitophagy receptors, such as optineurin and NDP52 (Lazarou et al., 2015), through the interaction between mitophagy receptors and LC3 via the LC3-binding LIR motif, leading to mitophagy induction.

However, the metabolic role of mitophagy is not fully understood. KO of either *Bnip3* or *Fundc1* specifically in the liver or adipose tissue leads to damaged mitochondria accumulation, excess lipid deposition, and systemic insulin resistance and obesity (Glick et al., 2012; Wu et al., 2019). However, by contrast, although skeletal muscle-specific *Fundc1* KO mice also show accumulation of damaged mitochondria and increased levels of reactive oxygen species in skeletal muscle as whole-body and adipose tissue-specific *Fundc1* KO mice (Fu et al., 2018), they have ameliorated insulin resistance, hepatic steatosis and obesity in response to high-fat diet challenge, due to enhanced secretion of FGF21 from muscle, a stress-induced metabolically beneficial myokine (muscle-secreted hormone). In line with this, skeletal muscle-specific *Atg7* deletion also induces mitochondrial dysfunction and increases Atf4-dependent FGF21 secretion, resulting in increased fatty acid β-oxidation, browning of white adipose tissue, and systemic energy metabolism improvement in response to high-fat diet treatment (Kim et al., 2013). Thus, FUNDC1, or FUNDC1 and ATG7-mediated mitophagy, may have different overall effects on systemic metabolism in different metabolic tissues via both cell autonomous and non-cell autonomous mechanisms.

Notably, physical exercise is a potent autophagy inducer in skeletal muscle (Rocchi and He, 2017). During exercise, mitochondrial oxidative stress in the contracting muscle is significantly increased, leading to activation of mitophagy (Laker et al., 2017). Exercise-induced mitophagy plays an important role in both mitochondrial quality control in the muscle and exercise training-induced metabolic adaptation. In response to energetic stress induced by acute exercise, specific isoforms of AMPKs (AMPKα1, α2, β2 and γ1) are found to be localized and activated on the mitochondrial outer membrane (Drake et al., 2021). Activated AMPK then phosphorylates and activates ULK1, which activates the downstream autophagy machinery and mitophagy. In line with this, KO of ULK1 specifically in the muscle abolishes the metabolic adaptations to exercise (Laker et al., 2017).

ER-phagy

The endoplasmic reticulum (ER) plays pivotal roles in protein synthesis and folding, calcium ion storage, and lipid synthesis and lipid droplet formation. The ER is also known as the primary membrane supplier for the isolation membrane. Misfold proteins, saturated fatty acids, and reactive oxygen spices induce ER stress, which impairs ER function and cellular homeostasis and is implicated in obesity and diabetes progression. There are two major ways of ER quality control, one is the unfolded protein response (UPR), and the other is ER-phagy (specific degradation of the ER by macroautophagy) (**Figure 1**). UPR is triggered by ER stress and activates several transcriptional factors including ATF6, ATF4, CHOP and XBP1, which upregulate downstream genes encoding chaperones and ER-associated degradation proteins to eliminate causes of ER stress (Yang et al., 2021). Notably, these transcriptional factors also upregulate the expression of ATG genes, including BECN1 and ATG7, in mouse embryonic fibroblasts (MEFs) (B'Chir et al., 2013). The ATG proteins participate in ER-phagy to regulate ER homeostasis. *In vivo Atg7* knockdown by adenovirus results in increased ER stress in the liver and disruption of systemic glucose

metabolism (Yang et al., 2010). In the liver of obese mice, the levels of key autophagy proteins, such as BECN1, ATG7 and ATG5, are decreased. Conversely, autophagy activation by overexpression of Atg7 or knock-in of the hyperactive mutant $BECN1^{F121A}$ reduces ER stress and improves both liver energy homeostasis and systemic insulin sensitivity (Yang et al., 2010; Yamamoto et al., 2018). Consistent with these studies, systemic administration of the autophagy activator rapamycin to Akita mice, a type 1 diabetes model with misfolded proinsulin accumulation, ameliorates diabetic phenotypes through activating autophagy and reducing ER stress and β cell apoptosis (Bachar-Wikstrom et al., 2013).

So far, eight ER-phagy receptors have been identified, FAM134 A/B/C, SEC62, RTN3, CCPG1, TEX264 and ATL3 (Khaminets et al., 2015; Fumagalli et al., 2016; Grumati et al., 2017; Smith et al., 2018; Chen et al., 2019; Chino et al., 2019). All the identified ER-phagy receptors are ER membrane proteins and are able to interact with LC3/GABARAP through the LIR motif. Among them, FAM134B is the best characterized ER-phagy receptor. ER stress induces FAM134B phosphorylation through activated CAMK2B, which enhances FAM134B oligomerization. Activation of FAM134B leads to enhanced fragmentation of ER membranes, followed by subsequent degradation via ER-phagy (Jiang et al., 2020). The other two FAM134 family proteins, FAM134A and FAM134C, also function as ER-phagy receptors, but there are a number of differences among the three. FAM134B is functional under both basal and stress conditions (Khaminets et al., 2015), whereas FAM134A and FAM134C are inactive under basal conditions and only activated by stress stimuli, and thus are key for stress-induced ER fragmentation and autophagic degradation (Kumar et al., 2021; Reggio et al., 2021). Furthermore, although all three FAM134 proteins possess the LIR domain, for pro-collagen I homeostasis, FAM134A functions also in a LIR-independent manner and compensates for the loss of FAM134B or FAM134C. In comparison, FAM134C is unable to compensate for the loss of other FAM134 proteins and functions coordinately with FAM134B (Reggio et al., 2021).

Specifically, ER-accumulated aggregates of prohormones, such as *Akita* mutant proinsulin, are eliminated via RTN3-dependent ER-phagy (Cunningham et al., 2019). In *Akita* mice, the C96Y mutation in *Insulin 2* ($Ins2^{C96Y}$) blocks protein folding and induces proinsulin aggregation in the ER, resulting in type 1 diabetes progression. These insoluble aggregates are unable to be removed by the ER luminal chaperon glucose-regulated protein 170 (GRP170); instead, they are cleared by RTN3-mediated ER-phagy. The RTN3-dependent ER-phagy pathway is also involved in the elimination of other misfolded or aggregated prohormones, including pro-opiomelanocortin (POMC) and pro-arginine-vasopressin (Pro-AVP) (Cunningham et al., 2019). Prohormone aggregates are captured by the transmembrane ER protein progesterone receptor membrane component 1 (PGRMC1) via its luminal domain, and delivered to the RTN3-mediated ER-phagy pathway for clearance (Chen et al., 2021).

In addition, a genome-wide CRISPRi screening reveals that mitochondrial oxidative phosphorylation proteins are important for ER-phagy (Liang et al., 2020), suggesting that different from bulk autophagy, ER-phagy requires normal mitochondrial metabolism and function for execution. Despite the link between ER stress and diabetes, how ER-phagy regulates energy metabolism and metabolic disease progression is largely unknown. Further studies are needed to demonstrate both the cell autonomous and the non-cell autonomous roles of ER-phagy and ER-phagy-related proteins in energy metabolism.

REGULATION OF TISSUE DEVELOPMENT AND HOMEOSTASIS

Adipose Tissue Development and Function

White adipocytes are occupied by lipid droplets and are the main storage of body neutral lipids, such as triacylglycerol. In addition to functioning as a fat storage organ, adipose tissue has also been recognized as an endocrine organ in the past 2 decades. Adipose tissues regulate systemic energy metabolism through secreting various adipokines (adipose-derived hormones), such as adiponectin, leptin and resistin. Both *in vitro* and *in vivo* evidence demonstrate that autophagy is required for adipogenesis (Baerga et al., 2009; Singh et al., 2009b; Zhang et al., 2009). In MEFs and 3T3-L1 fibroblast cells, genetic (Atg7 or Atg5 knockdown) or pharmacological (3-methyladenin or chloroquine treatment) inhibition of autophagy blocks adipocyte differentiation, via either promoting apoptosis, suppressing expression of the adipogenesis master regulator PPARγ, or enhancing PPARγ degradation via the ubiquitin-proteasome system (Baerga et al., 2009; Singh et al., 2009b; Zhang et al., 2013). *Atg7* deficiency at early development stages of white adipose tissue increases the mitochondrial content, the fatty acid β-oxidation rate and the number of multilocular lipid droplets, resulting in browning of the white adipocytes. As a result, adipocyte-specific Atg7 KO mice show decreased body weight and white adipose tissue mass, and increased systemic insulin sensitivity (Singh et al., 2009b; Zhang et al., 2009). Similarly, deletion of *Atg7* in brown adipose tissue also causes an increase in mitochondrial contents and β-oxidation, leading to elevated brown adipose tissue mass (Kim et al., 2019). Although the impact of *Atg7* deletion in mature adipocytes remains to be determined, other studies have shown that autophagy also plays an important role in the regulation of post-developmental functions and homeostasis of mature adipocytes. Different from KO of Atg7 during adipose development stages, adipocyte-specific KO of *Becn1* during adulthood increases adipose tissue inflammation, ER stress and apoptosis, and decreases insulin sensitivity (Jin et al., 2021). Similarly, post-developmental adipose-specific deletion of *Atg3* or *Atg16L1* leads to dysfunctional mitochondria accumulation followed by increased lipid peroxides in adipose tissue, although fat mass and body weight appear to be unaffected. Increased lipid peroxides enter the circulation and affect liver energy homeostasis, resulting in systemic insulin resistance (Cai et al., 2018).

In comparison to white adipose tissue, brown adipose tissue functions as a thermogenic organ through non-shivering

thermogenesis, and is characterized by high mitochondria contents, UCP1 (uncoupling protein 1) expression levels and fatty acid β-oxidation rates. There are also beige adipocytes, which are brown adipocyte-like cells present in white adipose tissue induced by browning of white adipocytes. Similar to brown adipocytes, beige adipocytes have multilocular lipid droplets, increased mitochondrial contents and upregulated β-oxidation. Activation of adipocyte browning is beneficial for systemic metabolism and potentially a therapeutic target in metabolic disorders, while beige-to-white transition has opposite effects. During beige-to-white adipocyte transition, autophagy is activated and is involved in mitochondrial clearance (Altshuler-Keylin et al., 2016). Accordingly, pharmacological or genetic inhibition of autophagy by chloroquine treatment, or *Atg5*-or *Atg12*-deletion, prevents beige-to-white adipocyte transition and protects against diet-induced obesity and systemic insulin resistance (Altshuler-Keylin et al., 2016).

β Cell Maintenance

Pancreatic β cells regulate systemic glucose metabolism by sensing circulating glucose and secreting insulin into the bloodstream. Under steady-state conditions, the autophagy activity in β cells is low, and autophagosome formation is upregulated in high-fat diet-induced and genetic mouse models of obesity and diabetes (Ebato et al., 2008; Chu et al., 2015; Sheng et al., 2017). Treatment of free fatty acids in cultured β cells effectively induces autophagy via the JNK1 signaling pathway (Komiya et al., 2010), indirectly explaining the *in vivo* data. β cell-specific *Atg7* deletion decreases β cell mass, intracellular insulin contents and glucose-stimulated insulin secretion, due to accumulated defective organelles, elevated ubiquitinated protein aggregates and increased apoptosis (Ebato et al., 2008; Jung et al., 2008). *Atg7* deletion in β cells results in hyperglycemia and impaired glucose tolerance in both regular and high-fat diet-fed mice. Thus, the basal autophagy activity in β cells plays a critical role in maintaining β cell function and systemic glucose homeostasis.

Yet too much autophagy in β cells can be metabolically detrimental. Although pharmacological activation of autophagy by rapamycin injections in Akita mice improves glucose metabolism, potentially via decreased β cell ER stress and apoptosis (Bachar-Wikstrom et al., 2013), chronic autophagy activation reduces insulin storage in β cells. Insulin granules are an unusual autophagy cargo via autophagic degradation of secretory vesicles (vesicophagy) (**Figure 1**). Chronic constitutive activation of autophagy by $BECN1^{F121A}$ leads to excessive degradation of insulin granules in β cells, resulting in decreased insulin storage and secretion and impaired systemic glucose tolerance in response to high-fat diet feeding, even though systemic insulin sensitivity is better than wild-type mice (Yamamoto et al., 2018). The vesicophagy degradation process is different from crinophagy, with the latter representing the direct fusion of secretory vesicles with lysosomes. Although the key autophagy proteins ATG5, BECN1 and ATG7 are potentially involved in vesicophagy, the molecular mechanism is largely unknown and needs further investigation. Furthermore, due to their insulin-secreting function, pancreatic β cells are more sensitive to autophagy level changes than other metabolic organs and cells; accordingly, the autophagy activity needs to be tightly regulated in β cells to maintain their homeostasis and function.

Liver Lipid Metabolism

The liver is an important metabolic organ regulating systemic energy homeostasis through gluconeogenesis and lipogenesis. In the liver, in addition to bulk autophagy, various selective autophagy pathways, including mitophagy and lipophagy, regulate liver energy homeostasis (Ueno and Komatsu, 2017). Overnutrition causes excess ectopic lipid accumulation in the liver, which has a causal link to the pathogenesis of non-alcoholic fatty liver disease (NAFLD), steatohepatitis (NASH) and hepatocellular cancer. In the liver of obese mice, the expression of several key ATG proteins, including ATG5, BECN1 and ATG7, is decreased, suppressing the autophagy flux (Yang et al., 2010). Pharmacological and genetic activation of autophagy by rapamycin treatment or *Atg7* overexpression in the liver decreases lipid accumulation, alleviates ER stress and improves insulin action, resulting in enhanced systemic glucose homeostasis. Conversely, KO of ATG genes, including *Atg7*, *FIP200* and the PI3KC3 *Vps34*, leads to accumulation of ubiquitin-positive aggregates and deformed organelles in the liver. These KO mice show hepatomegaly, hepatic steatosis, and exacerbated systemic glucose intolerance and insulin resistance (Komatsu et al., 2005; Yang et al., 2010; Jaber et al., 2012; Ma et al., 2013). Notably, many of these ATG gene KO mice have reduced glycogen contents but increased lipid storage levels in the liver, raising the possibility that hepatic autophagy deficiency may induce a shift of energy substrates from lipids to carbohydrates through altering gene expression. Indeed, autophagy is reported to regulate metabolic gene expression in the liver by degrading CRY1, an inhibitor of master circadian rhythm transcription factors CLOCK and BMAL1, via several LC3-interacting LIR motifs in CRY1 (Toledo et al., 2018). The expression of many energy metabolic genes is regulated by the circadian rhythm. Autophagy deficiency leads to hepatic accumulation of CRY1, but not other circadian rhythm-related proteins such as PER1, BMAL1 and CLOCK, resulting in the disruption of the circadian rhythm and nutrient metabolism.

CANCER METABOLISM IN METABOLIC TISSUES

Metabolic organs, including liver and pancreas, develop a variety of malignancies. Adipose tissues and muscles rarely develop cancer likely due to their largely quiescent status. Depending on tumor types, stages and genetic context, autophagy acts as a double-edged sword and has both tumor-suppressing and oncogenic roles in cancer development. Neoplasia has been found in the liver and pancreas of various autophagy-deficient mouse models, including *Becn1* heterozygous mice (Qu et al., 2003; Yue et al., 2003), systemic mosaic *Atg5* deletion mice (Takamura et al., 2011), and liver- or pancreas-specific *Atg5* or

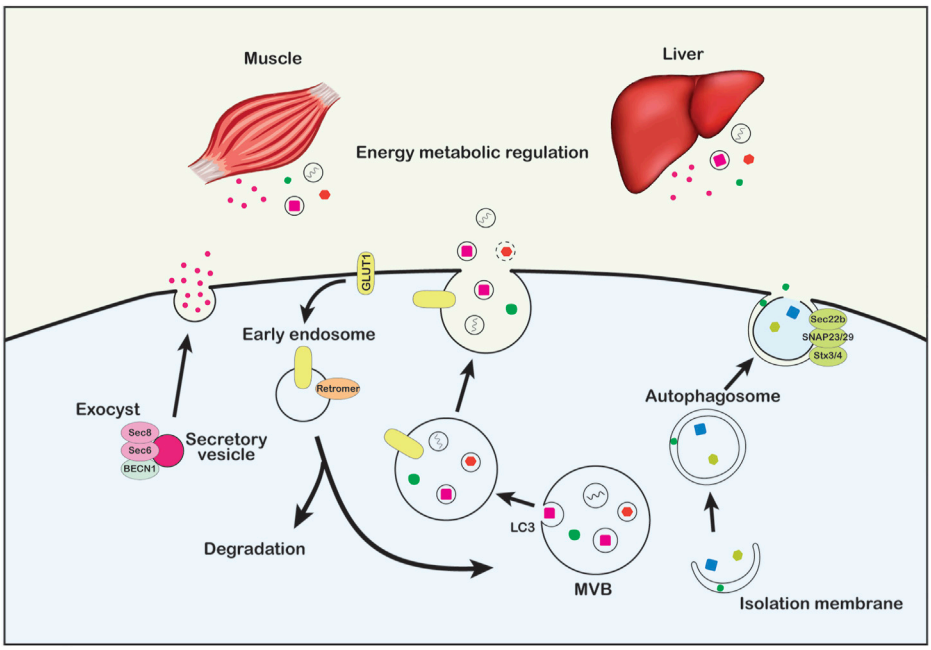

FIGURE 2 | Non-canonical functions of the autophagy machinery in vesicle trafficking and secretion. ATG proteins regulate systemic metabolic and energy balance via non-degradative functions in vesicle trafficking and secretory protein release, including adiponectin secretion through direct interaction between BECN1 and the exocyst protein (left), retromer-driven GLUT1 recycling and ATG5- and LC3-dependent exosome secretion through the endosomal and MVB pathway (middle), and secretory autophagy (right).

Atg7 deletion mice (Inami et al., 2011; Takamura et al., 2011; Rosenfeldt et al., 2013; Yang et al., 2014). Autophagy suppresses tumor initiation by maintaining cellular homeostasis through quality control of proteins, organelles and chromosomal stability (Mathew et al., 2007). However, lesions in autophagy-deficient mice are benign or premalignant, and do not progress to high-grade malignancy (Qu et al., 2003; Takamura et al., 2011; Rosenfeldt et al., 2013). Rather, in established tumors, genetic or pharmacological inhibition of autophagy suppresses pancreatic tumor growth (Yang et al., 2011; Yang et al., 2014; Yang et al., 2018). The tumor-promoting role of autophagy can be explained by two mechanisms. First, in established cancer cells, inhibition of autophagy causes the accumulation of defective mitochondria, which leads to a reduction in oxidative phosphorylation and suppression in tumor growth (Guo et al., 2011; Yang et al., 2011). Second, the autophagy-lysosomal pathway, regulated by the MiT/TFE family of transcription factors, plays an important role in maintaining cellular amino acid pools, which is essential for pancreatic cancer cell growth and proliferation under stress conditions (Perera et al., 2015). Thus, autophagy plays opposing roles in the initiation versus progression of tumors.

Tumors rely on nutrient supplies from the host. Autophagy is also important for tumor growth by regulating host energy metabolism and tumor environment (Kimmelman and White, 2017; Poillet-Perez and White, 2019). There are two main nutrient supply routes for tumors: one is the local tumor microenvironment, and the other is the host circulation. In the pancreatic tumor microenvironment, stroma-associated pancreatic stellate cells (PSCs) secrete alanine, a non-essential amino acid, through the autophagic pathway activated by signals released from cancer cells (Sousa et al., 2016). PSC-derived alanine serves as an alternative carbon source of pancreatic cancer cells. Host autophagy also maintains tumor growth by sustaining the level of circulating arginine, a semi-essential amino acid. In whole-body *Atg7* or *Atg5* KO mice, circulating arginine levels are decreased, resulting in suppressed tumor growth (Poillet-Perez et al., 2018). Mechanistically, liver damage and steatosis caused by autophagy deficiency in these mice promotes the release of arginase 1 (ARG1) into the bloodstream, which catalyzes the degradation of arginine into ornithine and subsequently decreases circulating arginine levels.

NON-DEGRADATIVE FUNCTIONS OF AUTOPHAGY

Conventional and Unconventional Secretion

Besides degradative functions, autophagy proteins promote protein secretion from both conventional and unconventional secretory pathways (**Figure 2**). The autophagy protein BECN1 facilitates the secretion of adiponectin, an insulin-sensitizing adipokine, independent of the canonical degradation function. In adipocytes, BECN1 interacts with several components of the exocyst complex (including SEC5, SEC6 and SEC8), a conserved octameric complex tethering secretory vesicles to the plasma membrane. The hyperactive $BECN1^{F121A}$ mutant shows

stronger binding to the exocyst components, leading to increased adiponectin secretion from adipocytes and improved systemic insulin sensitivity and energy homeostasis, via activation of AMPK signaling in metabolic organs by adiponectin (Kuramoto et al., 2021). Notably, different from adiponectin vesicles, vesicles containing several other adipokines, including leptin and resistin, are not efficiently recruited to the BECN1-exocyst complex, and their secretion is not regulated by BECN1. Thus, various types of secretory vesicles may occupy different subcellular locations and are sorted by distinct pathways from the trans-Golgi network to the plasma membrane. Further investigation is needed to reveal how BECN1 distinguishes adiponectin vesicles from other types of secretory vesicles. In addition, besides regulating hormonal secretion, the exocyst is also involved in the plasma membrane translocation of cell surface receptors such as the glucose transporter type 4 (GLUT4), which uptakes glucose in an insulin-responsive manner (Wang et al., 2019). Thus, it is possible that the autophagy machinery also plays a role in exocyst-dependent protein trafficking to the cell surface.

Most secretory proteins possess a canonical N-terminal signal peptide, which regulates secretory protein transport to the ER and the Golgi apparatus, and protein secretion via exocytosis. This pathway is known as the conventional secretory pathway. Over the past decade, a growing body of evidence shows that certain cytosolic proteins lacking the signal peptide, known as leaderless cargos, are secreted through unconventional secretory pathways, bypassing the ER-Golgi secretory pathway. There are four main types of unconventional secretory pathways: Type I is pore-mediated secretion; Type II is ABC transporter-mediated secretion; Type III is endocytic compartment-mediated secretion; and Type IV is Golgi-bypassed secretion (Rabouille, 2017; Padmanabhan and Manjithaya, 2020). Several ATG proteins, such as ATG5, ATG16L1, BECN1 and LC3, are involved in the Type III unconventional secretory pathway, termed secretory autophagy (**Figure 2**). A number of leaderless cargo proteins of secretory autophagy have been identified, including IL-1β and FABP4 (fatty acid binding protein 4). In response to lysosomal damage, IL-1β is reported to translocate into the intermembrane space or inner lumen of LC3-positive autophagosome-like vesicles (Dupont et al., 2011). These LC3-positive vesicles escape from Syntaxin (Stx) 17-dependent autophagosome-lysosome fusion, and fuse with the plasma membrane through the Sec22b-SNAP23/29-Stx3/4 complex for IL-1β secretion (Kimura et al., 2017). In Atg5 KO bone marrow-derived macrophages, IL-1β secretion is decreased compared with wild-type macrophages (Dupont et al., 2011).

Recent studies show that FABP4 is also secreted by the unconventional secretory autophagy. FABP4, also known as adipocyte protein 2 (aP2), is highly expressed in adipocytes and secreted as an adipokine. FABP4 functions as a lipid chaperone protein, and plays a key role in intracellular fatty acid metabolism, hepatic glucose production and insulin secretion (Prentice et al., 2019). FABP4 secretion from white adipocytes requires early components of the autophagy pathway such as FIP200, ULK1 and BECN1, but not ATG5 (Josephrajan et al., 2019). The molecular mechanism of FABP4 secretion through the autophagic secretory pathway is not fully elucidated, but it appears that it is different from ATG5-and LC3-dependent IL-1β secretion.

Recently, leaderless cargos have also been reported to be translocated into the ER-Golgi intermediate compartment through TMED10, a protein channel, and are then secreted along the secretory pathway (Zhang et al., 2020). TMED10 regulates the secretion of multiple leaderless cargos, including IL-1β, HSPB5, galectin1/3 and Tau. After the chaperone HSP90A unfolds the leaderless cargos in the cytosol, TMED10 binds and trans-locates the cargos into the ER-Golgi intermediate compartment for secretory vesicle packaging (Zhang et al., 2015; Zhang et al., 2020). How TMED10-mediated secretion crosstalks with the autophagy pathway remains unclear.

In addition, many studies have shown that autophagy proteins can indirectly regulate the secretion of neurotransmitters, neuropeptides, cytokines and secretory enzymes via modulating cellular stress responses, receptor metabolism and lipid metabolism (Saitoh et al., 2008; Kaushik et al., 2011; Bel et al., 2017; Kim et al., 2021). Thus, autophagy plays versatile roles in metabolism not only cell autonomously by degradation, but also non-cell autonomously via protein secretion.

Cell-Surface Receptor Recycling

The stability of cell-surface proteins, such as receptors and transporters, is important in maintaining cellular functions. The endosome-Golgi retrieval pathway plays a critical role in regulating the level of plasma membrane proteins, determining whether they are recycled to the plasma membrane or transported to the lysosome for degradation. The retromer is the key membrane protein sorting complex at the endosome, composed of Vacuolar Protein Sorting (VPS) 26, VPS29 and VPS35 (Chandra et al., 2021). The retromer sorts membrane proteins to the plasma membrane or trans-Golgi network, and prevents their degradation by lysosomes. The cell-surface level of glucose transporter 1 (GLUT1), also known as Solute Carrier Family 2 Member 1 (SLCA1) for glucose uptake, is regulated by the autophagy machinery via TBC1D5, a retromer-associated GTPase-activating protein specifically for RAB7 (Jimenez-Orgaz et al., 2018) (**Figure 2**). Inhibition of TBC1D5 activates RAB7, and promotes membrane association of the retromer and retromer-mediated receptor recycling. Under metabolic conditions relying on glycolysis, such as Ras transformation, hypoxia or glucose starvation, TBC1D5 binds with LC3 through a canonical LIR motif and re-localizes to LC3-positive autophagosomes from the retromer complex. This leads to retromer activation, increased GLUT1 recycling to the plasma membrane, and increased glucose uptake in autophagy-competent cells, but not in Atg5 or Atg7 KO cells (Roy et al., 2017). These findings suggest that GLUT1 recycling is dependent on the autophagy pathway. Given GLUT1 is ubiquitously expressed and its level is increased in many types of cancer cells, targeting autophagy may limit glucose metabolism in cancer cells and have therapeutic potential against tumors (Ancey et al., 2018). Notably, even though TBC1D5 is incorporated inside autophagosomes, the total TBC1D5 protein level is unchanged under the glucose starvation condition. Further investigation is

needed to reveal the mechanism by which TBC1D5 escapes from autophagic degradation.

Exosome Secretion

The autophagy machinery has recently been reported to regulate the secretion of extracellular vesicles and exosomes (**Figure 2**). Exosomes are small extracellular vesicles with a diameter of 50–150 nm, containing various types of cargos including microRNAs, lipids, enzymes and cytokines. Exosomes regulate a variety of physiological processes, such as tumor metastasis and energy metabolism, through cell-to-cell signaling (Guay and Regazzi, 2017). Exosomes are derived from invaginated vesicles inside multivesicular bodies (MVBs) in the endosomal pathway. MVBs face two fates: one is degradation via fusion with the lysosome, and the other is secretion as exosomes by translocation to the plasma membrane. Several key autophagy proteins, including ATG5, ATG16L1 and LC3, regulate the fate determination of MVBs. LC3 interacts with ATPase H^+ Transporting V1 Subunit E1 (ATP6V1E1), a component of the vacuolar ATPase (V-ATPase) involved in acidification of MVBs. LC3 and ATP6V1E1 are internalized into MVBs through an ATG5-dependent manner, resulting in increased MVB pH and exosome secretion (Guo et al., 2017). Certain exosomal cargos, such as RNA-binding proteins, can also interact with LC3 for packaging into exosomes (Leidal et al., 2020). This exosomal secretory mechanism is independent of additional canonical autophagy proteins, besides those required for LC3-PE conjugation. However, the role of the ATG5-and LC3-dependent exosomal secretion in metabolic regulation is unclear. In addition, whether the LC3 conjugation machinery is involved in the sorting of other MVB cargos towards secretion, and how synergism is achieved between classical MVB sorting proteins (such as RAB proteins) and the LC3 conjugation system, also need further investigation.

SUMMARY AND UNANSWERED QUESTIONS

Autophagy-related proteins play complex roles in metabolism, regulating not only the canonical autophagic degradation pathway, but also vesicle trafficking and proteins secretion. Compared to the degradative functions of ATG proteins, their roles in the regulation of cross-talk between metabolic organs via non-canonical, non-degradative, pathways are poorly understood. Autophagy may play more critical roles in endocrine organs than previously thought. For canonical autophagy, although various types of selective autophagy have thus far been discovered, such as mitophagy, lipophagy and ER-phagy, the molecular mechanisms and cargo receptors, which determine the degradation specificity of selective autophagy, remain to be fully elucidated. Furthermore, many single nucleotide polymorphisms (SNPs) on ATG genes have been identified to associate with the onset of metabolic diseases by genome-wide association studies, for example, rs10512488 in *Becn1* (Tamargo-Gómez et al., 2020), but their potential functions and mechanisms in the regulation of autophagy activity and energy metabolism are still unknown. Lastly, because autophagy plays an essential role in organ development and homeostasis, spatiotemporal-specific inhibition or activation of ATG proteins is needed to better understand how autophagy and ATG proteins regulate nutrient and energy homeostasis.

AUTHOR CONTRIBUTIONS

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Unexpected Phenotype Reversion and Survival in a Zebrafish Model of Multiple Sulfatase Deficiency

Angeleen Fleming[1,2,3*†], *Low Zhe Xuan*[2], *Gentzane Sanchez-Elexpuru*[1,2,3†], *Sarah V. Williams*[1,2,3], *Dylan Windell*[1,2,3†], *Michael H. Gelb*[4], *Zackary M. Herbst*[4], *Lars Schlotawa*[5†] *and David C. Rubinsztein*[1,3*†]

[1]Department of Medical Genetics, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, United Kingdom, [2]Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, United Kingdom, [3]UK Dementia Research Institute, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, United Kingdom, [4]Department of Chemistry, University of Washington, Seattle, WA, United States, [5]Department of Paediatrics and Adolescent Medicine, University Medical Centre Goettingen, Goettingen, Germany

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***Correspondence:**
Angeleen Fleming
af425@cam.ac.uk
David C. Rubinsztein
dcr1000@cam.ac.uk

†ORCID:
Angeleen Fleming
orcid.org/0000-0003-3721-7126
Gentzane Sanchez-Elexpuru
orcid.org/0000-0002-2104-2686
Dylan Windell
orcid.org/0000-0002-8188-6573
Lars Schlotawa
orcid.org/0000-0002-7415-4905
David Rubinsztein
orcid.org/0000-0001-5002-5263

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Multiple sulfatase deficiency (MSD) is a rare recessively inherited Mendelian disorder that manifests with developmental delay, neurodegeneration, skeletal deformities, facial dysmorphism, congenital growth retardation, and other clinical signs. The disorder is caused by mutations in the *SUMF1* gene, which encodes the formylglycine-generating enzyme (FGE), and responsible for the activation of sulfatases. Mutations in *SUMF1* result in reduced or absent FGE function with consequent compromised activities of its client sulfatases. This leads to an accumulation of enzyme substrates, such as glycosaminoglycans and sulfolipids, within lysosomes and subsequently impaired lysosome function and cellular pathology. Currently, there are no disease modifying therapeutic options for MSD patients, hence the need for more suitable animal models to investigate the disorder. Here, we describe the characterisation of a *sumf1* null zebrafish model, which has negligible sulfatase activity. Our *sumf1*$^{-/-}$ zebrafish model successfully recapitulates the pathology of MSD such as cranial malformation, altered bone development, an enlarged population of microglia, and growth retardation during early development but lacks early lethality of mouse *Sumf1*$^{-/-}$ models. Notably, we provide evidence of recovery in MSD pathology during later developmental stages, resulting in homozygous mutants that are viable. Hence, our data suggest the possibility of a unique compensatory mechanism that allows the *sumf1*$^{-/-}$ null zebrafish to survive better than human MSD patients and mouse *Sumf1*$^{-/-}$ models.

Keywords: multiple sulfatase deficiency, formylglycine-generating enzyme, lysosome, zebrafish, SUMF1

INTRODUCTION

Multiple sulfatase deficiency (MSD) is a rare, autosomal recessive disorder that encompasses the clinical characteristics of individual sulfatase deficiencies like mucopolysaccharidosis (MPS) and metachromatic leukodystrophy (MLD) (Schlotawa et al., 2020). Notably, MSD is part of a larger group of diseases classified as lysosomal storage disease (LSD). MSD is caused by the lack of post-translational modification of sulfatases due to a mutation in the Sulfatase Modifying Factor 1 (SUMF1) gene (Dierks et al., 2009). SUMF1 encodes the formylglycine-generating enzyme (FGE),

which uniquely converts the cysteine residue into a C-formylglycine residue at the sulfatase catalytic site. This conversion into C-formylglycine is essential for the sulfatase enzymatic activity (Schmidt et al., 1995). Since there is no other enzyme which can perform this modification, MSD patients have reduced activities of sulfatases (Dierks et al., 2009).

Sulfatases play the unique biochemical role of hydrolysing sulfate ester bonds of glycosaminoglycans (GAGs), like heparan sulfate, dermatan sulfate, keratan sulfate, chondroitin sulfate, and hyaluronan (Sardiello et al., 2005), steroid hormones (e.g., dehydroepiandrosterone 3-sulfate) and sulfolipids (e.g., cerebroside-3-sulfate) (Dierks et al., 2009). GAGs are characterized by their long, unbranched polysaccharide chain with O-sulfate groups, and are derived from proteoglycan degradation. These highly negatively charged groups interact with the positive charge of surrounding protein ligands and signalling molecules (Holmborn et al., 2012). Proteoglycans, like heparan sulfate proteoglycans and chondroitin sulfate proteoglycans, consist of a core protein coupled with varying numbers, and types of GAGs. These proteoglycans are abundant in the extracellular matrix (ECM) and at the cell surface. During degradation, the proteoglycans are internalised by endocytosis and the protein core is proteolytically removed [reviewed in (Freeze et al., 2015)]. The remaining glycosaminoglycan chain is then completely degraded by a suite of lysosomal enzymes (Freeze et al., 2015). Unsurprisingly, inactive sulfatases caused by *SUMF1* mutations lead to GAG accumulation within the lysosome, which is associated with impaired lysosome function and apoptosis. Indeed, accumulation of glycosaminoglycan is observed in both mouse *Sumf1*−/− models and human MSD patients (Settembre et al., 2007; Kobolák et al., 2019). Storage of sulfolipids results from the lack of arylsulfatase A (ARSA) activity and causes demyelination of the central and peripheral nervous system in MLD and MSD (Diez-Roux and Ballabio, 2005). In addition, non-lysosomal sulfatases modulate heparan sulfate dependent cell signalling pathways (Dhoot et al., 2001; Lamanna et al., 2007).

MSD is estimated to occur in one in 500,000 individuals worldwide (Cappuccio et al., 2020) with more than 143 cases of MSD recorded in the scientific literature (Schlotawa et al., 2020). Most MSD patients carry hypomorphic mutations, resulting in some residual sulfatase activity, although there are rare cases where FGE function is completely abrogated due to nonsense mutations (Schlotawa et al., 2019). A complete loss of FGE function leads to neonatal onset of MSD pathology and early mortality (Schlotawa et al., 2019). Presently, there are no disease modifying therapeutic options for MSD. This makes the generation of suitable animal models important for the development of potential treatment strategies. A *Sumf1* knockout mouse model (*Sumf1*−/−) with no sulfatase activity has been established (Settembre et al., 2007). These mice display similar clinical and biochemical features to MSD patients, such as scoliosis, facial malformation, congenital growth retardation, reduced sulfatase activities, and GAG storage in tissues and organs. However, mouse models are limited by the stock maintenance cost and slower drug screening efficiency when compared to smaller animal models. Furthermore, since MSD usually has an early onset, this suggests that most, if not all, deformities occur during early development (Schlotawa et al., 2020). Unfortunately, the ability to study early development in mouse models is impeded by their internal embryonic development and lack of transparency. Despite the evolutionarily conserved nature of *sumf1*/Fge, *Drosophila* are not an ideal animal model since they lack vertebrate-specific organs and structures such as the skeleton and multichambered heart, both of which have distinctive defects in MSD.

The zebrafish has emerged as an attractive model for studying childhood onset disorders as they undergo external development and are transparent during early development, allowing early morphogenesis to be observed under a light microscope. Their relatively small size and transparency also lends itself to confocal microscopy to allow assessment of cells and tissues *in vivo*, using a range of fluorescent markers and reporters. Two zebrafish *sumf1* null lines have been identified from mutagenesis screens (**Figure 1**). The first line (sumf1_la015919Tg), was identified from a mutagenesis screen using random insertion of a murine leukaemia-based retrovirus (Wang et al., 2007). The retroviral insertion occurs in exon 1 resulting in a frameshift and stop codon that truncates the FGE protein enzyme rendering it inactive (Varshney et al., 2013). The second line (sumf1_sa31531) was generated by the Zebrafish Mutation Project using random ENU mutagenesis (Kettleborough et al., 2013). This line has a C > A mutation in exon 2, which also leads to a nonsense mutation. We confirmed an absence of sulfatase activity and an accumulation of GAGs in both lines but, surprisingly, found that *sumf1*−/− zebrafish were viable as adults unlike the *Sumf1* knockout mouse model, which display high mortality at 3 months (Settembre et al., 2007). This suggests the possibility that *sumf1* mutation affects zebrafish differently compared to its mammalian counterpart. We went on to characterise cartilage and bone development, microglial cell populations, and lysosome abundance and activity in the viral insertion *sumf1*−/− zebrafish model. Similar to what has been observed in mouse and human patients, the *sumf1*−/− zebrafish model displayed altered bone development, cranial shape deformation, and an increase in macrophage and microglia populations during early development, suggesting that our model recapitulates many clinical features seen in MSD human patients (Schlotawa et al., 1993). Surprisingly, these deformities show recovery at later stages indicating that possible compensatory mechanisms exist in zebrafish but not in their mammalian counterparts. However, there was no recovery of sulfatase activity and no evidence of a block in autophagic flux, indicating that zebrafish have alternative mechanisms to survive the accumulation of GAGs and sphingolipids. Taken together, this work demonstrates that *sumf1*−/− zebrafish recapitulate aspects of the early developmental defects associated with MSD and observed in *Sumf1* null mice. Strikingly however, the novel observation of disease recovery and viability in the zebrafish model suggests the

FIGURE 1 | Protein alignment and mutation sites in human and zebrafish *SUMF1*/FGE. Human and zebrafish protein sequences share 59.94% sequence identify, increasing to 71.9% over the PFAM domain for FGE-sulfatase activity (yellow highlight). The viral insertion in the sumf1_la015919Tg line occurs in exon one and results in a premature stop codon (blue label). The point mutation in the sumf1_sa31531 line occurs in exon 2 and results in a nonsense mutation and premature stop codon (purple label).

presence of compensatory mechanisms which warrant further exploration.

MATERIALS AND METHODS

Zebrafish Stock Maintenance and Embryo Production

All zebrafish procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act with appropriate Home Office Project and Personal animal licences and with local Ethics Committee approval. Experiments were performed in accordance with ARRIVE guidelines. Zebrafish were maintained on a 14 h light: 10 h dark cycle under standard conditions (Westerfield, 2000). Embryos were collected from natural spawnings, staged according to established criteria (Kimmel et al., 1995) and reared in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM Mg_2SO_4, 5 mM HEPES) at 28.5°C (hereafter referred to as E3M). The density of embryos was kept constant with embryo medium replenished daily. The sumf1_la015919Tg strain (ZFIN ID: ZDB-ALT-120806-11568) was used for the majority of experiments (hereafter referred to as *sumf1*$^{-/-}$), unless otherwise stated. The sumf1_sa 31531 was used in initial validation experiments to confirm that defects observed in the sumf1_la015919Tg strain were also observed in an independent line. The *sumf1* mutant lines were maintained on the Tuebingen Longfin (TL) background and this wild-type strain was used as the control line in all experiments. In all post-mortem analysis, zebrafish were culled by immersion in 1 mg/ml 3-amino benzoic acid ethyl ester (MS222, also known as tricaine) prior to tissue processing.

Cartilage Staining

Zebrafish were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), hereafter called PFA, overnight at 4°C. After three washes in dH_2O, samples were stained with 0.1% Alcian Green overnight then differentiated in acid/alcohol (0.3% HCL, 70% Ethanol) and rehydrated by gradual replacement with dH_2O. Subsequently, samples were digested using trypsin at room temperature for 10 min (for larvae) or 37°C overnight (for adult specimens) then bleached with 3% H_2O_2 in 1% KOH to remove all pigment, followed by 3 washes of 1% KOH. KOH was gradually replaced with glycerol until the samples had equilibrated and cleared.

TABLE 1 | Western blotting reagents.

Antibody target	Primary/Secondary antibody	Concentration	Supplier
LC3 II (Rabbit)	Primary	1:500	Novus
Cathepsin D (Mouse)	Primary	1:500	Abcam
β-Actin (Mouse)	Primary	1:5000	Sigma-Aldrich
α-Tubulin (Mouse)	Primary	1:5000	Sigma-Aldrich
Anti-rabbit (Goat)	Secondary	1:5000	Dako
Anti-mouse (Goat)	Secondary	1:5000	Dako

In vivo Bone Staining

Craniofacial and axial bones were detected by staining the zebrafish in E3M containing 0.1% Alizarin Red S (from a saturated solution in dH_20) and incubated in the dark at 28.5°C for 2–5 h depending on the age of the zebrafish. Fish were then washed three times in E3M, anaesthetised, and viewed using a dsRed filter set on a Leica M205A microscope.

Wax Embedded Samples

Samples were fixed in Bouin's fixative at RT for up to 6 months. The samples were dehydrated by immersing in the following series of solutions for 30 min (juvenile) and up to 60 min (adults): 70% Ethanol, 87% Ethanol, 95% Ethanol, 100% Ethanol, 100% Ethanol, 100% Ethanol/Histoclear (1:1), and Histoclear (2 washes in an oven set to 68.5°C). The histoclear was then gradually replaced with molten wax. The processed samples were placed in a sectioning mould and mounted using molten wax. Samples were sectioned at 10 μM using a Leica microtome (RM2125RT).

Transverse sections were used for the Alcian Blue staining. The sections were rehydrated through a graded alcohol series, stained in 1% Alcian Blue in 3% acetic acid (pH 2.5) for 30 min, rinsed and counterstained in 0.1% Nuclear Fast Red solution for 5 min. Sections were then dehydrated and mounted in Depex medium.

For Haematoxylin & Eosin (H&E) staining, the sections were rehydrated through a graded alcohol series, stained with Mayer´s Haematoxylin solution for 10 min, rinsed and dipped in Eosin (1% Eosin Y in dH_2O) 12 times. Sections were then dehydrated and mounted in Depex medium.

Protein Lysate Preparation

Larvae and adults were anaesthetized, excess liquid was removed, and lysis buffer containing 1% octylglucoside, complete protease inhibitor cocktail, and PhosSTOP™ tablets added. Samples were sonicated then centrifuged at 7000 rpm for 5 min at 4°C to obtain pure protein lysate. Total protein for each lysate was determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific™). Supernatants were diluted in 2 × Laemmli Buffer at a 1:1 dilution and boiled for 10 min before loading.

Western Blotting

Samples were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis using the BioRad mini-PROTEAN Tetra Electrophoresis System and transferred to the PVDF membranes. The membranes were blocked in 5% non-fat dry milk in PBST (PBS +0.1% Tween20) for 1 h at RT before staining in primary antibodies as denoted in **Table 1**. Membranes were incubated with primary antibodies overnight at 4°C (rocking). After three washes of PBST, membranes were incubated for one hour with secondary antibodies (**Table 1**) then washed three times in PBST. Immunoreactive bands were detected using ECL™ (GE Healthcare Bioscience) on a LI-COR Odyssey Fc Imager (LI-COR Biosciences) and processed with FIJI software (ImageJ).

Wholemount Staining for Microglia/Macrophage

Staining was carried out as described (Astell and Sieger, 2017). Briefly, the paraformaldehyde-fixed samples were washed twice using PBStx (0.2% Triton X-100 in 0.01 M PBS) and blocked in 1% goat serum blocking buffer (1% normal goat serum, 1% DMSO, 1% BSA, and 0.7% Triton X-100 in 0.01M PBS) for 2 h. Subsequently, the samples were incubated with mouse anti-4C4 primary antibody (1:50; Abcam) overnight at 4°C. After two washes of PBStx, the samples were incubated with goat anti-mouse Alexafluor 488 in secondary antibody (1:1000; Thermo Fisher Scientific) in blocking buffer overnight at 4°C. After three washes of PBStx, the samples were stored in 70% glycerol at 4°C until the final mounting.

Microscopy and Image Processing

Images for *in vivo* bone staining, cartilage staining, wholemount immunostaining were acquired using a fluorescence stereomicroscope (Leica; M205 FA) equipped with a Leica DFC7000T digital camera using the Leica Application Suite (LASX). Histological and immunostained sections were imaged using a Zeiss AxioPlan 2 Motorized Microscope equipped with a QImaging 2000R digital camera using QS imaging software. For wholemount immunostaining of macrophages and microglia, z-stacks were taken through the entire fish and LASX deconvolution was applied to the maximum intensity projection. For all images, the threshold was adjusted to 185 (min.) and 255 (max.) and particles within the z-stacks were counted.

Lysotracker Staining

Larvae at 2 d.p.f, 5 d.p.f, and 10 d.p.f were incubated with 0.5% LysoTracker™ Red DND-99 (Invitrogen) in embryo medium for 45 min. 10 d.p.f larvae were treated from 1 d.p.f. with EM containing 0.03% phenylthiourea to prevent pigmentation. After staining, larvae were then anaesthetized, mounted in 1% low melting agarose in embryo medium and viewed using a Leica SP8 laser confocal microscope. Images were then processed with FIJI software (ImageJ).

Sulfatase and Lysosomal Hydrolase Activity Assays

Lysis of fish larvae and tissues for activity assays was done as described above for western blot analysis. Instead of octylglucoside buffer lysis was done in presence of PBS (pH 7.4) and protease inhibitors (1% v/v,

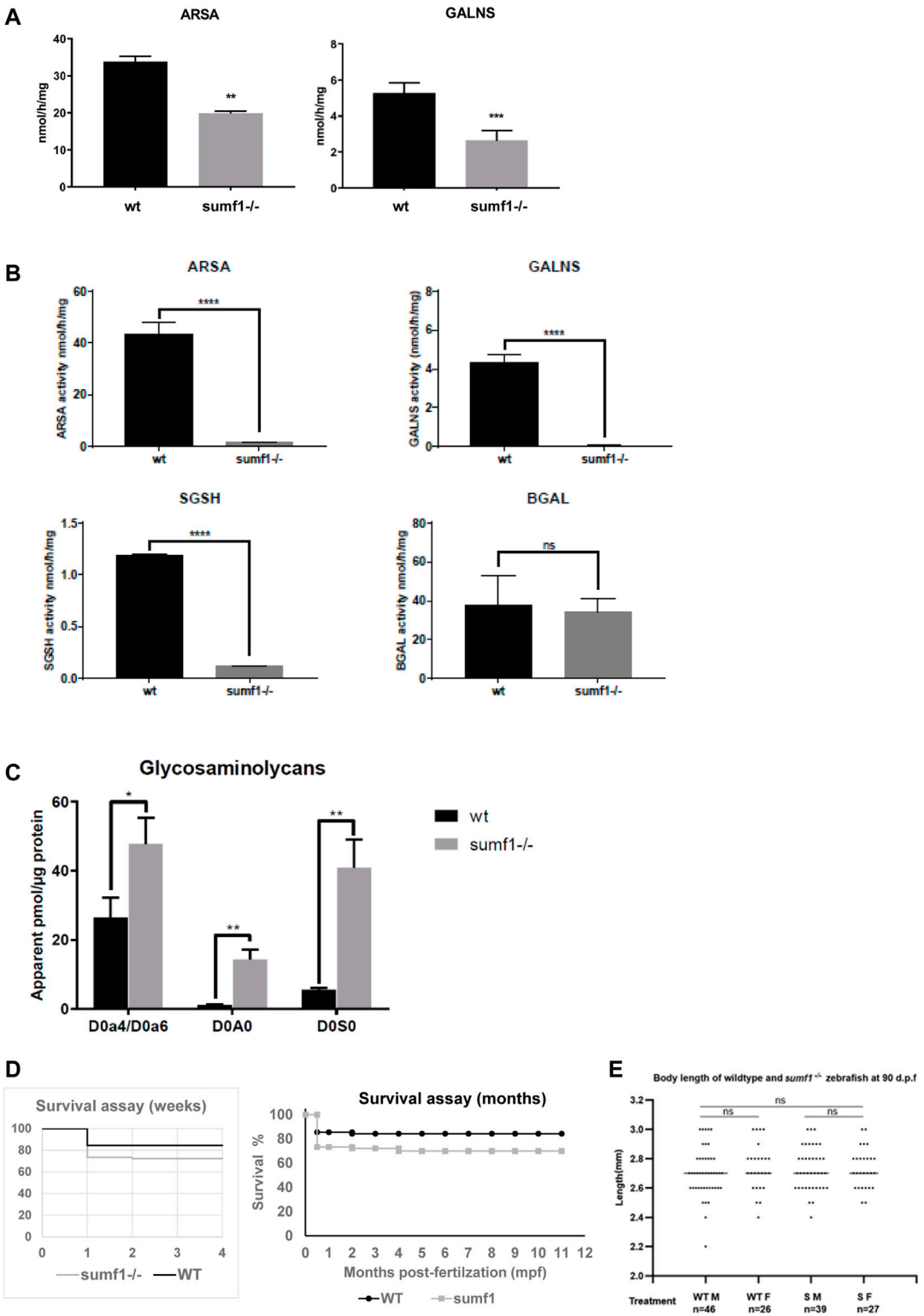

FIGURE 2 | Sulfatase activity, GAG accumulation and viability of *sumf1*$^{-/-}$ zebrafish. **(A)** Activities of arylsulfatase A (ARSA) and N-acetylgalactosamine-6-sulfatase (GALNS), enzymes that are dependent on FGE function for their activation. Enzymatic activities were measured at 5 d.p.f. in larvae from in-crosses of *sumf1_LA*$^{+/-}$ adults. A significant reduction in sulfatase activity was confirmed in *sumf1*$^{-/-}$ larvae, indicating a loss of FGE function. **(B)** Since larvae from heterozygous females may retain maternal mRNA and/or protein, enzymatic activity assays were performed on *sumf1*$^{-/-}$ larvae from *sumf1*$^{-/-}$ parents and compared to wildtype larvae from a wildtype (TL) background. No or negligible levels of ARSA, GALNS, and N-Sulfoglucosamine sulfohydrolase (SGSH) activity could be detected in *sumf1*$^{-/-}$ larvae at

(*Continued*)

FIGURE 2 | 5 d.p.f. indicating an absence of FGE activity. Beta-galactosidase was included as a control as this enzyme is a lysosomal hydrolase which is not dependent on FGE. **(C)** Glycosaminoglycan levels were significantly elevated in *sumf1*$^{-/-}$ larvae at 5 d.p.f. relative to wildtype larvae of the same age. Chondroitin/Dermatan Sulfate (CS/DS) and Heparan Sulfate (HS) apparent levels were calculated using Chondrosine as an internal standard. D0a4: Chondroitin/Dermatan Sulfate Internal Disaccharide 4-sulfation (CS-A, DS) = unsaturated UA-GalNAc (4S); D0a6: Chondroitin Sulfate Internal Disaccharide 6-sulfation (CS-C) = unsaturated UA-GalNAc (6S); D0A0: Heparan Sulfate Internal Disaccharide n-sulfated = unsaturated UA-GlcNS; D0S0: Heparan Sulfate Internal Disaccharide no sulfation = unsaturated UA-GlcNAc. Apparent pmols were calculated using Chondrosine as an internal standard for mass spectrometry-based detection. **(D,E)** *sumf1*$^{-/-}$ zebrafish and wildtype fish were reared concomitantly and initially assessed weekly (for 1 month) and then monthly to assess survival. Although an initial drop in viability was observed in *sumf1*$^{-/-}$ larvae at 14 d.p.f., no further differences in viability were observed up to 12 months of age **(D)** and no differences in body length were observed in either sex at 30 d.p.f., suggesting that growth was normal **(E)**. For **(A–C)**, graphs show mean values (±SD) from at least 3 biological replicates. Statistical analysis was performed using two-tailed t-test. $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, and ***: $p \leq 0.001$.

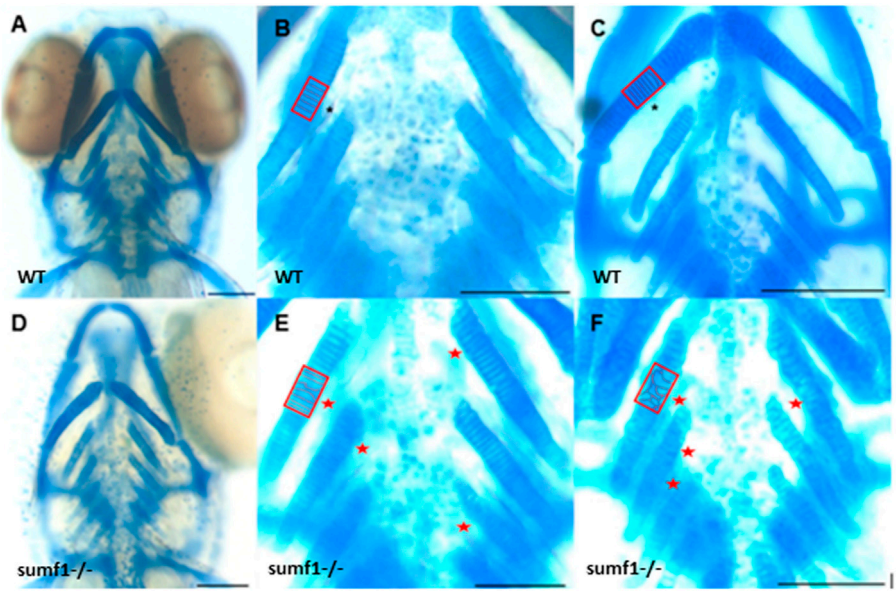

FIGURE 3 | Intercalation of chondrocytes in the craniofacial skeleton. Alcian blue stained samples of **(A,B)** wildtype ($n = 4$) and **(D,E)** *sumf1*$^{-/-}$ zebrafish ($n = 6$) at 5 d.p.f. The chondrocytes of wildtype larvae **(B)** at 5 d.p.f. were neatly stacked (asterisk) indicating complete intercalation whereas intercalation in *sumf1*$^{-/-}$ larvae **(E)** was disrupted in some areas (outlined) and stacked elsewhere (red star). **(C,F)**. At 10 d.p.f. (C&F, $n = 3$ per genotype), the intercalation of chondrocytes was disrupted in **(F)** *sumf1*$^{-/-}$ larvae (asterisk and red box) when compared to **(C)** wildtype (asterisk). Scalebar represents 200 µm.

1% Protease inhibitor mix, Roche, Mannheim, and Germany). Arylsulfatase A, N-acetylgalactosamine-6-sulfatase (GALNS) activity and N-sulfoglucosamine sulfohydrolase (SGSH) activity were determined following previously published protocols (Baum et al., 1959; Steckel et al., 1983; van Diggelen et al., 1990; Karpova et al., 1996). For β-galactosidase, lysates (2 µg) were diluted with substrate buffer (0.1 M citrate-phosphate pH 4.5, 2 mM 4-MU-β-D-galactopyranoside) Calbiochem Merck, Darmstadt, and Germany) to 40 µl final volume in wells of a black 96-well plate. A standard product dilution serious with 4-MU (Sigma Aldrich Merck, Darmstadt, Germany) diluted in H_2O and 0.05 M Tris pH 8.0 was added. After incubation of 30 min at 37°C, the reaction was stopped with 150 µl stop buffer (0.17 M glycine-carbonate), and plates were centrifuged for 15 min at 1160 g. Readout was done using a fluorescent plate reader (Synergy Mx, BioTek, Winooski, United States) with excitation at 360 nm and emission at 460 nm. Activities were determined referring changes in OD or fluorescence respectively to total protein amounts and incubation time.

GAG Analysis

Lysates of zebrafish larvae and adult brains were prepared as described for sulfatase and lysosomal hydrolase activity assays and analysed using the internal disaccharide method as described in (Herbst et al., 2020).

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism or Excel. Unpaired or Welch's t-test were used for the analysis of two samples. One-way ANOVA followed by Tukeys multiple comparison test was used for comparing multiple samples.

RESULTS

Sulfatase Activity and Survival

In-crosses of heterozygous fish for both the *sumf1_LA* and *sumf1_sa* lines produced viable offspring with the expected Mendelian distributions of wildtype, heterozygous and

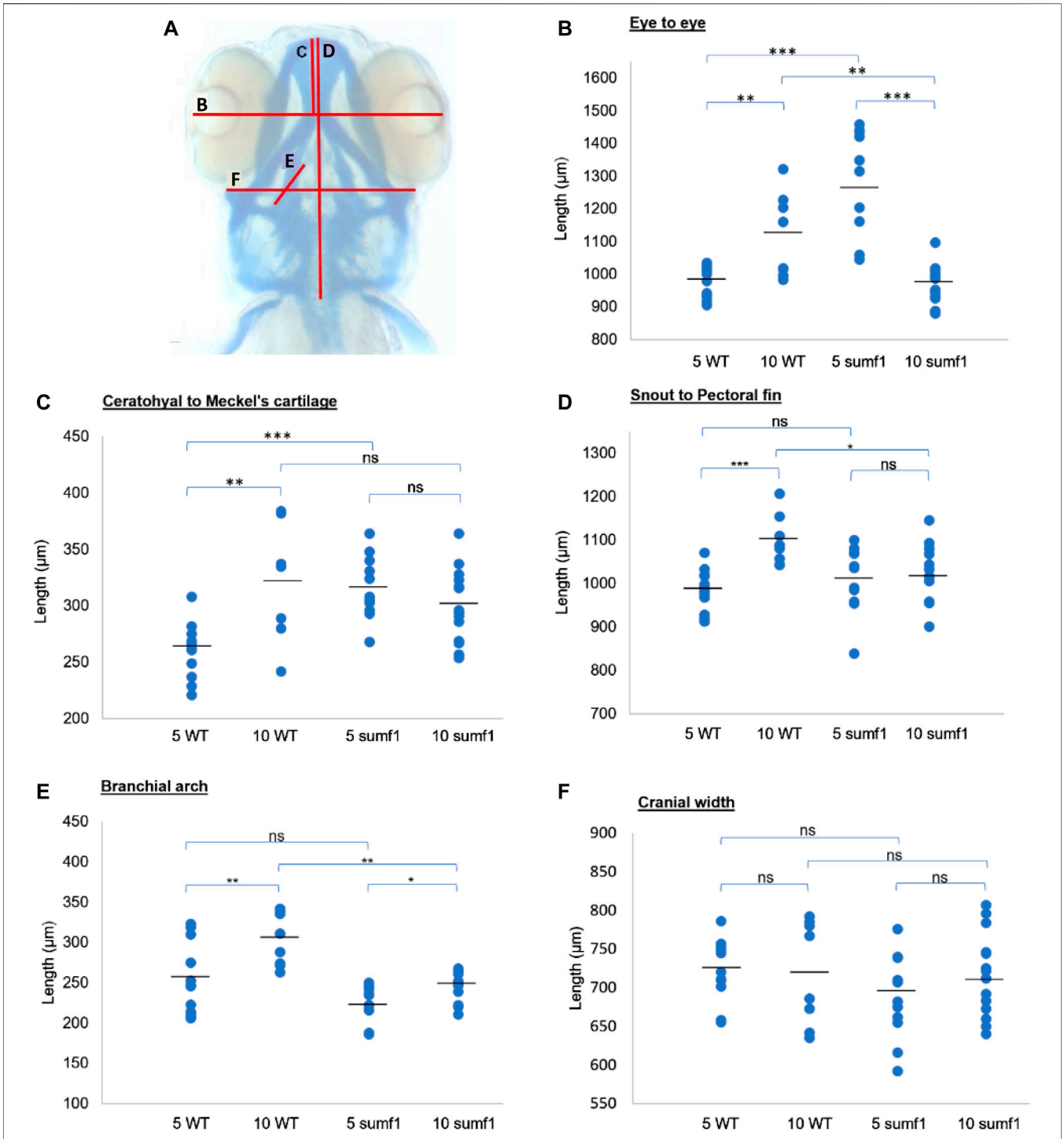

FIGURE 4 | Cranial cartilage measurements of wildtype and *sumf1* mutant zebrafish at 5 and 10 d.p.f. **(A)** Schematic diagram indicating measurements of cartilage element representing the following: The distance between the eyes **(B)**, Meckel's cartilage and ceratohyal **(C)**, snout and the pectoral fin **(D)**, the length of the first branchial arch **(E)**, cranial width **(F)**. Differences in the mean of each measurement was analysed using Welch's t-test (wildtype and *sumf1*$^{-/-}$; 5 d.p.f. and 10 d.p.f.) and the *p*-values are denoted as following: ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, and ***: $p \leq 0.001$. Means values are indicated by the line.

homozygous embryos and larvae. Embryos and larvae were observed daily up to 10 days post-fertilisation (d.p.f.), with no overt morphological differences observed between the genotypes.

To confirm that the mutations in each line truly resulted a loss of FGE function, we analysed the activities of 2 different sulfatases that rely on FGE modification to become active (arylsulfatase A,

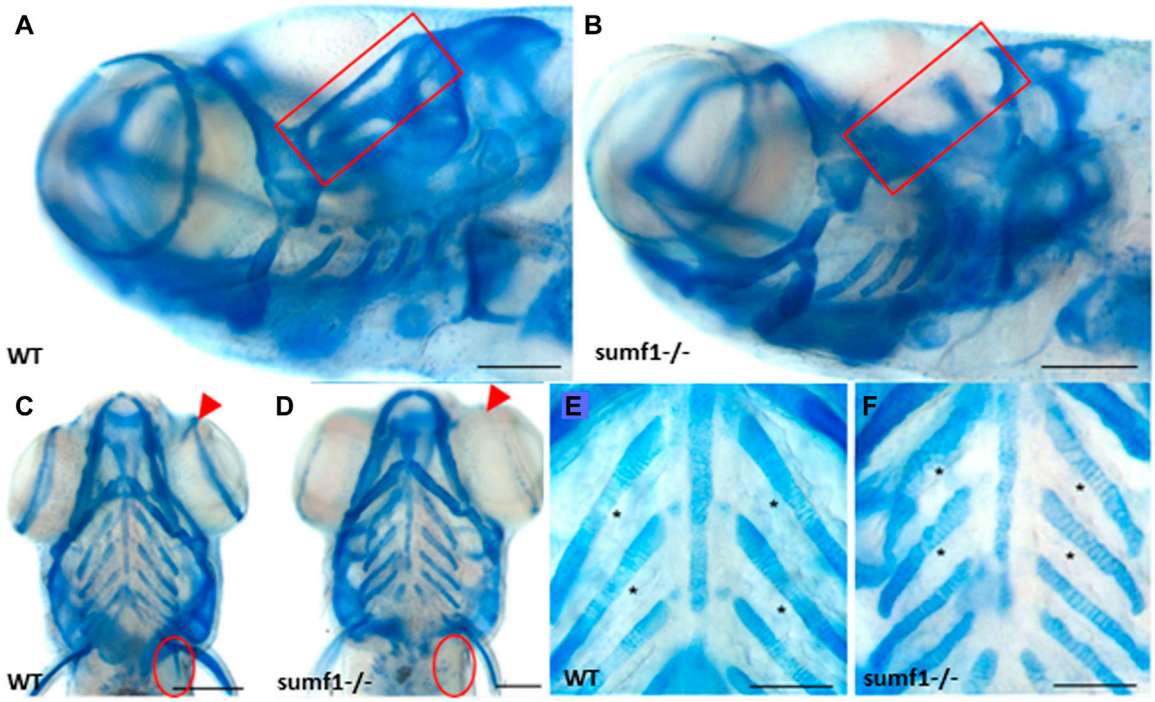

FIGURE 5 | Analysis of craniofacial cartilage at 30 d.p.f. The semi-circular canal (red box) and the scapulocorocoid (red circle) were present in wildtype **(A,C)** were missing in *sumf1*$^{-/-}$ larvae **(B,D)**. Similarly, supraorbital cartilage (red arrow) was well developed in wildtype larvae **(C)** but retarded in *sumf1*$^{-/-}$ larvae. Notably, chondrocytes in the branchial arches were neatly stacked (intercalated) in wildtype larvae **(E)**, whereas the intercalation of the chondrocytes was disrupted in *sumf1*$^{-/-}$ larvae **(F)**. Scalebar represents 200 μm. Representative images of $n = 3$ samples for each genotype.

ARSA and N-acetylgalactosamine-6-sulfatase, GALNS) in wildtype and homozygous siblings from in-crosses of *sumf1_LA*$^{+/-}$ adults at 5 d.p.f. A significant reduction in sulfatase activity was confirmed in *sumf1*$^{-/-}$ larvae, indicating a loss of FGE function (**Figure 2A**). Offspring from in-crosses of heterozygous fish were raised to adulthood and genotyped at 3 months post-fertilisation (m.p.f.). Normal Mendelian distributions of genotypes were observed for both *sumf1_LA* and *sumf1_sa* lines, hence longevity was analysed up to 2 years old and no differences were observed between genotypes. We therefore tested whether in-crosses of *sumf1*$^{-/-}$ adults produced viable offspring, since these females would produce eggs lacking maternally-deposited FGE protein and *sumf1* mRNA and therefore may be expected to have a more severe phenotype than those from *sumf1*$^{+/-}$ parents. All embryos and larvae from this cross were viable and with the expected Mendelian distributions of genotypes. To confirm that the offspring lacked FGE, we performed sulfatase activity assays on larvae at 5 d.p.f., comparing *sumf1*$^{-/-}$ larvae from *sumf1*$^{-/-}$ parents to wildtype larvae from a wildtype (TL) background. In *sumf1*$^{-/-}$ larvae, no or negligible levels of ARSA, GALNS and N-Sulfoglucosamine sulfohydrolase (SGSH) activity could be detected (**Figure 2B**), suggesting absent FGE activity. Beta-galactosidase was included as a control as this enzyme is a lysosomal hydrolase which is not dependent on FGE.

One would predict that an absence of sulfatase activity would result in an accumulation of glycosaminoglycans and, indeed, this was observed in *sumf1*$^{-/-}$ larvae relative to wildtype larvae of the same age (**Figure 2C**). Next, we examined the viability of *sumf1*$^{-/-}$ larvae from in-crosses of *sumf1*$^{-/-}$ adults and, although a drop in survival was observed at 14 d.p.f., we found no difference in long-term survival (**Figure 2D**), no difference in the size of *sumf1*$^{-/-}$ fish compared to wildtype fish reared in parallel (**Figure 2E**) and no overt histological differences were observed in muscle, brain or bone of *sumf1*$^{-/-}$ adults at 12 months old (**Supplementary Figure S1**).

Cartilage and Bone Formation

These finding were surprising since the mouse *Sumf1*$^{-/-}$ model and human MSD patients with null mutations often suffer from early mortality and growth retardation. We therefore looked in detail at the development of *sumf1*$^{-/-}$ embryos and larvae to determine whether they displayed any of the morphological defects observed in *Sumf1*$^{-/-}$ knockout mouse model and human MSD patients. Analysis of craniofacial cartilage development revealed a defect in the intercalation of chondrocytes in the branchial arches in larvae at 5 d.p.f. and this persisted at 10 d.p.f. (**Figures 3A–F**). In addition, we measured a range of craniofacial dimensions to determine whether *sumf1*$^{-/-}$ larvae displayed the same widening of the head and growth retardation as observed in mouse models and human MSD patients (**Figure 4A**). Analysis of larvae at 5 and 10 d.p.f. revealed differences in measurements between wildtype and *sumf1*$^{-/-}$

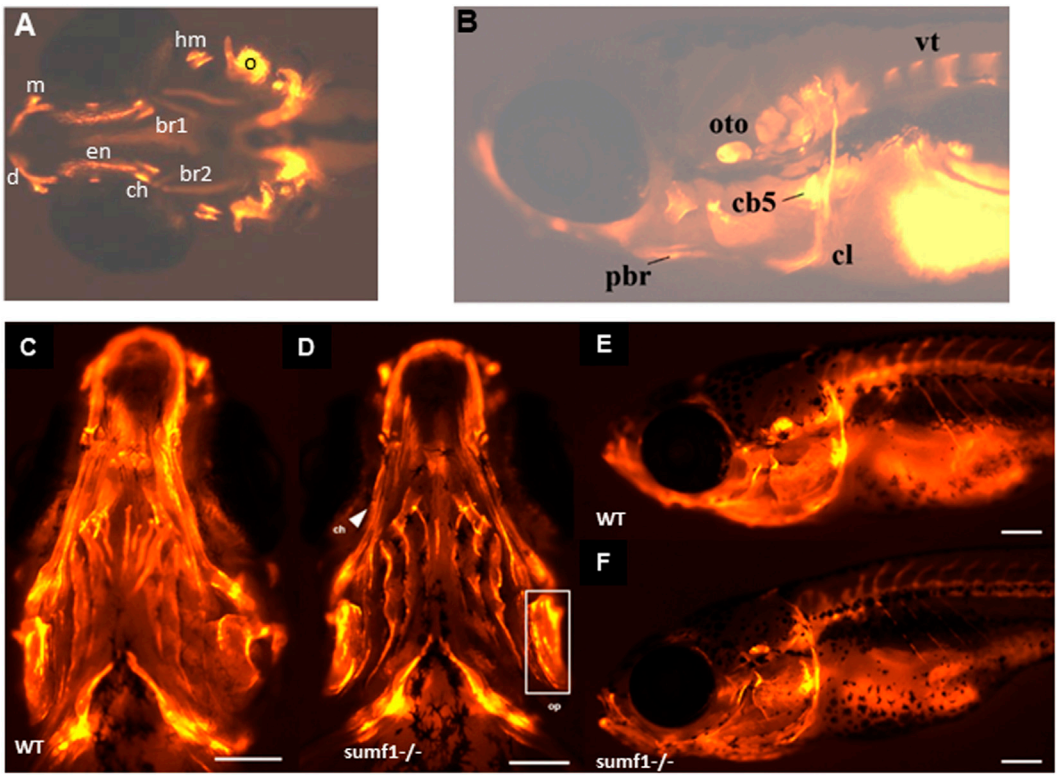

FIGURE 6 | Development of ossification centres in different craniofacial elements. **(A,B)** Alizarin red stained larvae were imaged at 10 and 15 d.p.f. and scored according to the size of the ossification centre (growth) and intensity of the fluorescent stain. Ossification centres in the vertebrae (vt), otoliths (oto), primary ossification center (oc) for the hyomandibular (hyo), and ceratohyal (ct), maxilla (m), dentary (d), cleithrum (cl), posterior branchiostegal ray (pbr), ceratobranchial 5 (cb5), operculum (op), entopterygoid (en), branchiostegal ray (bcr) 1 and 2 were assessed (see **Table 2**). At 30 d.p.f., no overt differences in the mineralisation/ossification of the craniofacial skeleton could be identified between wildtype **(C,E)** and *sumf1*$^{-/-}$ larvae (D&F). No differences were found in the fluorescent intensity of the operculum (white box) and ceratohyal (white arrow). The scalebar represents 500 μm. Figure shows representative images of n = 3 samples for each genotype.

TABLE 2 | Quantification of ossification centres in different craniofacial elements.

Treatment	vt	Oto	Oc (hyo)	Oc (ct)	m	d	cl	pbr	Cb5	op	en	Br 1	Br 2	Total (mean)
Day 10 WT (n = 6)	*	**	*	*	**	**	**	**	**	**	**	*	*	13.67
Day 10 sumf1 (n = 9)	*	*	*	*	**	*	*	*	*	*	*	*	*	10.11
Day 15 WT (n = 7)	***	***	****	****	***	****	***	***	***	****	****	****	****	39.29
Day 15 sumf1 (n = 5)	**	**	*	*	***	**	***	***	***	***	**	**	**	22.40

*The ossification centres of the different craniofacial elements (**Figure 6A**) were analysed at 10 and 15 d.p.f. and scored according to the size of the ossification centre (growth) and intensity of the fluorescent stain (degree of mineralisation), using a 4-point scoring system, for 13 different ossification centres. Ossification centres in the vertebrae (vt), otoliths (oto), primary ossification center (oc) for the hyomandibular (hyo) and ceratohyal (ct), maxilla (m), dentary (d), cleithrum (cl), posterior branchiostegal ray (pbr), ceratobranchial 5 (cb5), operculum (op), entopterygoid (en), branchiostegal ray (bcr) 1 and 2 were assessed. The mean scores for each element were summarised as *≤1, **≤2, ***≤3, ****≤4.*

larvae at these timepoints, as well as differences in the growth of these elements between the two groups (**Figures 4B–F**). The greatest difference observed between wildtype and *sumf1*$^{-/-}$ larvae at 5 d.p.f. was the eye-to-eye width (**Figure 4B**) and length from the ceratohyal to Meckel's cartilage (**Figure 4C**). These measurements indicate that the cranial shape of this region was narrower and shorter in *sumf1*$^{-/-}$ larvae at 5 d.p.f. compared to wildtype, which is consistent with the cranial deformation seen in MSD patients and mouse models. In addition, these two measurements, as well as the snout to pectoral fin length (**Figure 4D**) increased significantly in wildtype larvae between 5 and 10 d.p.f. (indicative of growth/elongation of the head), whereas no growth was observed in *sumf1*$^{-/-}$ larvae suggesting that development and growth of the facial cartilages of *sumf1*$^{-/-}$ zebrafish was retarded compared to wildtype larvae. The change in eye-to-eye width in *sumf1*$^{-/-}$ larvae between 5 and 10 d.p.f. was surprising and is likely to result from a mechanism

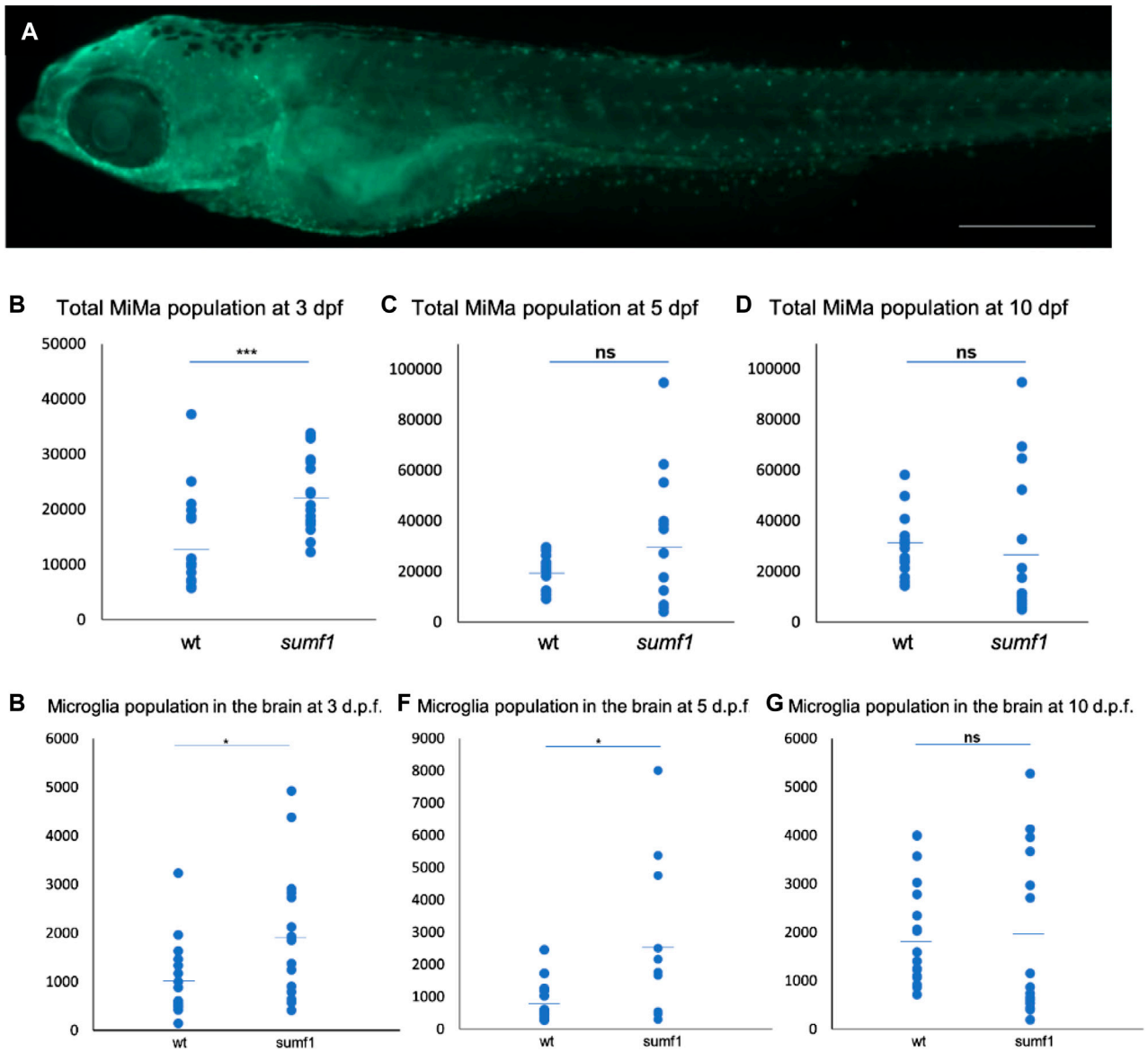

FIGURE 7 | Quantification and distribution of microglia and macrophages. **(A)** Wildtype and *sumf1*$^{-/-}$ larvae were immunostained with an antibody to detect microglia and macrophages. Scalebar represents 200 μm. The total number of microglia and macrophages (Mi/Ma) was quantified at 3, 5, and 10 d.p.f. by analysing maximum intensity projections of a Z-stack through the entire larva. **(B)** At 3 d.p.f., there was a significant increase in the Mi/Ma population in *sumf1*$^{-/-}$ larvae compared to wildtype larvae. However, these differences did not persist at **(C)** 5 d.p.f. and **(D)** 10 d.p.f. **(E–G)** The brain resident population of labelled cells (assumed to by microglia) was quantified at 3, 5, and 10 d.p.f. The brain was selectively quantified by selecting the same Region of Interest (ROI) targeting the frontal and the dorsal brain in all images. There was a significant increase in the microglia population in *sumf1*$^{-/-}$ larvae at **(E)** 3 d.p.f. and **(F)** 5 d.p.f. but no difference at 10 d.p.f. **(G)**. Two-tailed t-test with *p*-values are denoted as follows: ns: $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, and ***: $p \leq 0.001$. Means values are indicated by the line.

different to cartilage development since it resolves over time (e.g., interim oedema), as one would not expect a reduction in this measurement during development unless it were coupled with growth in perpendicular axis (e.g., ceratohyal to Meckel's cartilage or ceratohyal to Meckel's cartilage).

We next explored whether the defects in the cartilaginous elements of the craniofacial skeleton persisted into adulthood and whether defects in bone formation were observed in zebrafish larvae at 10, 15, and 30 d.p.f. The most striking differences in the cartilaginous skeleton at 30 d.p.f. was the lack of specific cartilaginous structures in *sumf1*$^{-/-}$ zebrafish, namely the otic capsule (n = 3; 67%) (**Figures 5A,B**) and the scapulocorocoid, and reduction of staining in the supraorbital cartilage, indicative of retarded formation (**Figures 5C,D**). The intercalation of chondrocytes into the branchial arch elements in wildtype fish remained uniform, with narrow cells

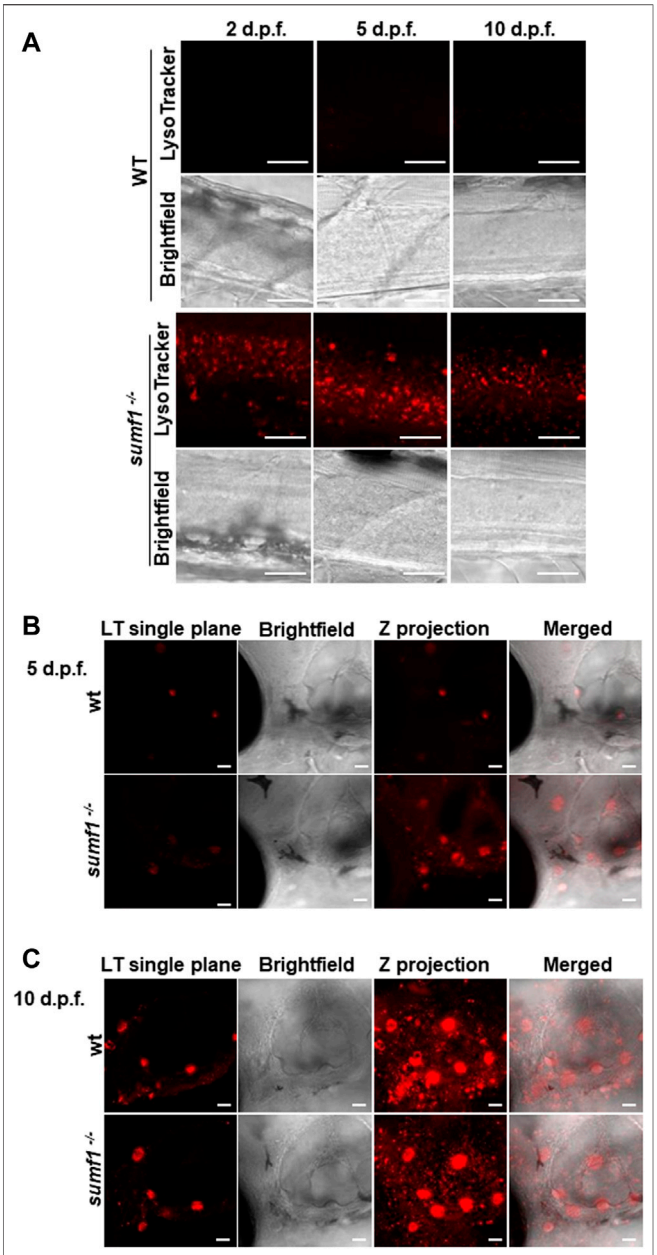

FIGURE 8 | Imaging of lysosomes. *In vivo* lysotracker staining was imaged using confocal microscopy. **(A)** A striking increase in lysotracker staining was observed in the spinal cord of *sumf1*$^{-/-}$ larvae compared to wildtype (wt) larvae at 2 d.p.f., 5 d.p.f., and 10 d.p.f. **(B)** An increase in lysotracker staining was observed in and around the otic vesicle of *sumf1*$^{-/-}$ larvae compared to wt larvae at 5 d.p.f., whereas no differences between the two groups were observed at 10 d.p.f. **(A–C)** Representative images of the lysotracker staining, brightfield and the z projection. At least 5 fish were analysed for each group. Scale bar represents 30 μm for all images.

appearing neatly stacked (**Figure 5E**). In contrast, in *sumf1*$^{-/-}$ zebrafish, the chondrocytes showed some degree of stacking but were not as tightly stacked as seen in wildtype (**Figure 5F**) leading to distortion (bending) of the branchial arches due to the irregular round cell shape.

In zebrafish, ossification of endochondral bone in the craniofacial region begins at 3 d.p.f. but occurs at different timepoints in different elements and it is only from 14 d.p.f. that all ossification centres are present (Cubbage and Mabee, 1996). To examine whether these changes in the cartilage of the endochondral skeleton affected ossification, we performed *in vivo* bone staining using Alizarin red. The ossification centres of the different craniofacial elements (**Figures 6A,B**) were analysed at 10 and 15 d.p.f. and scored according to the size of the ossification centre (growth) and intensity of the fluorescent stain (degree of mineralisation), using a 4-point scoring system, for 13 different ossification centres. The results for each ossification centre are summarised in **Table 2** with the mean score for all elements. At 10 d.p.f., *sumf1*$^{-/-}$ zebrafish showed a significant reduction in mean score of all the ossification centres ($p < 0.05$, Welch's t-test) when compared to wildtype. However, when looking at the ossification of individual elements, only the otoliths were significantly more developed ($p < 0.05$, Welch's t-test) in wildtype, compared to *sumf1*$^{-/-}$ zebrafish, indicating that the mean score represents cumulative modest changes in ossification between wildtype and *sumf1*$^{-/-}$ larvae.

Similarly, the mean ossification score of *sumf1*$^{-/-}$ was also reduced at 15 d.p.f. ($p < 0.05$, Welch's t-test), with significantly less development of both the hyomandibular and the ceratohyal primary ossification centers ($p < 0.05$, Welch's t-test) in *sumf1*$^{-/-}$ compared to wildtype zebrafish. Furthermore, ossification of non-endochondral elements (i.e., dermal bones), such as the branchiostegal rays, were also significantly less developed in the *sumf1*$^{-/-}$ zebrafish. While these observations suggested that the retardation in bone development was still present at 15 d.p.f., the variation in different elements suggests that the effect of *sumf1*$^{-/-}$ null mutation on bone development was not uniform with longer bone elements, like the branchiostegal rays and entopterygoid significantly affected ($p < 0.05$, Welch's t-test) at 15 d.p.f., whereas wider bone elements like operculum and ceratobranchial 5 were not significantly different ($p > 0.05$, Welch's t-test).

Since these data suggested that cartilage deformities do translate into retarded bone ossification in *sumf1*$^{-/-}$ zebrafish, we next examined bone formation at 30 d.p.f. No overt differences were observed in qualitative comparisons of the Alizarin red staining between wildtype and *sumf1*$^{-/-}$ fish in any of the structures in terms of bone shape and intensity of fluorescent staining (**Figures 6C–F**). To support this observation, quantification of fluorescence intensity was performed on two bones; the ceratohyal (long bone element) and operculum (wide bone element). No significant differences were observed between wildtype and *sumf1*$^{-/-}$ fish, suggesting that the early defects in cartilage formation did not persist into adulthood.

Immune Cell Responses

One of the most evident features of pathology in the *Sumf1–/–* mouse model was the presence of highly vacuolated macrophages and activated microglia (Settembre et al., 2007). Indeed, activated microglia appear to contribute to neurodegeneration in mouse models of many lysosomal

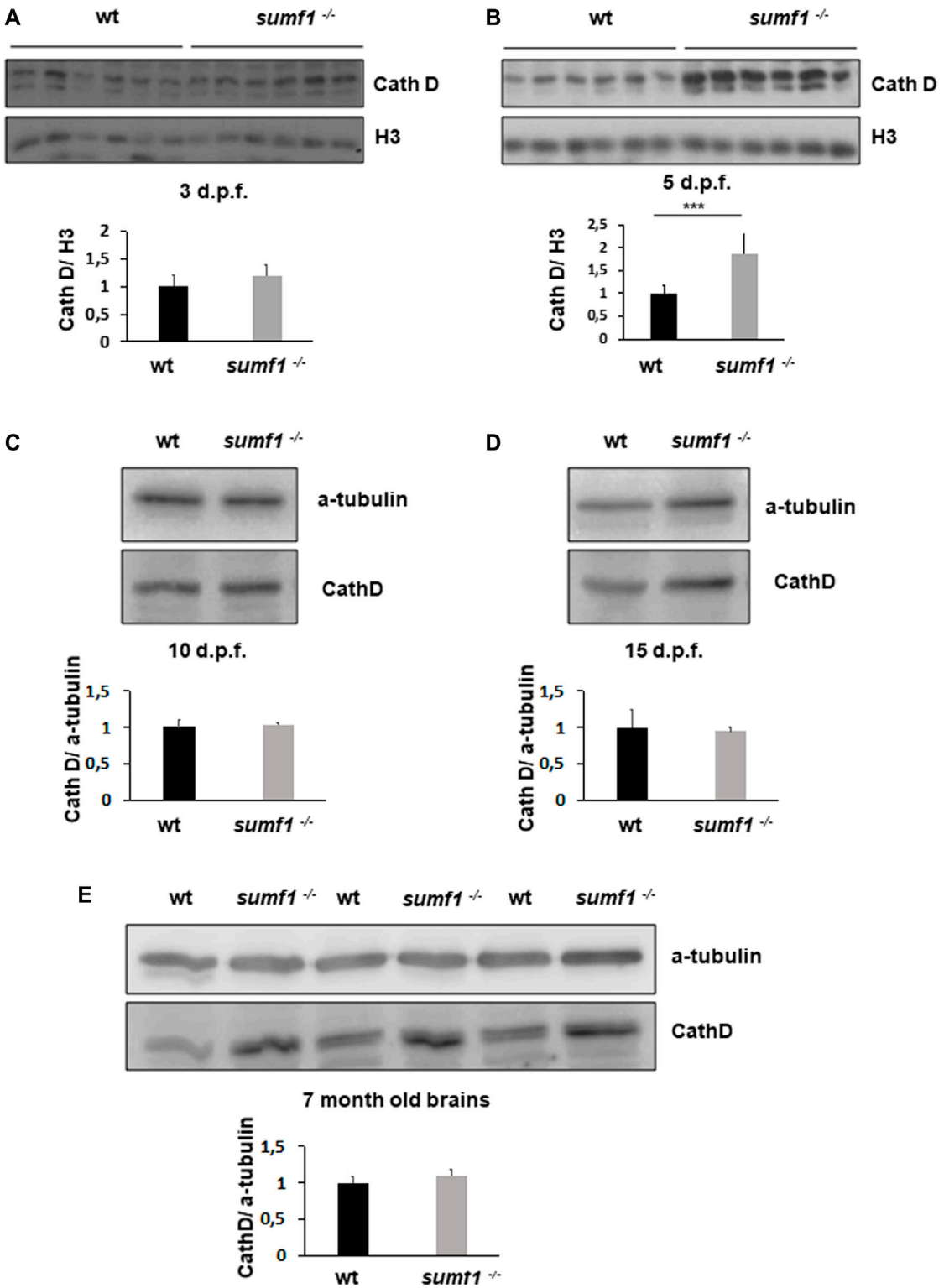

FIGURE 9 | Cathepsin D protein levels as an indicator of lysosome digestive capacity. Western blotting was performed in larvae of different ages (3, 5, 10, and 15 d.p.f.) and on brains of adult zebrafish to measure the levels of the mature form of cathepsin D (37 kDa band presented) as an indicator of the digestive capacity of lysosomes. **(A)** No differences in cathepsin D were observed between wildtype (wt) and *sumf1*$^{-/-}$ fish at 3 d.p.f. The levels of cathepsin D are significantly increased at 5 d.p.f. in the *sumf1*$^{-/-}$ fish relative to wt fish **(B)** whereas no differences were observed between the different genotypes at 10 d.p.f. **(C)**, 15 d.p.f. **(D)** and in the brains of adult fish (7 months old), **(D)**. Graphs show mean values (± SEM) of densitometry of cathepsin D normalised to histone 3 (loading control at 3 d.p.f and 5 d.p.f.) and α-tubulin (loading control at 10 d.p.f. and 15 d.p.f. larvae and 7 months old brain) from at least 3 independent experiments. Statistical analysis was performed using a two-tailed t-test. ***$p < 0.001$.

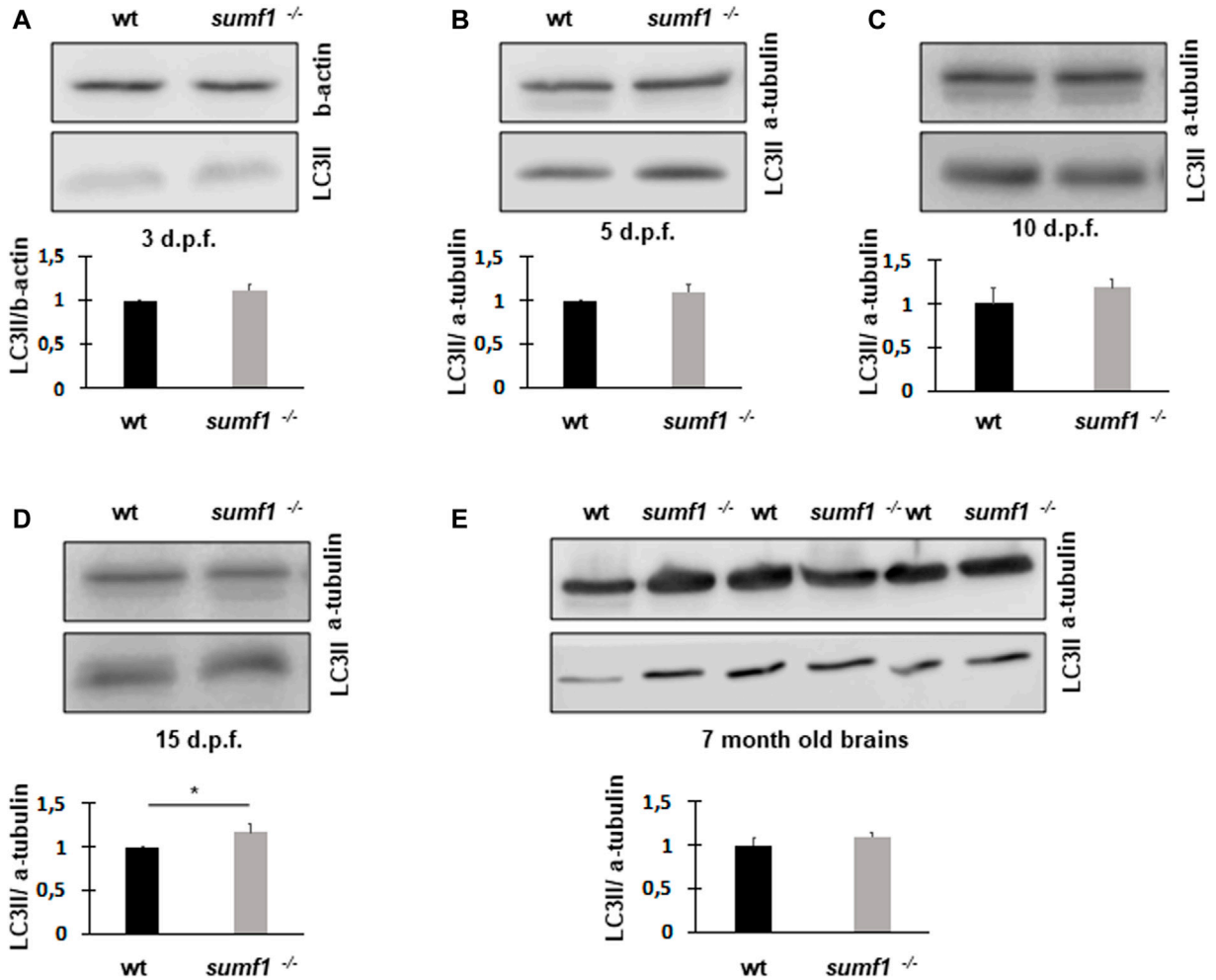

FIGURE 10 | LC3-II protein levels as an indicator of autophagosome formation and flux. Western blotting was performed in larvae of different ages (3, 5, 10, and 15 d.p.f.) and on brains of adult zebrafish to measure the levels of LC3-II as a measure of autophagosome number. No differences in LC3-II levels were observed between wildtype (wt) and *sumf1*$^{-/-}$ fish at 3 d.p.f., 5 d. p.f., 10 d.p.f. Although a modest increase (1.28x) increase in LC3-II was observed at 15 d.p.f. this difference was not evident in the brains of adult fish (7 months). Graphs show mean values (±SEM) of densitometry of LC3-II normalised to β-actin (loading control at 3 d.p.f) and α-tubulin (loading control at 5 d.p.f., 10 d.p.f., 15 d.p.f. and 7 months) from at least 3 independent experiments. Statistical analysis was performed using a two-tailed t-test. *$p < 0.05$.

storage diseases (Bellettato and Scarpa, 2010). Based on these observations in mammalian models, we examined the number and distribution of microglia (Mi) and macrophages (Ma) (hereafter referred to as total Mi/Ma population) in our *sumf1*$^{-/-}$ zebrafish model (**Figure 7A**) at different time points. Quantification of wholemount antibody staining demonstrated that the total Mi/Ma population at 3 d.p.f. was significantly higher ($p < 0.05$, Welch's t-test) in *sumf1*$^{-/-}$ than wildtype zebrafish (**Figure 7B**). However, no significant difference was observed at 5 and 10 d.p.f. (**Figures 7C, D**) suggesting that the expansion of the population observed at 3 d.p.f. does not persist. To investigate the growth in the total MiMa population throughout the 10 days, we compared the population size between consecutive days (3–5 d.p.f. and 5–10 d.p.f.) for each genotype. The Mi/Ma population increased in wildtype larvae from 3–10 d.p.f. ($p < 0.05$, Welch's t-test), whereas this population, whilst initially higher in *sumf1*$^{-/-}$ zebrafish, did not increase significantly ($p > 0.05$, Welch's t-test) over that same duration.

To investigate the possibility that the brain resident microglia population changes in a different manner to the total population, we selectively counted the microglia within the brain of the zebrafish (**Figures 7E–G**). A significant increase ($p < 0.05$, Welch's t-test) in microglia in *sumf1*$^{-/-}$ larvae was observed at 3 d.p.f. (**Figure 7E**). and persisted at 5 d.p.f. (**Figure 7F**) but became similar to that of wildtype larvae at 10 d.p.f. (**Figure 7G**). Taken together, these data suggest that early differences in microglial and macrophage populations normalise over time, which was consistent with the cartilage and bone malformation data described earlier.

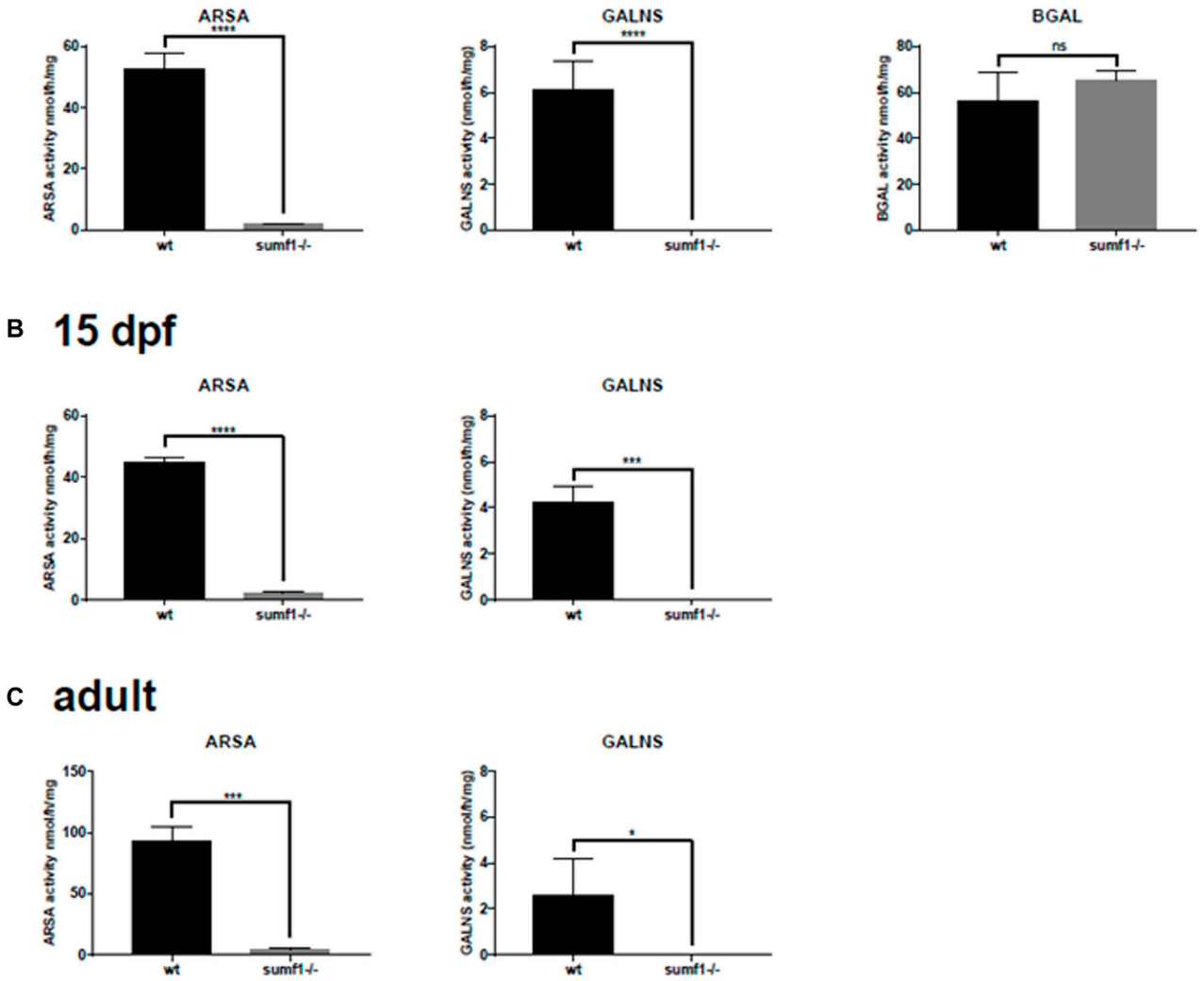

FIGURE 11 | Sulfatase activity in older larvae and adult brains. Larvae at 5 d.p.f. showed no or neglible sulfatase activity (**Figure 2B**). However, since pathological phenotypes in *sumf1*$^{-/-}$ larvae resolved with age and adult *sumf1*$^{-/-}$fish were viable, we measured sulfatase activity at older timepoints. No or negligible levels of ARSA and GALNS activity could be detected in *sumf1*$^{-/-}$ larvae at 10 and 15 d.p.f., and in the brains of adult fish. Beta-galactosidase was included as a control as this enzyme is a lysosomal hydrolase which is not dependent on FGE. Graphs show mean values (±SD) from at least 3 biological replicates. Statistical analysis was performed using a two-tailed t-test. $p > 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, and ***: $p \leq 0.001$.

Lysosome Characterisation and Autophagy

In humans, MSD results in the accumulation of undigested GAGs within the lysosome which is associated with a lysosomal storage deficiency. Therefore, we assessed abundance and activity of lysosomes over a range of ages in our zebrafish *sumf1*$^{-/-}$ model. Lysotracker imaging of larvae from 2–10 d.p.f. revealed a marked increase in staining at all larval ages examined (**Figure 8**). We analysed staining in the spinal cord region (**Figure 8A**), since lysosome accumulation is known to occur in the CNS in other models and around the otic capsule (**Figure 8B**) because this region showed defects in cartilage and bone formation. The increase in Lysotracker staining may reflect an increase in lysosome numbers or increases in their size. Therefore, we examined cathepsin D levels as a marker of lysosome digestive capacity and as a surrogate marker for lysosome abundance. We observed a significant increase in cathepsin D levels in *sumf1*$^{-/-}$ larvae relative to wildtype controls at 3 and 5 d.p.f. (**Figures 9A,B**) but no difference at later larval ages (10 and 15 d.p.f., **Figures 9C,D**) nor in the brains of adult *sumf1*$^{-/-}$ fish. Interestingly, these increases in cathepsin D expression coincide with the earliest onset of defects that we noted in craniofacial development and increases in the Mi/Ma population in *sumf1*$^{-/-}$ larvae and the normalisation of cathepsin D levels correlates with recovery of the early morphological and cell population defects.

Next, we measured LC3-II levels as a marker of autophagy, since this degradative pathway is known to be compromised in lysosomal storage diseases. Since LC3-II levels correlate with the numbers of autophagosomes and autolysosomes, one would expect the marker to increase in lysosome storage disorders where autophagosomes cannot be degraded by dysfunctional lysosomes. Surprisingly, we found no changes in LC3-II levels between wildtype and *sumf1*$^{-/-}$ larvae at 3, 5, and 10 d.p.f. (**Figures 10A–C**). and only a modest (1.28 x increase) in LC3-II at 15 d.p.f (**Figure 10D**). In addition, LC3-II levels in the brains of adult (7 month old fish) were also not affected (**Figure 10E**).

We postulated that the recovery in pathological phenotypes observed in *sumf1*$^{-/-}$ fish could be caused by two alternative mechanisms. Firstly, it may be caused by a recovery of sulfatase activity. Alternatively, it may be caused by the upregulation of unknown pathways that allow zebrafish to tolerate high levels of substrate accumulation or pathways that allow the degradation or removal of such substrates. To investigate this, we measured sulfatase activity in older larvae, at times when pathological phenotypes resolve, and in the brains of adult fish. In all ages investigated, no or negligible levels of ARSA and GALNS activity could be detected in *sumf1*$^{-/-}$ fish (**Figures 11A–C**), suggesting an absence of FGE activity with no change in Beta-galactosidase (used as a control).

DISCUSSION

The aim of our work was to characterise a zebrafish *sumf1*$^{-/-}$ null mutant with the expectation that this would recapitulate the severe features of MSD pathology seen in mouse and *Drosophila* models, notably early lethality, but with the benefit of being a vertebrate model organism that was amenable to high-throughput chemical and genetic screens, to identify targets for therapeutic intervention. Whilst young *sumf1*$^{-/-}$ larvae displayed some aspects of MSD pathology, namely facial dysmorphia, elevated microglia population and growth retardation, the recovery of these defects, and the survival of *sumf1*$^{-/-}$ to adulthood was unexpected. This, coupled with the normalisation of cathepsin D levels and no evidence of a block in autophagic flux suggests that the *sumf1*$^{-/-}$ zebrafish have compensatory mechanisms that allow normal lysosomal function in the absence of enzymes that, in all other organisms, are required to degrade GAGs and sulfolipids. This demonstrates that it is possible for a vertebrate organism to be viable and healthy in the absence of FGE function and when all cellular sulfatases are inactive. Our findings suggest that zebrafish possess a mechanism to circumvent the accumulation of lysosomal substrates that result in severe lysosomal disorders in mammals, perhaps by compensating for dysregulated intracellular pathways and lipid membrane turnover, features that are characteristics of lysosomal disorders in non-vertebrates (Platt et al., 2012). Despite the fact that this model is not suitable for viability screens, it offers the potential for identifying alternative intracellular signalling pathways which, if existing and targeted in man, could be therapeutic strategies for the treatment of MSD and possibly other LSDs.

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 authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Cambridge.

AUTHOR CONTRIBUTIONS

AF conceived study, co-ordinated scientific work, drafted manuscript. LX, GS-E, SW, DW, MG, ZH, AF, and LS performed experiments and analysed data. AF, LS, and DR acquired funding. DR conceived and supervised study. All authors reviewed and commented on drafts of the paper.

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CREB Ameliorates Osteoarthritis Progression Through Regulating Chondrocytes Autophagy *via* the miR-373/METTL3/TFEB Axis

Haibin Zhang[1†], *Xilei Li*[2,3†], *Yusheng Li*[1], *Xucheng Yang*[1], *Runzhi Liao*[1], *Haoyi Wang*[1] and *Junxiao Yang*[1,3*]

[1]Department of Orthopedics, Xiangya Hospital, Central South University, Changsha, China, [2]Department of Anesthesiology, Xiangya Hospital, Central South University, Changsha, China, [3]National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, China

Osteoarthritis (OA) is a degenerative joint disease characterized by articular cartilage degradation. Dysregulated autophagy is a major cause of OA. However, the underlying mechanism is unclear. Here, we found that the expression of element-binding protein (CREB) was downregulated in both cartilage tissues of OA patients and mouse OA model. In tert-butyl hydroperoxide solution-treated chondrocytes, increased apoptosis and autophagic blockage were attenuated by CREB overexpression. Mechanically, MiR-373 directly targeted the 3′UTR of methyltransferase-like 3 (METTL3) and led to its downregulation. METTL3 epigenetically suppressed TFEB. The upregulation of miR-373 by CREB overexpression induced the release of TFEB from METTL3 and restored the autophagy activity of chondrocytes. Taken together, our study showed that CREB alleviates OA injury through regulating the expression of miR-373, which directly targeted METTL3, and finally relieved TFEB from METTL3-mediated epigenetic suppression. The CREB/miR-373/METTL3/TFEB axis may be used as a potential target for the treatment of OA.

Keywords: osteoarthritis, autophagy, CREB, METTL3, miR-373

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Carmine Settembre,
Telethon Institute of Genetics and Medicine (TIGEM), Italy
Shu Li,
Northeast Agricultural University, China

****Correspondence:***
Junxiao Yang
yangjunxiao@csu.edu.cn

[†]These authors have contributed equally to this work and share first authorship

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HIGHLIGHTS

1 CREB is decreased in OA while the regaining of this protein attenuates OA-caused chondrocytes apoptosis and autophagy blockage
2 The expression of miR-373 is maintained by the binding of CREB to its promoter
3 METTL3 expression is suppressed by miR-373, which is necessary for TFEB-mediated autophagy and survival of chondrocytes in OA

Abbreviations: CREB, cAMP response element-binding protein; CQ, chloroquine diphosphate; CSTB, cystatin B; LC3B, microtubule-associated proteins 1A/1B light chain 3B; LAMP2, lysosome-associated membrane protein-2; METTL3, methyltransferase-like 3; OA, Osteoarthritis; ULK1, Unc-51–like kinase 1; PDGF-BB, platelet-derived growth factor BB; TBHP, tert-butyl hydroperoxide.

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by articular cartilage degradation in synovial joints. Since the cartilage serves as a firm and tenacious pad protecting the ends of long bones at the joint, the destruction of the tissues at this site always leads to physical disability to a different degree (Wang et al., 2011). It has long been believed that "wear and tear" is the etiological factor for the initiation and progression of this condition (Aigner et al., 2004) since the OA lesion is often localized to weight-bearing cartilage or sites of injury (Dieppe and Kirwan, 1994). With more in-depth knowledge about OA, OA is now considered a heterogeneous group of disorders with various pathogenic factors, but all lead to similar patterns of cartilage degeneration.

The main structural protein of cartilage is type II collagen, which provides a basic framework that receives stabilization from other collagen types and non-collagenous proteins, including but not limited to Collagens type IX, X, XI, aggrecan, decorin, biglycan, and fibromodulin (Roughley and Lee, 1994; Sophia Fox et al., 2009). As the most dominant cell type in cartilage, chondrocytes are in full responsibility in the generation of the complex cartilage architecture and the regulation of the biochemical composition (Woo et al., 1989; Alford and Cole, 2005). Changes in the chemical, mechanical, or cytokine exposure in the articular environment arouse responses from chondrocytes, which is mainly presented as the altered balance between catabolic and anabolic activities in the cartilage maintenance (Martel-Pelletier et al., 2008; Guo et al., 2017). Based on the understanding of the cartilage cellularity supported by chondrocytes, it is now generally accepted that the OA progression is an outcome of imbalanced chondrocyte activity conducted by abnormal biomechanical, biochemical, and genetic factors (Hering, 1999; Liu et al., 2016).

Autophagy is an essential homeostatic process by which cells break down their components to maintain survival, differentiation, development, or homeostasis in various tissues or organs including cartilage (Mizushima et al., 2008). It has been proved that the dysregulation of several key autophagy genes has a causative relation to OA pathogenesis (Sasaki et al., 2012; Caramés et al., 2013). In OA samples, the expression of the most upstream autophagy inducer, Unc-51–like kinase 1 (ULK1), autophagy structural and functional factor, microtubule-associated protein 1 light chain 3 (LC3B), and key autophagy regulator Beclin1 are all reduced. More than that, inhibition of autophagy would cause OA-like gene expression changes, while artificial induction of autophagy reduced MMP13 and ADAMTS5 expression induced by interleukin (IL)-1β, a major pro-inflammatory cytokine implicated in OA (Kapoor et al., 2011; Zhang et al., 2015). Many potential OA therapies are working as autophagy inducers to promote the survival and activity of chondrocytes, such as rapamycin (Pal et al., 2015). By inducing lysosome biogenesis and promoting autophagosome formation and lysosome fusion, TFEB turns to be a master regulator of the autophagic flux (Sardiello et al., 2009; Settembre et al., 2011). It has been proved that TFEB overexpression protects chondrocytes *in vitro* from tert-butyl hydroperoxide solution (TBHP)-induced lysosome dysregulation and attenuates the surgically induced OA by promoting autophagy in cartilage tissues (Zheng et al., 2018). However, how TFEB is regulated in the related process is still far from clear.

As a great variety of stimuli can modulate autophagy, it is not surprising that a growing number of signaling pathways have been shown to control this process, like tyrosine kinase receptors, casein kinase II (Holen et al., 1993), MAP kinases (Haussinger et al., 1999; Ogier-Denis et al., 2000), and calcium (Gordon et al., 1993), and cAMP response element-binding protein (CREB) pathway is a novel member in this family. CREB is a well-known transcription factor that plays a positive role in cell survival and proliferation while protecting cells from harmful stimuli and apoptosis (Teich et al., 2015; Saura and Cardinaux, 2017). Recently, its role in autophagy regulation has been proved, showing that CREB inhibition directly leads to blockage of autophagy activation (Chong et al., 2018). More than that, this transcriptional activator upregulates autophagy genes, including ATG7, ULK1, and TFEB (Seok et al., 2014). Inspiringly, accumulating evidence implies that the CREB pathway is also engaged in the regulation of chondrocytes activity and cartilage maintenance (Bui et al., 2012). However, the underlying mechanism is far from being well studied.

In the present study, we identified that CREB directly binds to micro-RNA-373 to activate its expression in chondrocytes. The increased miRNA-373 dramatically represses the amount of its target, methyltransferase-like 3 (METTL3), an essential epigenetic regulator, which releases TFEB from the suppression of METTL3-conducted epigenetic modification. This discovery may not only bring a new angle of view to the understanding of OA pathology but also provide several new targets for the potential treatment of this disorder.

MATERIALS AND METHODS

Human Cartilage Tissues

This study was performed with the approval of the Human Ethics Review Committees of The Xiangya Hospital of Central South University. For material collection, informed consent was obtained in writing from each subject or family of the donor. OA cartilage samples were obtained from OA knee joints of patients within 4 h after surgery. The diagnosis of OA was based on the criteria for knee OA of the American College of Rheumatology. Control cartilage samples were obtained from nonarthritic knee joints from traumatic amputee donors. The donors had no known history of joint disease, and the normality of the joint was confirmed macroscopically at the time samples were obtained.

Chondrocytes Isolation and Culture

To isolate the chondrocytes, the cartilage shavings were minced into small chips (0.5–1 mm) and then incubated with recombinant collagenase class II (300 U/ml) and thermolysin (1 mg/ml) for 6 h at 37°C, 5% CO_2. Filter the collagenase/chips solution through a 20-μm nylon filter membrane to obtain

dissociated chondrocytes. Centrifuge to collect and wash the chondrocytes. Then, chondrocytes were seeded in a cell culture plate and cultured in DMEM/F12 supplemented with 10% FBS and 1% antibiotic in 5% CO_2 at 37°C. To establish an OA model *in vitro*, chondrocytes were treated with oxidative stress inducer tert-butyl hydroperoxide (0, 6.25, 12.5, and 25 μM, TBHP) (Sigma, 458139) for 24 h.

Animal and DMM Model Establishment

All experimental protocols were approved by the Institutional Animal Care and Use Committee of The Xiangya Hospital of Central South University. Male 6-week-old BALB/c wild-type mice were purchased from the Laboratory Animal Center of the Institute of Genetics in Beijing and maintained under specific pathogen-free conditions with 12/12-h light/dark cycles, at 25°C, with fodder (Rat & Mouse Maintenance Diet 1022, HFK Bio-tech, China) and water *ad libitum*.

For DMM surgery, mice were anesthetized with 300 mg/kg intraperitoneal Tribromoethanol and the knees were prepared for aseptic surgery. Buprenorphine (Buprenex®, Reckitt and Coleman Products, Kingston-Upon-Hull, United Kingdom) was provided peri-operatively at 0.09 mg/kg. Surgery was operated under direct visualization and strictly followed the details described in a previous study (Glasson, Blanchet, and Morris 2007).

Immunohistochemistry

Cartilage tissues were fixed in 10% formalin for 24 h and embedded in paraffin. Then, blocks were sectioned into 5 μm thick for immunohistochemical (IHC) examination. Sections on slides were dewaxed in xylene and rehydrated in ethanol gradient. Antigen recovery was accomplished in boiling 0.01% sodium citrate buffer. Primary antibody, CREB (ProteinTech, 12208-1-AP), was diluted in antibody dilution buffer (3% BSA, 1% normal donkey serum in Tris-buffered saline) for incubation. Slides were then incubated with the primary antibody overnight at 4°C. Slides were processed using the biotin-streptavidin HRP detection system (SP-9002, ZSGB-BIO, Beijing, China) followed by 3,3′-diaminobenzidine (DAB) staining, with counter-staining by hematoxylin. Slides were sealed in neutral resins with coverslips for subsequent detection. Images of the tissue sections were captured by a Zeiss imaging system (Zeiss, Germany).

Autophagic Flux Assay

The assay was operated according to a protocol from the manufacturer for the application of LC3B antibody (NB100-2220, Novus Bio, Colorado United States). In detail, chloroquine diphosphate (CQ) (10 mM) was added to the culture dishes at designated time points to a final concentration of 50 μM and incubated overnight (16 h). Cells were harvested and subjected to immunocyto-fluorescence staining. Dilute primary antibody in 1% BSA in PBS. Incubate samples with 5 μg/ml rabbit anti-LC3B primary antibody at 4°C overnight. After wash, apply an appropriate dilution of fluorophore-conjugated anti-rabbit secondary antibody in 1% BSA. After wash and counter-staining with DAPI, use a fluorescence microscope to examine and image the cells.

TUNEL Assay

The assay was accomplished by commercial kit according to the manufacturer's manual (ab66108, Abcam, Cambridge, United Kingdom). In detail, harvest cells at designated time points and wash with PBS and then spin onto slides. Fix cells with cold formaldehyde for 4 min and then wash with PBS. Add ice-cold 70% ethanol and incubate for 30 min. Wash with wash buffer twice and then incubate with staining solution for 60 min at 37°C. Add rinse buffer wash samples twice. Resuspend cells in DAPI/RNase A solution and incubate for 30 min at room temperature. Then, analyze results under a fluorescence microscope.

LysoTracker Staining

The staining procedure was accomplished by a LysoTracker™ kit (L7528, Invitrogen, California United States). When cells have reached the desired confluence, remove the medium from the dish and add the prewarmed (37°C) probe-containing medium. Incubate the cells for 1 h. Then, cells were washed with fresh medium and harvested for ICC analysis under a microscope.

Quantitative Real-Time PCR

RNA from the cartilage tissues or chondrocytes was extracted by TRIZOL Reagent (Invitrogen, Life Technologies, United States). According to the manufacturer's suggestion, 1.5-fold suggested volume of isopropanol was used to make sure RNA in small size, including miRNA, could be well precipitated. The TRIZOL-extracted RNA samples were then applied to Turbo DNase treatment to remove possible genomic DNA contamination. For mRNA quantification, 1 μg of purified total RNA was used for reverse transcription (Promega Reverse Transcription System) following the manufacturer's protocol. The iTaq Universal SYBR Green Supermix (Bio-Rad, 1725120) was used for the mRNA qPCR reaction. To quantify miRNA expression change, the TaqMan microRNA assay kit (Applied Biosystems) was used for the reverse transcription and qPCR following the manufacturer's protocol. qPCR reactions were operated in triplicate and analyzed by the Applied Biosystems QuantStudio 6 Pro real-time PCR system (Life Technologies, CA, United States). The comparative Ct ($2^{-\Delta\Delta Ct}$) method was used to determine the relative gene expression change. GAPDH was used as an endogenous reference for mRNA quantification and U6 was used for miRNA quantification. The primer sequences were as follows: CREB-F: 5′-AGTTTGACGCGG TGTGTTAC-3′; CREB-R: 5′-TACCTGGGCTAATGTGGCAA-3′; miR-373-F: 5′-CGCGAAGTGCTTCGATTTTG-3′; miR-373-R: 5′-GTGCAGGGTCCGAGGT-3'; METTL3-F: 5′-GAGTGC ATGAAAGCCAGTGA-3′; METTL3-R: 5′-CTGGAATCACCT CCGACACT-3'; TFEB-F: 5′-TGATCCACTTCTGTCCACCA-3′; TFEB-R: 5′-GCAGGTGGCTACTTCACACA-3'; GAPDH-F: 5′-CTGACTTCAACAGCGACACC-3′; GAPDH-R: 5′-GTG GTCCAGGGGTCTTACTC-3′; U6- F: 5′-CTCGCTTCGGCA GCACA-3'; U6-R: 5′-AACGCTTCACGAATTTGCGT-3′.

Western Blotting

The total protein of cartilage tissues or chondrocytes was prepared by homogenizing the tissues in WIP Tissue and Cell lysis solution (CellChip Beijing Biotechnology Company, Beijing, China) following the manufacturer's instructions. Protein samples (10 μg each) were loaded on 10% SDS–PAGE and transferred to polyvinylidene fluoride membranes (IPVH00010, Millipore, MA, United States). Membranes were incubated overnight at 4°C with corresponding antibodies. The secondary antibody (IRDye 680RD, LI-COR Biosciences, United States) was diluted at 1:10,000 in Tween-Tris-buffered saline. Blottings were visualized by Odyssey CLx Imaging System (LI-COR Biosciences, United States). GAPDH was used as an endogenous reference. The density of the bands was normalized to that of GAPDH.

Antibodies used for blotting in this study are as follows: BAX (Santa Cruz, sc-7480), Bcl-2 (Abcam, ab196495), Cleaved Caspase-3 (Cell Signaling Tech, #9664), CREB (ProteinTech, 12208-1-AP), GAPDH (Abcam, ab9485), LAMP2 (Cell Signaling Tech, #49067), LC3I (Novus Bio, NB100-2331), LC3II antibody (Novus Bio, NB100-2220), METTL3 (Abcam, ab195352), P62 (Cell Signaling Tech, #5114), and TFEB (Cell Signaling Tech, #4240).

Plasmids, Transfection, and RNA Knockdown

cDNAs of human CREB and METTL3 were generated by PCR and cloned into *Asc*I and *Fse*I sites of the pCS2-CMV expression plasmid. Primer sequence: CREB, forward primer—5′-GGGTTTGGCCGGCCAATGACCATGGAA-3′, reverse primer—5′-GGGTTTGGCGCGCCT TAATCTGAT-3'; METTL3, forward primer—5′-GGGTTTGGCCGGCCATGTCGGACA-3′, reverse primer—5′-GGGTTTGGCGCGCCCTATAAATTC-3'. Transfection of plasmids was performed by using Effectene Transfection Reagent (Qiagen, 301425) according to the manufacturer's instructions.

Short hairpin RNA targeting CREB (sh-CREB) or scrambled control sequence (sh-NC) were designed and produced by GenePharma (Shanghai, China). Cells were transfected with sh-CREB or sh-NC using Lipofectamine 3000 following the manufacturer's instructions.

For the miRNA mimics and inhibitor transfection, cultured cells were transiently transfected with miR-373 mimic (GenePharma), miR-373 inhibitor (GenePharma, Shanghai, China), or corresponding negative control (GenePharma, Shanghai, China) using Lipofectamine 3000. The total RNA and protein were extracted for further investigation as indicated.

Dual-Luciferase Reporter Assay

To verify the interaction and the effect of CREB onto the miR-373 promoter, the luciferase reporter vectors containing the promoter region of miR-373 or its three different mutants were constructed. The mutations were conducted by using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent). The wild-type or mutant promoter reporter vectors together with CREB-overexpression plasmid were co-transfected into cultured normal chondrocytes, which used empty plasmid as control.

Likewise, to verify the interaction between miR-373 and METTL3, the predicted target site for miR-373 on METTL3 RNA transcripts or its mutant were integrated into the luciferase reporter vector. miR-373 mimics were co-transfected with the reporter vectors into the cells. The reporter gene activity was determined by measuring the luciferase activity using Dual-Luciferase®Reporter Assay System (Promega, E1910). The luciferase activity was normalized with the activity of the internal control enzyme, Renilla luciferase on the same vector, to adjust the transfection efficiency between different groups.

Quantification of TFEB m6A Modification

Briefly, total RNA was isolated by using TRIzol as mentioned above in the method of RT-qPCR. Total RNA (3 mg) was well mixed with 3 fmol spike-in and equally split for three following processes: m6A/m-RIP, IgG control, and input. m6A/m-RIP and IgG control samples were incubated in parallel with 1 mg of anti-m6A/m antibody (Synaptic Systems, l202 003) or 1 mg of rabbit IgG in immunoprecipitation buffer with RNasin Plus (Promega, N2111). Then, the mixture was incubated with washed Dynabeads M-280 (Thermo Fisher, 11203) to capture the antibody–RNA complexes. The bead-bound antibody–RNA complexes were recovered on a magnetic stand and the RNA was extracted again by TRIzol. The RNA products, including input samples, were reverse-transcribed by following the mentioned method in qRT-PCR. The abundance of TFEB was then quantified by following the method in qRT-PCR. The measurements of m6A-TFEB for each treatment were normalized to their corresponding input amount.

Chromatin Immunoprecipitation

Briefly, approximately 50 mg of chondrocyte homogenates was first cross-linked with 2 mM disuccinimidyl glutarate (DSG, Thermo Fisher Scientific) and 1 mM $MgCl_2$ for 40 min, and second cross-linked with 1% formaldehyde for 10 min at room temperature. Extracted nuclei constituents were sonicated to fragment DNA to approximately 0.5 kb. Fragmented DNA was immunoprecipitated with CREB antibodies (ProteinTech, Rosemont, IL, 12208-1-AP) overnight at 4°C. Protein G beads (Sigma, St. Louis, MO, P3296) were added and rocked for 3 h at 4°C. The beads were washed and reverse-crosslinked in elution buffer overnight at 65°C. DNA was purified by phenol/chloroform. Anti-rabbit IgG antibody (Santa Cruz Biotechnology, Dallas, TX, sc-2027) was used as a negative control. Then, qPCR was conducted to measure following the method mentioned above.

Experimental Design and Statistical Analyses

All the data were expressed as mean ± SD with each model performed in triplicate. Appropriate statistical significance was chosen based on the experimental strategy using GraphPad Prism. Data were analyzed either by *t*-test or ANOVA. Values of $p < 0.05$ were considered statistically significant.

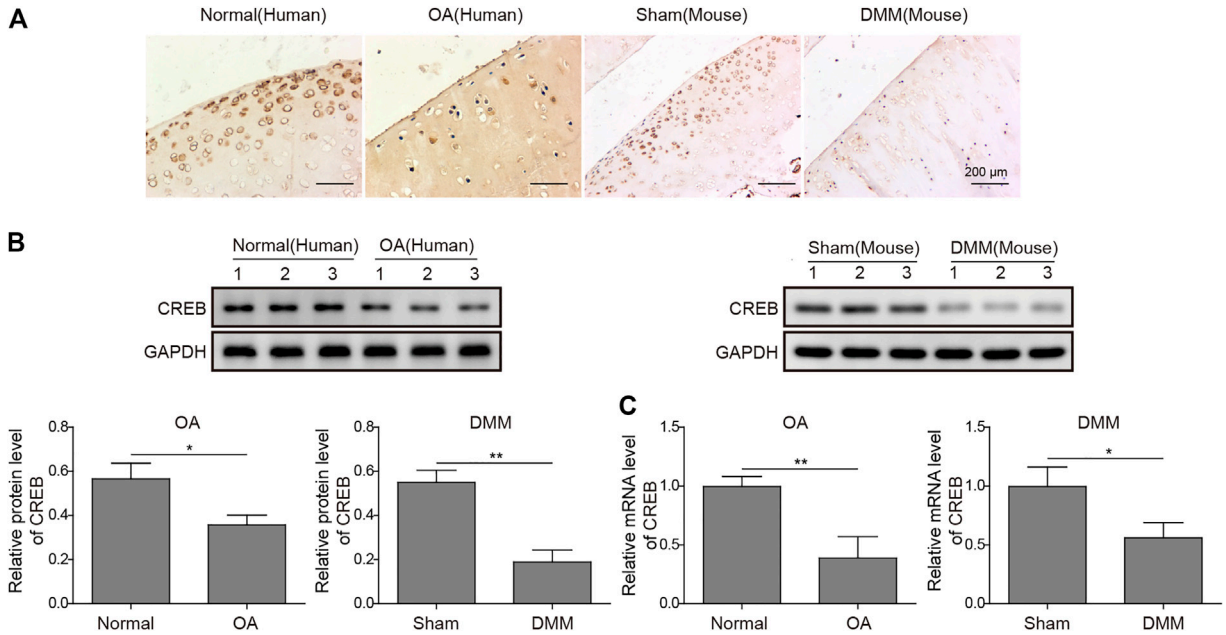

FIGURE 1 | Abnormal expression of CREB in clinic human OA tissues and surgically induced mouse OA model. **(A)** The *in situ* expression changes in human OA samples and mouse-induced OA tissues were examined by IHC. Scale bar = 200 μm. **(B)** The protein level of CREB was determined by Western blotting and then quantified. CREB signal was normalized to GAPDH intensity. **(C)** The expression of CREB mRNA was determined by qRT-PCR. GAPDH was used as the endogenous reference for normalization. $p < 0.05$ was marked as *, $p < 0.01$ was marked as **.

RESULTS

CREB is Dramatically Decreased With the Occurrence of OA

To build up the connection between CREB and OA pathogenesis, we first examined the CREB expression in both normal and OA clinical specimens of humans. As detected by IHC, the CREB staining was clear and perfectly colocalized with the chondrocytes (**Figure 1A**). In contrast, the chondrocytes in OA samples showed a much weaker CREB signal compared to normal tissues (**Figure 1A**). To confirm that this was a conservative phenomenon among different species, we then employed one of the most conventional OA mouse models, the DMM model, to examine the CREB expression. Similar to the result in human tissues, CREB was also decreased in DMM mice cartilage tissues. This decrease of CREB was also confirmed at the whole protein and mRNA level. As shown, in both human and mouse samples, the mRNA and protein of CREB were significantly decreased (**Figures 1B,C**, $p < 0.01$ or $p < 0.05$). These results indicated that the CREB expression level was closely connected to the occurrence of OA. Meanwhile, the reproduction of this phenomenon in the induced OA mouse model also proved its rationality in the study on CREB's role in OA.

Complemental CREB Under TBHP Prevents Apoptosis and Autophagic Flux Blockage

To clarify the causative relation between CREB level and OA development, we treated *in vitro* cultured human chondrocytes with TBHP, a widely used OA model *in vitro*. In this model, TBHP steadily releases oxidative stress to mimic the biomechanically induced oxidative stress regulating mechanosensitive signals in chondrocytes (Zheng et al., 2018). Interestingly, CREB was decreased along with the increasing dosage of TBHP. Under the highest concentration of TBHP (25 μM), CREB protein was decreased by 81.7% (**Figure 2A**, $p < 0.001$). Considering that the CREB/CRE transcriptional pathway is widely regarded as essential protective machinery for cells under oxidative pressure, we presumed that a complemental CREB might help alleviate the TBHP-induced OA phenotype. To prove this idea, a CREB-overexpression vector was constructed and transfected into *in vitro* cultured chondrocytes (CREB group) with the empty vector as a control (vector group). Apoptosis was determined by the TUNEL assay. In the control group, the apoptosis signal was barely detected while there was a marked increase in apoptosis caused by the TBHP treatment (**Figure 2B**). Vector groups still showed a comparable number of apoptotic cells to TBHP individual treated groups, but CREB groups had much fewer TUNEL positives. The quantitative analysis further supported the cytological result, showing a significant difference between vector and CREB groups ($p < 0.05$). Health cartilage relies on active and well-orchestrated autophagy to effectively remodel the extracellular matrix in the tissues and further prevent chondrocyte death, OA-like changes in gene expression, as well as cartilage degeneration. As shown in the *in situ* autophagic flux assay by LC3B immunocyto-fluorescence

staining (**Figure 2C**), TBHP largely diminished the LC3B fluorescence signal and empty vector transfection of chondrocyte did not affect this decrease. However, with the overexpression of CREB, the level of LC3B was restored almost to the level observed in controls (**Figure 2D**). Meanwhile, we also examined whether or not lysosomes, as another essential participant in autophagy flux, were under the regulation of CREB. Similar to the condition of autophagosome, LysoTracker-indicated lysosomes were also largely removed under TBHP treatment but recovered with the CREB overexpression. Consistent with these results, CREB overexpression reversed all the TBHP-induced changes of proteins functioning in various categories (**Figure 2E**). In detail, pro-apoptosis indicators/factors cleaved caspase-3 and Bax, autophagic flux blockage marker P62 (Tang et al., 2017), were promoted by TBHP but the increase was attenuated by CREB overexpression. Apoptosis inhibitor Bcl-2, active autophagy-lysosome pathway markers including LC3-II, lysosome-associated membrane protein-2 (LAMP2), and cystatin B (CSTB) were all decreased by TBHP, while overexpression of CREB rescued their amount. Collectively, the results above imply that the CREB level is positively correlated with the viability and normal function of chondrocytes under oxidant pressure.

CREB Controls Chondrocyte Apoptosis and Autophagy Through miR-373

To explore the role of miR-373 as well as its regulation in OA, we first conducted qRT-PCR was used to determine its expression in OA patients. We found that miR-373 was decreased by more than 50% in OA samples (**Figure 3A**, $p < 0.05$). To test the interaction between CREB and miR-373, CHIP assay was done by incubating cloned miR-373 promoter fragments in immunoprecipitated CREB–chromatin complexes. As a result, the promoter fragment was effectively pulled down by the CREB-specific antibody after the incubation with chondrocyte lysate (**Figure 3B**). To confirm the binding specificity of CREB onto miR-373 promoter, we constructed three different mutant promoters (Mut 1-3; **Supplementary Table S1**) for dual-luciferase reporter assay. It showed that all mutations exhibited weaker luciferase activity than wild type to different extents (**Figure 3C**, $p < 0.05$ or $p < 0.01$). Next, we examined the effects of CREB overexpression/knockdown on the expression of miR-373 in chondrocytes. The results showed that CREB overexpression induced the expression of miR-373, while CREB knockdown repressed its expression (**Figure 3D**). Then, we used a miR-373 inhibitor to prove its role in the CREB-rescued OA *in vitro* model. We found that CREB overexpression significantly reduced TBHP-caused chondrocyte apoptosis indicated by TUNEL staining. During the TBHP treatment, transfected inhibitor NC had no impact on the reduced TUNEL counting but miR-373 inhibitor transfection led to a significant increase of chondrocyte apoptosis (**Figure 3E**). A similar reversive effect by miR-373 inhibitor was also observed in the aspects of autophagy flux and related proteins. In detail, the CREB-enhanced autophagosome (LC3B signal, **Figure 3F**) and lysosome activity (LysoTracker signal, **Figure 3G**) under TBHP treatment were reversed by miR-373 inhibition. Likewise, the cleaved Caspase-3, Bax, Bcl-2, and P62 were decreased while LC3II, LAMP2, and CTSB were increased in CREB overexpressed groups, and this function can be inversed by co-transfection with miR-373 inhibitor (**Figure 3H**). These results proved that CREB cannot rescue the survival and activity of chondrocytes under oxidative pressure partially without miR-373.

METTL3 is a Direct Target of miR-373

By analyzing the target information of miR-373 from StarBase, a miRNA–mRNA interaction prediction database (Yang et al., 2011), we found that METTL3 turned out to be one of the prior candidates. Both mRNA and protein expressions of METTL3 were significantly increased in OA (**Figures 4A,B**). More than that, treatment of miR-373 mimic onto chondrocytes caused a significant decrease of METTL3 mRNA and protein while miR-373 inhibitor promoted its expression even higher than control/NC (**Figures 4C,D**). To further confirm the interaction between METTL3 and miR-373, the predicted miR-373-recognized sequence on METTL3 was mutated at 16 bases so that this mutation (MUT) should be resistant to the miRNA. Then, both WT- and MUT-METTL3 were fused to a fluorescence reporter for luciferase assay. Indeed, luciferase activity tagged with WT was dramatically reduced by miR-373 mimic but that of MUT was almost intact (**Figure 4E**), indicating METTL3 is a direct target of miR-373.

METTL3 is Part of the CREB/miR-373 Axis Regulating the Autophagic Flux in Chondrocyte

To prove the role of METTL3 in OA development and the CREB-regulated pathway, we examined its change in the TBHP-induced OA cell model. We found that the overexpression of CREB significantly down-regulated METTL3. However, the downregulation of METTL3 by CREB was reversed by transfection of miR-373 inhibitor (**Figure 5A** Left). Together with the CREB-induced miR-373 increase result, it further proved that METTL3 was a part of the CREB-miR-373 regulation axis. We found that the autophagic flux master regulator TFEB was increased with the overexpression of CREB but decreased when miR-373 inhibitor co-transfection. Similarly, METTL3 overexpression also effectively suppressed the CREB-induced TFEB expression (**Figure 5A** Right). At the protein level, the changes of METTL3 and TFEB in chondrocytes under different treatments were consistent with their mRNA change (**Figure 5B**). This inverse correlation between TFEB and METTL3 inspired us to check the epigenetic changes on TFEB. As shown in the m6A quantification result, overexpression of CREB caused a decrease of m6A modification on the mRNA of TFEB (**Figure 5C**), which was coincident with the decrease of METTL3 expression and a consequent increase of TFEB expression itself (**Figure 5A**). The miR-373 inhibitor transfection under CREB overexpression led to an increase of TFEB m6A modification. Co-transfection of METTL3 and CREB overexpression vector increased TFEB m6A level as compared with CREB overexpressed

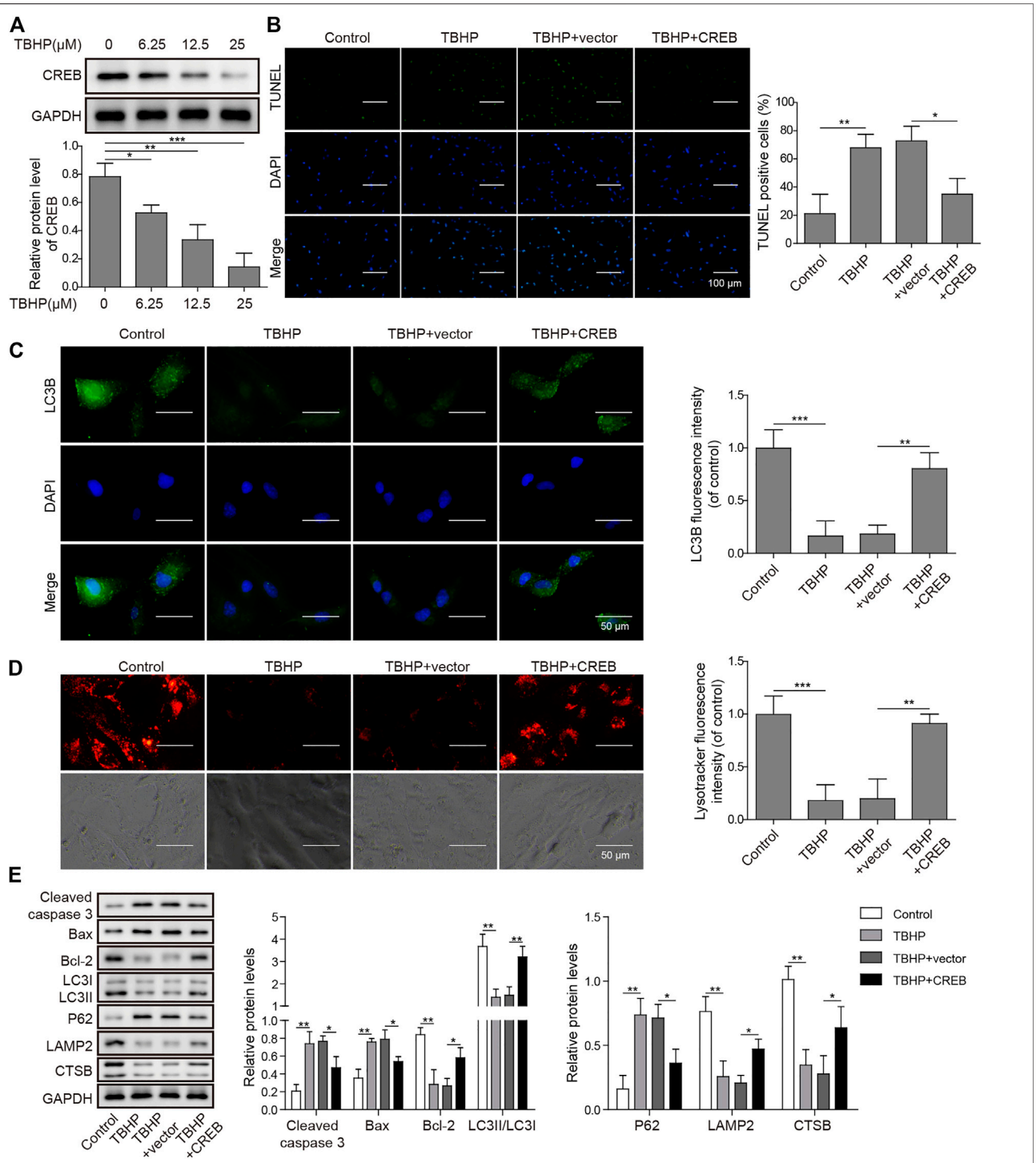

FIGURE 2 | Overexpression of CREB-attenuated TBHP-induced apoptosis and blockage of autophagic flux in cultured human chondrocytes. **(A)** The change in CREB protein under different dosages of TBHP (0, 6.25, 12.5, and 25 μM) was determined by Western blotting and then the intensity of bands was quantified. **(B)** The apoptosis caused by either TBHP alone or together with CREB overexpression was determined by TUNEL assay. The empty vector in the same concentration as CREB-overexpression plasmids was used as a control. TUNEL-positive cells were counted to quantify the outcome of each treatment. **(C)** The autophagic flux under treatments of either TBHP alone or together with CREB overexpression was determined by the immunocyto-fluorescence staining of autophagosome marker LC3B. LC3B fluorescence intensity was measured and data from all groups were normalized to the control group. **(D)** The change of lysosome activity under treatments above was determined by LysoTracker staining. LysoTracker fluorescence intensity was measured and data from all groups were normalized to the control group. **(E)** The impact of the treatments on the essential markers of apoptosis and autolysosome was determined by Western blotting. Quantified intensity of the bands was normalized to GAPDH to generate the column chart. $p < 0.05$ was marked as *, $p < 0.01$ was marked as **, $p < 0.001$ was marked as ***.

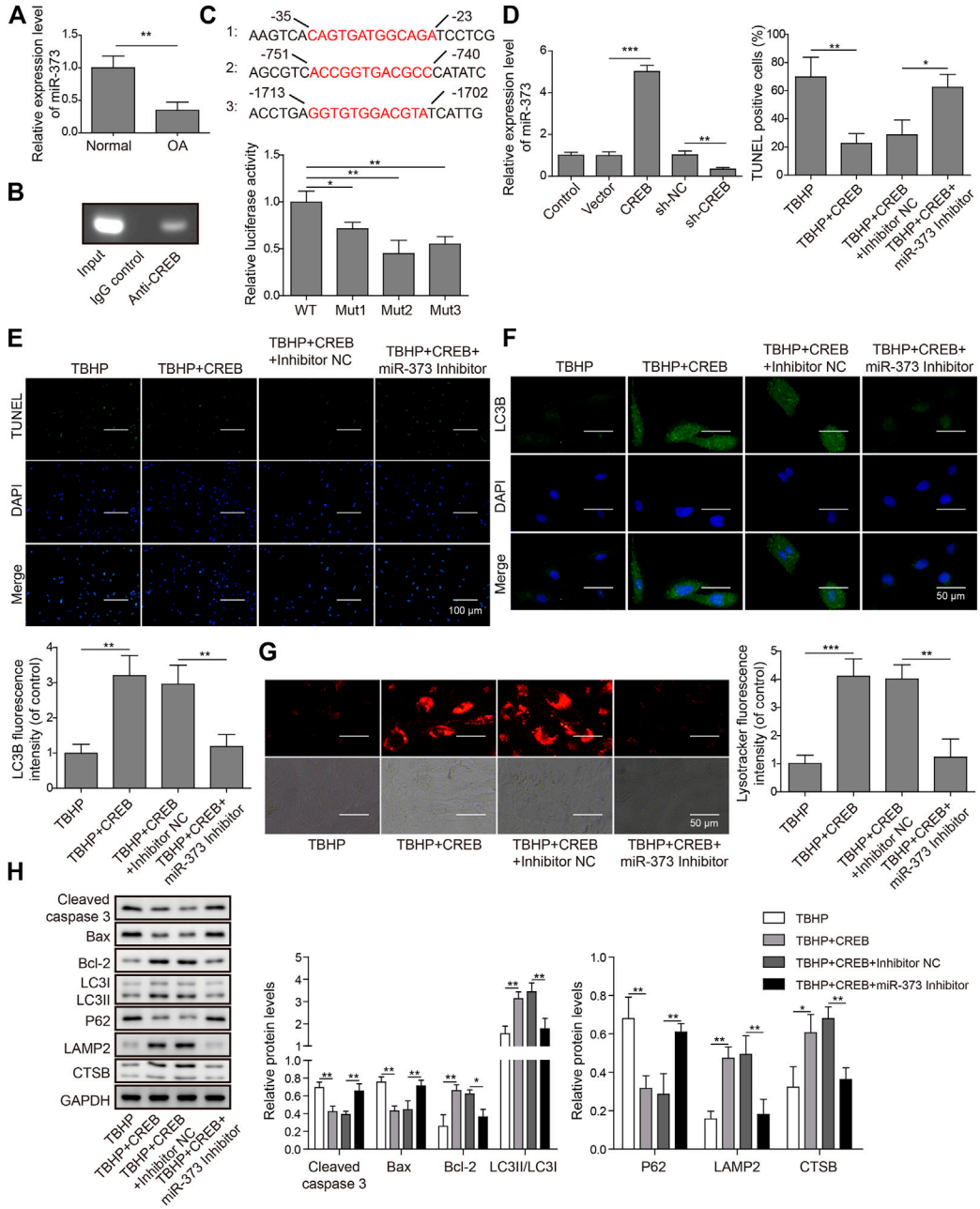

FIGURE 3 | CREB regulated apoptosis and autophagic flux through miR-373. **(A)** The expression of miR-373 in normal and OA human cartilage tissues was determined by qRT-PCR. **(B)** The binding of CREB at the miR-373 promoter was determined by ChIP assay. **(C)** A schematic diagram of CREB binding domain on the miR-373 promoter. The promoting activity of CREB to different mutated miR-373 promoters was measured by luciferase reporter assay. The signal data from each group were normalized to WT to generate the column chart. **(D)** Chondrocytes were transfected with or without CREB-overexpressed vectors (or empty vectors) or sh-CREB (or sh-NC). The expression of miR-373 after transfection was measured by qRT-PCR. **(E)** The effect of miR-373 inhibitor on apoptosis under CREB overexpression was determined by TUNEL assay. The TUNEL-positive cells were numbered to generate the column chart. **(F)** The effect of miR-373 inhibitor on the CREB-promoted autophagic flux in the TBHP-induced *in vitro* OA model was determined by the immunocyto-fluorescence staining of autophagosome marker LC3B. To generate the column chart, LC3B fluorescence intensity was measured and data from all groups were normalized to the TBHP group. **(G)** The change of lysosome activity in the same model was determined by LysoTracker staining. LysoTracker fluorescence intensity was measured and data from all groups were normalized to the TBHP group. **(H)** The impact of the treatments on the essential regulators or markers of apoptosis and autolysosome was determined by Western blotting. The quantified intensity of the bands was normalized to GAPDH to generate the column chart. $p < 0.05$ was marked as *, $p < 0.01$ was marked as **, $p < 0.001$ was marked as ***.

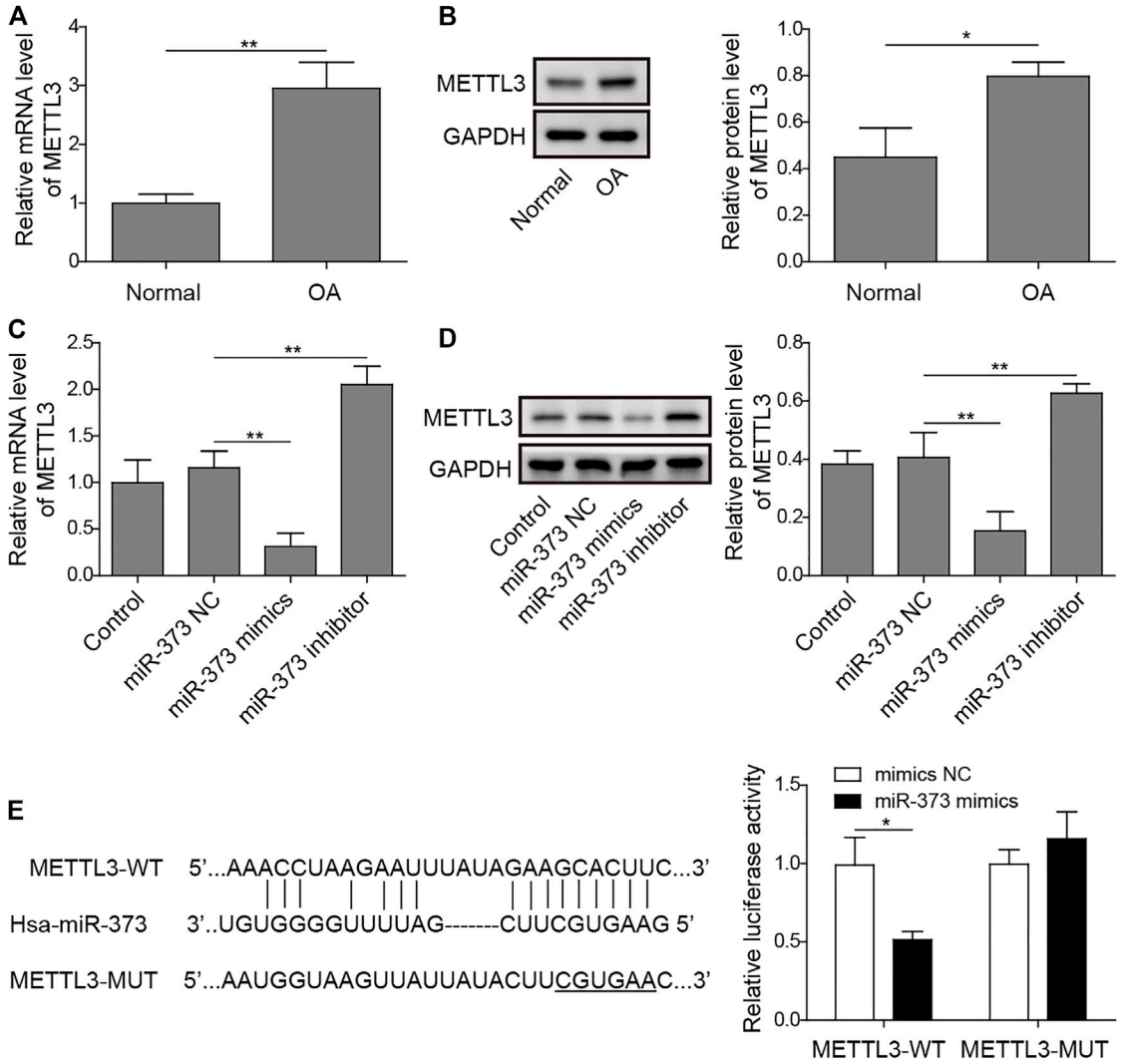

FIGURE 4 | METTL3 was a direct target of miR-373. **(A,B)** The expression of METTL3 mRNA and protein in normal and OA samples was determined by qRT-PCR and Western blotting. GAPDH was used as the endogenous reference for normalization. **(C,D)** The effect of miR-373 mimic or inhibitor on the expression of METTL3 mRNA and protein in cultured human chondrocytes was determined by qPCR and Western blotting. GAPDH was used as the endogenous reference for normalization. **(E)** The targeting specificity of miR-373 to METTL3 was verified by dual-luciferase reporter assay. A miR-373-resistant METTL3 was generated as the shown sequence. Luciferase activity based on either METTL3-WT or METTL3-MUT was normalized to the corresponding mimic NC control separately. $p < 0.05$ was marked as *, $p < 0.01$ was marked as **.

chondrocytes. The changes of TFEB m6A level further proved that TFEB expression was regulated through CREB/miR-373 axis by METTL3's epigenetic regulation. To confirm the effect exerted by this possible regulation axis, we examined the change of autophagic flux indicated by LC3B. Both immunostaining image and the quantification analysis of LC3B fluorescence strength showed that the overexpression of METTL3 dramatically suppressed the increase LC3B signal by CREB overexpression (**Figure 5D**). More than that, as revealed by Western blotting, CREB significantly increased LC3II, LAMP2, and CTSB but decreased P62, indicating a rescued autolysosomal function. However, the overexpression of METTL3 cut back the amount of these positive regulators and recovered P62 to the level as TBHP group (**Figure 5E**). Taken together, these results implied that the CREB/miR-373 axis-regulated autophagic flux in chondrocytes was partially by METTL3-mediated m6A modification of TFEB (**Figure 6**).

DISCUSSION

OA has long been considered a challenge to control due to its complex pathogenesis. Our study of this disorder has revealed a regulation axis initiated by CREB towards the TFEB-regulated chondrocyte autophagy in OA cartilage. We first found that CREB was significantly lower in clinic OA human cartilage samples than normal samples. A similar change was also found in the DMM mouse OA model. More than that, the

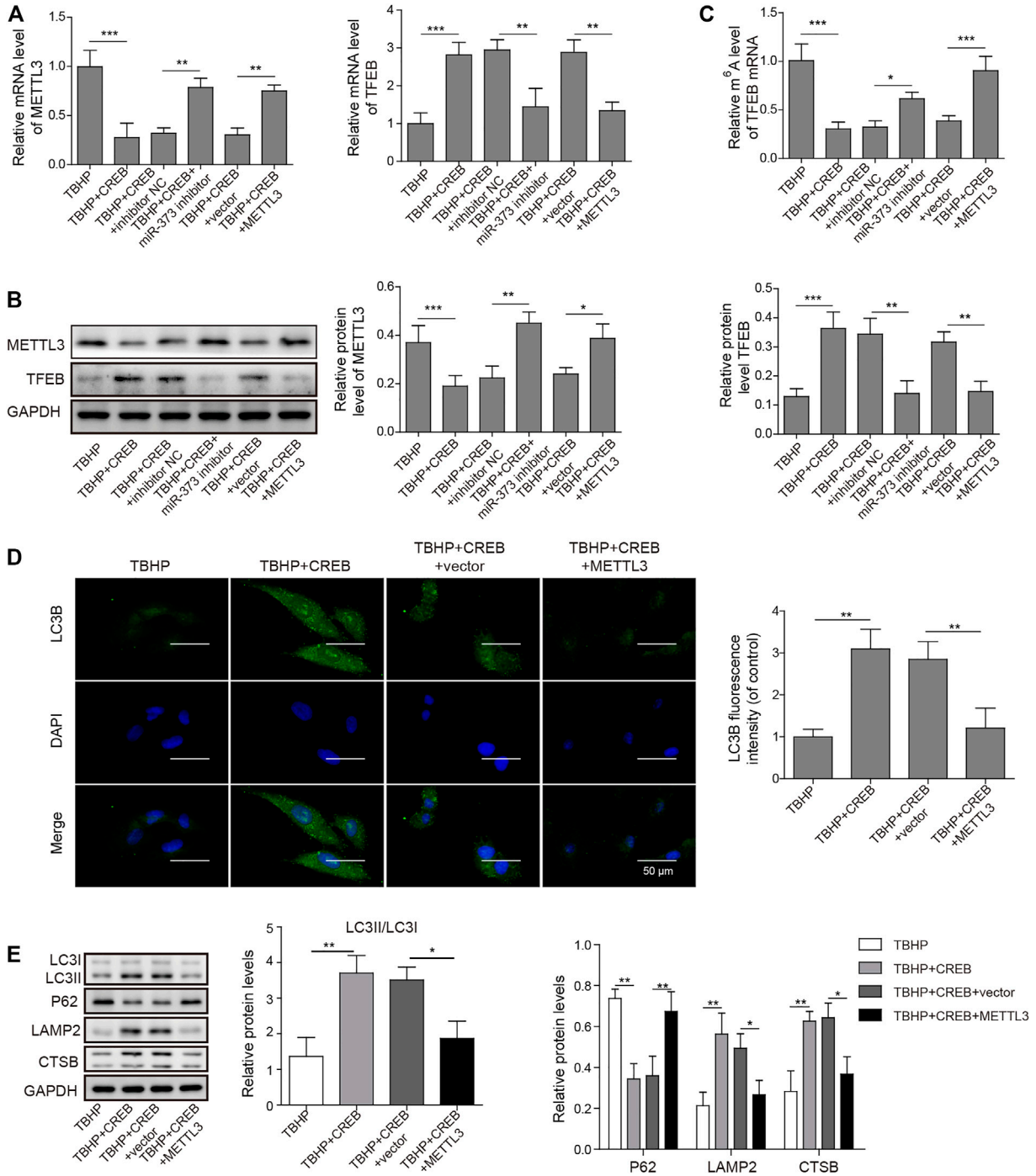

FIGURE 5 | Overexpression of METTL3 overturned the CREB-promoted autophagic flux. **(A)** The expression of METTL3 (left) and TFEB (right) were confirmed by qRT-PCR. GAPDH was used as an endogenous reference. **(B)** The changes of the protein of METTL3 and TFEB in the experiment above were determined by Western blotting. Quantified intensity of the bands was normalized to GAPDH to generate the column chart. **(C)** The changes of m6A of TFEB mRNA in different treatments above. **(D)** The impact on the autophagic flux was determined by the immuno-staining of LC3B. LC3B fluorescence intensity was measured and data from all groups were normalized to the TBHP group. **(E)** The changes of the essential regulators or markers of apoptosis and autolysosome were determined by Western blotting. Quantified intensity of the bands was normalized to GAPDH to generate the column chart. $p < 0.05$ was marked as *, $p < 0.01$ was marked as **, $p < 0.001$ was marked as ***.

overexpression of CREB in TBHP-treated human chondrocytes effectively attenuated the TBHP-induced apoptosis and the blockage of autophagic flux. This effect was proved to be dependent on miR-373, which is one of the most decreased miRNAs in OA cartilage (Cong et al., 2017). Next, we proved that METTL3 was targeted by miR-373 while this epigenetic

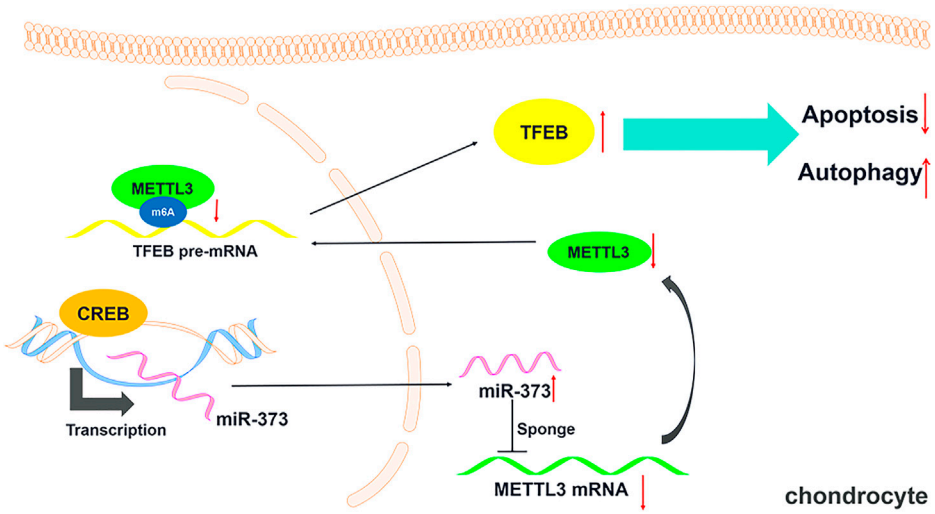

FIGURE 6 | Schematic diagram of this study. A schematic model showing that CREB regulating chondrocytes autophagy/apoptosis *via* the miR-373/METTL3/TFEB axis.

regulator strictly controlled one of the most essential autophagy regulators TFEB in cartilage tissues. Taken together, we found a novel regulation axis initiated by CREB. This axis maintains the autophagy activity of chondrocytes to support their survival. This discovery may provide a novel ideal in both the understanding of the pathogenesis and the development of the treatment for this disorder.

As one of the most classic regulation pathways, CREB is involved in a tremendous number of biological processes. The change of CREB in OA has been mentioned in some studies and controversy also exists. In most of the studies, the result is consistent with our finding in which CREB is downregulated in either protein or phosphorylation modification in OA (Wang et al., 2017a; Sun et al., 2017). However, some show an increase in phosphorylated CREB (Ji et al., 2019). It is understandable because of the heterogeneous etiologic factors for this disorder. A different model may involve different interruptions in different networks. It reminds us that the underlying mechanisms are much more complex than imagined. Other than that, the alternation of CREB expression has always been regarded as an inevitable consequence rather than a start point. However, in our study, we for the first time provided solid evidence in which artificially increased CREB level in OA was able to protect the chondrocytes from loss of activity and even cell death. This not only proved that CREB could manage the development as a major regulator independently but also provided a potential therapeutic target.

Furthermore, although the decreased miR-373 in chondrocytes of OA has been continuously noticed (Song et al., 2015; Jin et al., 2017; Zhang et al., 2018) and long non-coding RNA PART1 has been proved to be a post-transcriptional regulator of miR-373 in chondrocytes (Zhu and Jiang, 2019), the direct upstream regulator is still unknown. In our study, we first confirmed that the expression of miR-373 was indeed decreased in both human samples and surgically induced mouse OA model, which again implied the possible engagement of miR-373 in OA etiology. More than that, as an answer to what the direct regulator of miR-373 on the transcription level is, our findings proved that CREB increases miR-373 expression by directly binding to its promoter. Interestingly, this interaction has also been observed in pancreatic cancer (Zhang et al., 2013), which not only shows the essential role of autophagy regulation in human health but also implies that the regulation axis starting from CREB may serve as one of the top candidates for the treatments to other diseases. Meanwhile, it has been discovered that the introduction of miR-373 into mice DMM model significantly reduced cartilage destruction (Song et al., 2015), which is also consistent with our results. Based on this discovery, we proved that the protection of chondrocyte autophagy from CREB depended on miR-373 induction. This discovery first provided another line of evidence supporting the essential role of non-coding RNA regulation, especially miRNA, in OA development as well as other osteopathological process (Wang et al., 2017b; Liu et al., 2017). Other than that, with the discovery of diverse transportation of miRNAs among cells such as the delivery by exosome (Li J. et al., 2017), the miRNA-conducted regulation within, or even beyond, specific cell type would also be of interest and necessity. In addition, the deepened understanding of non-coding RNA's role in the development of OA may provide several novel diagnostic or therapeutic targets to strengthen the arsenal against bone-related diseases (Li Z. et al., 2017).

In addition to that, METTL3 was first identified as a direct target for miR-373. METTL3 is a key factor in a methyltransferase complex catalyzing the modification of N^6-methyladenosine (Liu et al., 2014). This modification is the most prevalent epigenetic modification of mRNA (Zhao et al., 2016). The role of METTL3 on OA etiology has been discussed recently. It has been reported that the expression of METTL3 was elevated in chondroprogenitor cells after IL1-β stimulation, and the consequent m6A increase resulted in OA development

through severe inflammatory and extracellular matrix degradation. Similarly, our research found that the overexpression of METTL3 promoted OA-like phenotype through the inhibition of autophagy flux. More importantly, we identified TFEB as one of the METTL3 targets, which was responsible for the effect of METTL3 in OA development. This is inspiring not only because TFEB was recently proved to be a target of METTL3 in cardiomyocytes (Song et al., 2019), but also because it is widely regarded as a core regulator in autophagic and lysosomal biogenesis (Settembre et al., 2011; Decressac et al., 2013; Lapierre et al., 2013). Moreover, TFEB has been reported to control autophagy of the endoplasmic reticulum in chondrocytes by inducing the expression of FAM134B, an endoplasmic reticulum autophagy receptor (Cinque et al., 2020), which might explain its beneficial effects on OA. Taken together, these findings integrate a major autophagy regulator into an epigenetic network, and it further consolidates the position of METTL3 in OA development and autophagy regulation within the condition. Besides, this may serve as a novel target for a more effective treatment. More interestingly, it should be emphasized that the pathway being studied in the previous and present research, either downstream or upstream, is diverse, implying that a regulation network centered by CREB/miR-373/METTL3 is still to be further unveiled.

In summary, our studies demonstrate that CREB plays a critical role in the pathogenesis of OA. As a transcription factor, it controls the expression of miR-373, which directly targets an important epigenetic regulator METTL3 and finally relieves TFEB from the METTL3-conducted epigenetic suppression. This regulation axis may serve as a novel target for the treatment of OA.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Ethics Review Committees of the Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of The Xiangya Hospital of Central South University.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to this study. XL and HZ contributed to study design, data collection, analysis and interpretation of the data, drafting of the manuscript, and final approval of the article. YL and XY contributed to study design, interpretation of the data, drafting and critical revision of the article for important intellectual content, and final approval of the article. JY provided the study materials, contributed to critical revision of the article for important intellectual content, and final approval of the article. RL and HW contributed to study design, critical revision of the article for important intellectual content, and final approval of the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.778941/full#supplementary-material

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REVIEW
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Targeting Autophagy in Thyroid Cancer: EMT, Apoptosis, and Cancer Stem Cells

Tammy M. Holm[1,2]*, *Syn Yeo*[2], *Kevin M. Turner*[1,2] *and Jun-Lin Guan*[2]*

[1]Department of Surgery, The University of Cincinnati, Cincinnati, OH, United States, [2]Vontz Center for Molecular Studies, Department of Cancer Biology, The University of Cincinnati, Cincinnati, OH, United States

Autophagy is a highly conserved recycling process through which cellular homeostasis is achieved and maintained. With respect to cancer biology, autophagy acts as a double-edged sword supporting tumor cells during times of metabolic and therapeutic stress, while also inhibiting tumor development by promoting genomic stability. Accumulating evidence suggests that autophagy plays a role in thyroid cancer, acting to promote tumor cell viability and metastatic disease through maintenance of cancer stem cells (CSCs), supporting epithelial-to-mesenchymal transition (EMT), and preventing tumor cell death. Intriguingly, well-differentiated thyroid cancer is more prevalent in women as compared to men, though the underlying molecular biology driving this disparity has not yet been elucidated. Several studies have demonstrated that autophagy inhibitors may augment the anti-cancer effects of known thyroid cancer therapies. Autophagy modulation has become an attractive target for improving outcomes in thyroid cancer. This review aims to provide a comprehensive picture of the current knowledge regarding the role of autophagy in thyroid cancer, focusing on the potential mechanism(s) through which inhibition of autophagy may enhance cancer therapy and outcomes.

Keywords: autophagy, thyroid cancer, EMT, apoptosis, cancer stem cells

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Monica Fedele,
Consiglio Nazionale Delle Ricerche (CNR), Italy

***Correspondence:**
Tammy M. Holm
tammy.holm@uc.edu
Jun-Lin Guan
guanjl@ucmail.uc.edu

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INTRODUCTION

Thyroid cancer is the most common endocrine malignancy. Worldwide, there were 586,202 new cases of thyroid cancer in 2020 (Sung et al., 2021). Well-differentiated thyroid cancers (WDTC) account for the majority of cases and generally exhibit a favorable prognosis with conventional treatment including surgery and possibly radioactive iodine (RAI) ablation. While WDTC is significantly more prevalent in women as compared to men, the underlying molecular biology driving this mutation has not yet been elucidated and recent epidemiologic studies suggest that the observed disparity between men and women is due to overdiagnosis (Song et al., 2013; Meng et al., 2014; Daskalaki et al., 2018; Gugnoni et al., 2021). Importantly, mortality from thyroid cancer increases significantly for patients with recurrent WDTC, which occurs in up to 20% of cases, or those with dedifferentiated, anaplastic thyroid cancer (ATC) (Mazzaferri and Jhiang, 1994). Therapeutic options for advanced, dedifferentiated disease remain limited.

The term "autophagy" from the Greek, self-devouring, was coined by the Belgian biochemist and cytologist, Dr. Christian de Duve. Initially recognized as a protective process induced to prevent starvation, and then a house-keeping pathway for the orderly degradation of unnecessary or dysfunctional cellular components, autophagy is now recognized to play an active role in a multitude of human diseases including cancer (Dikic and Elazar, 2018; Singh et al., 2018). In

TABLE 1 | Effects of autophagy modulation in thyroid cancer.

	Thyroid cancer cell lines	Effects of autophagy modulation	References
Autophagy inhibition			
CDH-6	• PTC: TPC-1, BCPAP	Autophagy inhibition drives DRP-1 mediated mitochondrial reorganization and induces EMT	Gugnoni et al. (2017)
BIRC7	• PTC: BCPAP, TPC-1, K1, IHH4	Autophagy inhibition via BECN1 and ATG5 repression induces EMT	Liu et al. (2020)
Lys05	• PTC: T32, T68 • FTC: FTC-133 • ATC: 8505c	Autophagy inhibition at the level of the lysosome inhibits EMT in all cell lines and induces apoptosis only in FTC-133 and 8505c cells	Holm et al. (2021)
BAG3	• PTC: KTC1 • ATC: FRO, KTC3	Autophagy inhibition in starved cancer cells induces apoptosis	Liu et al. (2013)
LDHA	• PTC: KTC1, BCPAP	Autophagy inhibition induces EMT and cell proliferation	Hou et al. (2021)
Autophagy induction			
BANCR	• PTC: IHH4	Autophagy induction inhibits apoptosis and induces cell proliferation	Wang et al. (2014)
HMGB1	• PTC: TPC-1 • FTC: FTC-133	Autophagy induction sustains thyroid cell differentiation (NIS expression)	Chai et al. (2019)
Dual therapy			
LDHA inhibition + HCQ	• PTC: BCPAP	LDHA inhibition induces autophagy inhibiting apoptosis. Dual LDHA and autophagy inhibition drives apoptosis, no effect on EMT	Hou et al. (2021)
Vemurafenib + HCQ	• PTC: BCPAP • ATC: 8505c	Autophagy induction with vemurafenib. Synergism with HCQ + vemurafenib increases cell death	Wang et al. (2017)
Apatinib + CQ/HCQ	• PTC: K1, KTC1 • ATC: KHM-5M, C643	Autophagy induction with apatinib. Synergism with CQ/HCQ + apatinib increases apoptosis	Feng et al. (2018) and Meng et al. (2020)
Sorafenib + CQ	• FTC: FTC-133 • ATC: 8505c	Autophagy and apoptosis induction with sorafenib. Dual therapy with sorafenib + CQ results in synergistic increase in apoptosis	Yi et al. (2018)
Radiation/doxorubicin + RAD001	• PTC: TPC1 • ATC: 8505c	Autophagy induced by radiation/doxorubicin. Dual therapy with potent autophagy inducer (RAD001) increased cell death independent of apoptosis	Lin et al. (2009); Lin et al. (2010)

PTC, Papillary thyroid cancer; FTC, Follicular thyroid cancer; ATC, Anaplastic thyroid cancer.

this review, we examine the current understanding of the role of autophagy in pathogenesis of thyroid cancer with a focus on EMT, apoptosis, and cancer stem cells (CSCs). Moreover, we provide a perspective on how these processes can be targeted for potential therapeutic approaches and summarize the most recent data on the role of autophagy inhibitors in thyroid cancer care (**Table 1**).

AUTOPHAGY AND EPITHELIAL-TO-MESENCHYMAL TRANSITION: INVASION AND STABILIZATION

Epithelial-to-mesenchymal transition (EMT) is considered to be crucial for the development of an invasive phenotype in tumor cells and therefore a hallmark of metastatic disease (Brabletz et al., 2018). Mesenchymal features are transiently adapted by epithelial cells which subsequently lose their apical basal polarity as well as cell-cell adhesion. Further dedifferentiation of epithelial cells results in higher cellular plasticity and motility, as well as resistance to apoptosis. Recently, EMT has been described as a spectrum of states, including intermediary hybrid-EMT states. The process of EMT is highly regulated and requires profound molecular and biochemical changes both within the tumor cell itself and with the surrounding microenvironment.

The SNAI family of transcription factors (TF) including Snail and Slug, together with the zinc-finger E-box-binding homeobox (ZEB) 1/2, and basic helix-loop-helix transcription factors Twist 1/2, are thought to be the drivers of EMT as they promote the expression of genes typically expressed in mesenchymal cells, namely: N-cadherin, vimentin, and fibronectin, while concomitantly suppressing the expression of epithelial markers: E-cadherin, cytokeratins, claudins, and occludins (Meng et al., 2014; Brabletz et al., 2018). The loss of E-cadherin leads to the activation of the Wnt/β-catenin pathway and subsequent transcription of genes promoting proliferation and migration. Consequently, cells undergoing EMT lose polarity and acquire a fibroblast-like morphology which allows for the degradation of the underlying basement membrane and subsequent migration from the epithelial layer and surrounding extracellular matrix. Activation of EMT is thought to be pivotal for both the acquisition of the malignant phenotype and metastases.

The tumor microenvironment is characterized in part by inflammation, with tumor cells recruiting activated fibroblasts and immune cells which in turn secrete tissue-specific soluble factors including transforming growth factor beta (TGF-β) and tissue necrosis factor alpha (TNF-α) (Song et al., 2013). Hypoxia

within the microenvironment also promotes EMT via hypoxia-inducible-factor 1-alpha (HIF1α) and increased mitochondrial production of reactive oxygen species (ROS). All of these factors, among several others, have also been shown to influence both the induction and suppression of autophagy (Daskalaki et al., 2018). This observation recently led to multiple studies seeking to describe the intricate relationship between these two processes and cancer. Notably, the effect of autophagy on EMT appears to be dependent on cell-type as well as the specific autophagic stimulus.

Because of the observation that EMT is often induced in cancers with more aggressive phenotypes and heightened metastatic potential, recent interest in the relationship between EMT and autophagy in thyroid cancer is focused on identifying markers that could help predict which thyroid cancers are more likely to progress to recurrent or advanced disease. Several recent studies have utilized large tumor repositories in concert with genomic datasets including The Cancer Genome Atlas (TCGA) to identify potential markers of advanced disease. Multiple studies have then endeavored to understand how these markers interact with autophagy to drive disease progression (Gugnoni et al., 2017; Gugnoni et al., 2021; Hou et al., 2021; Xu et al., 2021).

Cadherin-6 (CDH6) is a type 2, atypical cadherin which is aberrantly reactivated in several cancers (Shimazui et al., 1998; Köbel et al., 2008). Recently, CDH6 was found to be overexpressed in aggressive papillary thyroid cancer (PTC) both *in vitro* and in tissue derived from PTC patients (Ciarrocchi et al., 2011; Sancisi et al., 2013). The authors found that expression of CDH6 is localized to the invasive front of tumors and controlled by TGFβ induced RUNX2-regulated transcription of SNAI 1/2. Notably, CDH6 expression is specific to thyroid cancer cells as compared to wild-type thyrocytes. Follow-up work by Gugnoni and colleagues found that loss of CDH6 expression by siRNA attenuates the mesenchymal features of thyroid cancer cells by altering cytoskeletal architecture and intercellular associations (Gugnoni et al., 2017). Using a yeast-two hybrid screen and GST-pulldown experiments, they identified interactions between CDH6 and three autophagy related proteins, GABA type A receptor-associated protein (GABARAP), BCL2 interacting protein 3 (BNIP3), and BCL2 interacting protein 3 like (BNIP3L). Subsequent work demonstrated that CDH6 restrains autophagy and promotes cell motility through dynamin-related protein (DRP1) mediated mitochondrial reorganization. To further support a role for CDH6, the authors analyzed a large cohort of tissue specimens from patients with thyroid cancer, and found that through these interactions, CDH6 expression independently associated with distant metastases and decreased disease specific survival in PTC patients. In this study, inhibition of autophagy is thought to be a consequence of CDH6 binding to GABARAP with concomitant DRP-1 mediated mitochondrial fission, and subsequent cytoskeletal remodeling, cell migration, and EMT induction. The model suggests that induction of autophagy in PTC cells could negatively control and ultimately reverse EMT by promoting mitochondrial fusion and cytoskeletal stabilization.

Baculoviral IAP Repeat Containing 7 (BIRC7) is an inhibitor of apoptosis (IAP) family gene whose expression is linked to tumor progression and metastasis in lung and prostate cancer (Chen et al., 2012; Zhuang et al., 2015). BIRC7 has recently been shown to be upregulated in several validated PTC cell lines, as well as fresh tumor tissue from PTC patients, as compared to wildtype thyrocytes or benign thyroid tissue (Liu et al., 2020). In the same study, markers of EMT were significantly downregulated with knockdown of BIRC7 whereas overexpression markedly enhanced PTC migration and invasion *in vitro*. Conversely, markers of autophagy were increased with knockdown of BIRC7 and decreased with overexpression suggesting that BIRC7 works to suppress autophagy in PTC cells. To further dissect the link between autophagy and BIRC7, the authors assessed the effect of concomitant BIRC7 knockdown with either BECN1 or ATG5 knockdown in PTC cells and found that both migration and invasion were markedly increased, suggesting that the induction of autophagy resulting from BIRC7 knockdown is protective in PTC. They then compared the effect of rapamycin, a well-described drug known to induce autophagy and found that BIRC7 knockdown together with rapamycin synergistically reduced cell migration and invasion while increasing autophagy flux. Markers of EMT were similarly significantly decreased with dual rapamycin therapy with BIRC7 knockdown. Importantly, *in vivo* xenograft models of PTC metastasis with tail vein injection showed a significantly reduced burden of metastatic disease in animals injected with BIRC7 knockdown PTC cells co-treated with rapamycin. Once again, markers of autophagy activation were upregulated and markers of EMT were downregulated in mice injected with BIRC7 knockdown PTC cells and treated with rapamycin. Taken together, these findings suggest that BIRC7 promotes invasion and metastasis by driving EMT and inhibiting autophagy.

Notably, a recent study evaluating the effect of autophagy inhibition in thyroid cancer found that vimentin, a marker of mesenchymal cells, was significantly downregulated in both WDTC and ATC cells treated with Lys05, a potent and specific inhibitor of lysosomal autophagy (Holm et al., 2021). Directed autophagy inhibition with Lys05, chloroquine, or FIP200 (a mediator of canonical autophagy) knockdown decreased cell migration and invasion thereby attenuating the effects of EMT rather than promoting EMT progression. These findings contrast with those of Liu and Gugnoni where they found that restraining autophagy in the context of CDH6 and BIRC7 upregulation was a possible driver of the mesenchymal phenotype.

The relationship between EMT and autophagy in the pathogenesis of cancer continues to be controversial and incompletely defined. Multiple studies have demonstrated that both autophagy inhibition and induction may modulate EMT in conflicting ways (Gugnoni et al., 2017; Liu et al., 2020; Holm et al., 2021; Hou et al., 2021). The stimulus driving either EMT or autophagy, together with the specific tumor microenvironment likely affects the ultimate outcome. Interestingly, in colorectal cancer cells, both induction of autophagy by mTOR inhibition or inhibition of autophagy by BECN1 knockdown lead to inhibition

of EMT (Gulhati et al., 2011; Ren et al., 2015; Shen et al., 2018). Whether autophagy induction or inhibition is a consequence of EMT activation, or whether autophagy functions as a driver, or an inhibitor of EMT in thyroid cancer remains to be determined.

Apoptosis and Autophagy: to Die or to Devour?

Programmed cell death type 1, or apoptosis, is induced by multiple stimuli including cellular stress, DNA damage, and immune surveillance (Das et al., 2021). Dysregulation of apoptosis has been well described in cancer biology. Defective apoptosis can drive *de novo* tumor development resulting from a failure of normal cell turnover and also promote tumor progression and metastasis by supporting: 1) a more permissive environment for genetic instability, 2) resistance to immune cell and cytotoxic (chemo/radiation) therapies, and 3) tumor cell survival despite a hypoxic or detached/suspended (metastatic) state.

The relationship between autophagy and cell death is complex because autophagy can drive both an adaptive, pro-survival response as well as a cytotoxic, pro-death response (Das et al., 2021). Whether a given cell will initiate apoptosis or autophagy depends upon the cellular context, intensity of the stimulus, and the cell's threshold for either response. Cross-talk between autophagy and apoptosis is well-described (Das et al., 2021). As with EMT, both pathways are induced by many of the same stressors (reactive oxygen species, p53, death associated protein kinases, BH3-only proteins). Moreover, several canonical autophagy proteins including ATG5 and BECN1 directly and indirectly impact the regulation or execution of the apoptotic pathway (Das et al., 2021). Autophagy and apoptosis are for the most part mutually inhibitive but whereas apoptosis is purely a cell death pathway, autophagy can induce either cell survival or cell death.

Therapeutic strategies to induce apoptosis in cancer cells is of significant interest in cancer research. Both from the perspective that the apoptosis machinery is often mutated during tumorigenesis and because therapeutic resistance is frequently associated with ineffective apoptosis and/or necrosis of tumor cells. Suppression of apoptosis can induce autophagy, which in turn may promote tumor survival by supporting cell growth and proliferation in a nutrient-poor, hypoxic environment. Paradoxically, autophagy induction may also support tumor cell death by promoting tumor regression through type II programmed cell death (PCD) or type III cell death, necrosis. Multiple non-apoptotic programmed cell death pathways also exist which interact with autophagy, including necroptosis, ferroptosis, and pyroptosis (Miller et al., 2020). A deeper understanding of the relationship between cancer biology, autophagy, apoptosis, and necrosis is important for the development of novel, effective therapeutics.

Specific to thyroid cancer, the relationship between autophagy, apoptosis and cell necrosis is complex. There are a wealth of studies which demonstrate both: 1) a pro-tumorigenic effect of autophagy with increased cell proliferation and concomitant suppression of apoptosis and/or cell death and 2) a tumor suppressive effect of autophagy with decreased cell proliferation, induction of apoptosis and/or cell death (Li et al., 2014; Ren et al., 2015; Feng et al., 2018; Das et al., 2021). Regarding several autophagy modulators, findings with respect to upregulation vs. downregulation of autophagy as well as subsequent effects on apoptosis, cell proliferation, and cell death, often conflict across different thyroid cancer types and sometimes between different studies.

BRAF-activated long non-coding RNA (BANCR) was initially shown to be an overexpressed inducer of EMT in melanoma, colon cancer, and non-small cell lung cancer (Sunaga and Kaira, 2015; Yu et al., 2017; Hussen et al., 2021). Specific to thyroid cancer, BANCR was found to be uniquely expressed both in PTC cells *in vitro* and in tumor samples derived from PTC patients as compared to normal controls (Wang et al., 2014). Overexpression of BANCR in PTC cells markedly activated autophagy and inhibited apoptosis but there was no effect on cell migration or invasion. Knockdown of BANCR inhibited cell proliferation and increased apoptosis in PTC cells. Inhibition of autophagy stimulated apoptosis and abrogated the effects of BANCR on cell proliferation. These results suggest that autophagy stimulation induced by BANCR is tumor protective in thyroid cancer because induction of autophagy with BANCR led to increased cell proliferation and decreased apoptosis.

Another protein known to modulate autophagy and apoptosis in thyroid cancer is Bcl2-associated athanogene 3 (BAG3) (Li et al., 2014). BAG3 was initially identified as an anti-apoptotic protein and negative regulator of EMT expressed in multiple thyroid cancer cell lines (Chiappetta et al., 2007; Meng et al., 2014). Subsequent studies demonstrated a role for BAG3 in the activation of chaperone-mediated autophagy in HepG2 cells derived from a patient with hepatocellular carcinoma (Liu et al., 2013). In an elegant follow-up study aiming to understand whether BAG3 might also be involved in canonical autophagy in thyroid cancer cells, Li et al. (2014) sought to evaluate the effects of BAG3 expression under starvation conditions thought to mimic the tumor microenvironment. Under starvation conditions, they found that autophagy was induced and BAG3 expression inhibited. However, the forced expression of BAG3 under these same conditions suppressed autophagy and promoted apoptosis (Liu et al., 2013; Li et al., 2014). The duality of BAG3, promoting cell survival *via* upregulation of CMA (Liu et al., 2013) while also promoting apoptosis via inhibition of canonical autophagy, (Li et al., 2014) is likely reflective of the cellular context, the underlying autophagy stimulus, and the complex nature of autophagy modulation itself.

An additional study highlighting the contradictory nature of autophagy modulation in thyroid cancer progression was recently published by Hou et al. (2021). They identified lactate dehydrogenase A (LDHA), an enzyme overexpressed in aggressive PTC, as a driver of EMT gene transcription, cell proliferation and autophagy regulator (Hou et al., 2021). LDHA was identified as a potential diagnostic and therapeutic target for PTC following examination of the TCGA database. Overexpression of LDHA was confirmed in PTC tissues derived from patients with aggressive disease as well as multiple, well-established thyroid cancer cell lines. The authors found that

LDHA promoted EMT, as well as cell proliferation *in vitro* and *in vivo*, in part by inhibiting autophagy. They observed that LDHA overexpression led to the inhibition of AMPK-mediated protective autophagy and apoptosis, with concomitant increased tumor cell proliferation. Knockdown of LDHA or the LDHA inhibitor, FX11, induced autophagy and inhibited apoptosis. Dual therapy with the LDHA inhibitor, FX11, and the autophagy inhibitor, hydroxychloroquine (HCQ), had a synergistic effect on apoptosis *in vitro* and significantly reduced tumor growth in mouse models.

This study is one of many which highlight the Janus-faced nature of autophagy. While LDHA overexpression induced a pro-tumorigenic phenotype with autophagy inhibition, upregulation of EMT and cell proliferation, induction of autophagy from LDHA knockdown or FX11 also promotes a pro-tumorigenic phenotype by inhibiting apoptosis in tumor cells. Therapy induced autophagy is well described (Lin et al., 2009; Ma et al., 2011; Zhang et al., 2015) with several studies highlighting the effect of "enforced" autophagy resulting from therapeutic influence. The role of autophagy modulation and apoptosis in the context of therapeutic interventions for thyroid cancer is an active area of investigation.

Vemurafenib is a selective RAF inhibitor utilized in patients with BRAF-mutant melanoma (Wang et al., 2017). However, while the BRAFV600E mutation is common in aggressive forms of thyroid cancer, there is significant resistance to vemurafenib therapy in this patient population. An elegant study by Wang et al. (2017) found that this observation may result from the induction of autophagy by vemurafenib. They found that dual therapy with vemurafenib and the autophagy inhibitor HCQ augmented vemurafenib-induced cell death by blocking ER stress-response mediated autophagy both in cell culture and xenograft mouse models.

Similar work evaluating another well described tyrosine kinase inhibitor, apatinib, found that induction of autophagy by apatinib led to inhibition of apoptosis and therapeutic resistance (Feng et al., 2018; Meng et al., 2020). Dual therapy with apatinib and autophagy inhibition with either HCQ or CQ led to reduced tumor growth and augmented apoptosis both *in vitro* and *in vivo* in PTC and ATC models (Feng et al., 2018; Meng et al., 2020). In another study evaluating the effect of the multi-kinase inhibitor, sorafenib, in thyroid cancer found that dual therapy with the autophagy inhibitor CQ significantly suppressed tumor growth as compared to either therapy alone (Yi et al., 2018). Moreover, the authors demonstrated that while sorafenib alone increased both autophagic activity and apoptosis, the introduction of an autophagy inhibitor served to specifically augment apoptosis in both ATC and FTC cell lines and in xenograft models.

Notably, these studies contrast with older work which suggested that dual therapy with autophagy enhancement, as opposed to inhibition, and antitumor therapy served to augment tumor cell death in thyroid cancer. Lin et al. (2009) found that while autophagy was significantly induced by radiation or doxorubicin, inhibition of autophagy actually decreased both radio- and chemo-sensitivity. In follow up to this, they found that enhanced autophagy with RAD001, a potent activator of autophagy, sensitized PTC cells to cytotoxic chemotherapy with doxorubicin and radiation (Lin et al., 2010). Importantly, the authors found that the increased cytotoxicity stemming from dual therapy with RAD001 and either doxorubicin or radiation did not correlate with a concomitant increase in apoptosis but rather an attenuation of c-MET signaling. Whether the synergistic affect of autophagy induction with doxorubicin or radiation drives increased autophagic cell death, necrosis, or other non-apoptotic cell death remains to be determined.

A more recent study looking at the effects of autophagy inhibition alone found that there was no effect of autophagy inhibition on apoptosis in two PTC cell lines (Holm et al., 2021). However, in FTC and ATC cell lines, inhibition of autophagy with Lys05 was associated with induced apoptosis *in vitro* (Holm et al., 2021). These differences in the effects of autophagy modulation likely highlight the inherent distinctions in the pathogenesis of different thyroid cancer types as well as differences in native to the specific autophagy stimulus.

Cancer Stem Cells and Autophagy: Maintaining the Quietest Cell

Within solid tumors, cancer stem cells (CSCs) exist as a small subpopulation of cancer cells that retain the capacity for long-term self-renewal, proliferation, and multi-potent differentiation (Hardin et al., 2017; Nazio et al., 2019). The CSC hypothesis posits that CSCs are responsible for tumor heterogeneity, proliferation, invasion, and metastasis. In this model, tumorigenesis, recurrence, and therapeutic resistance are the result of a small but persistent CSC population which fails to respond to conventional anticancer regimens. Targeted therapy with complete elimination of CSCs would therefore address both continued tumor growth and relapse.

Autophagy has been proposed to play a role in the maintenance of CSC pluripotency as well as the CSC niche. Failure of autophagy in multiple cancer types: breast, pancreas, liver, ovarian, glioblastoma leads to the negative regulation of staminal markers of CSCs which ultimately influence their self-renewal ability (Gong et al., 2013; Song et al., 2013; Wolf et al., 2013; Yeo et al., 2016; Zhang et al., 2016; Nazio et al., 2019). Elegant work by Gong et al. (2013) identified a role for autophagy in the maintenance of CSCs in breast cancer. They found that autophagy was upregulated in breast CSCs as compared to bulk tumor. Inhibition of autophagy mediated by Beclin-1 shRNA significantly reduced breast CSC proliferation and self-renewal. Xenograft experiments with breast CSCs with or without shRNA induced silencing of beclin-1 demonstrated that autophagy is critical for tumorigenicity. Subsequent work in pancreatic and ovarian cancer have similarly identified autophagy as an essential player in the maintenance and tumorigenicity of CSCs (Yang et al., 2015; Buccarelli et al., 2018). Altogether, these studies found that combined therapy with autophagy inhibition and standard chemotherapy had a synergistic effect, eradicating or significantly reducing tumor development. As such, inhibition of autophagy, with its concomitant destabilization of the CSC population, provides an attractive therapeutic target.

Thyroid CSCs (TCSCs) are characterized by the expression of various biomarkers including ALDH and CD44 as well as their

ability to form thyrospheres *in vitro* and tumors *in vivo* (Todaro et al., 2010). They are highly tumorigenic and recapitulate the behavior of the parental tumor with respect to their ability to locally invade and metastasize. Across different thyroid cancer subtypes, CSCs are most common in ATC (~5%), followed by PTC (~2%) and FTC (1%–2%). As with most CSCs, TCSCs are quiescent and intrinsically resistant to conventional therapy. These features of TCSCs are thought to drive drug resistance, metastasis, and recurrence. Targeted therapeutics aimed to eliminate TCSCs could either directly eradicate the CSCs themselves or induce or promote terminal differentiation.

The underlying mechanisms which specifically support TCSC maintenance are an area of active investigation. A great deal of information has been inferred by observing how both the tumor microenvironment and pathways crucial to TCSC viability are modified. Within the tumor microenvironment, the TCSC niche is sustained by a complex, bidirectional network of chemokines/cytokines mediated by the surrounding stroma including cancer associated fibroblasts (CAFs), macrophages, and lymphocytes (Visciano et al., 2015; Parascandolo et al., 2017; Zheng et al., 2019). Dysregulation of the NF-kB, MAPK, and GSK3b pathways have been implicated in the maintenance of TCSCs themselves across multiple thyroid cancer subtypes (Ricarte-Filho et al., 2009; Li et al., 2013). Because EMT is a clear example of cellular plasticity, which is also a feature of CSCs, multiple studies have also sought to investigate the relationship between EMT and TCSCs. Interestingly, their work has revealed a correlation between EMT upregulation and increased TCSCs in PTC, FTC, and ATC (Liu and Brown, 2010; Lan et al., 2013).

Epigenetic modifications thought to maintain TCSC viability include: 1) DNA methylation 2) histone modification 3) non-coding RNA including miRNAs and lncRNAS (Vu-Phan and Koenig, 2014). DNA methylation has been implicated as a principal pathway because of the observation that components of the principal pathways involved in TCSC survival, MAPK, PI3K, and beta-catenin, are silenced by hypermethylation (Vu-Phan and Koenig, 2014). While the methylome profile of TCSCs is not yet defined, a role for DNA methylation in TCSC viability is hypothesized because of the focus on methylation of the promoters of thyroid-specific differentiation (Borbone et al., 2011). Histone deacetylase (HDAC) inhibitors are a well-known treatment for impairing cell growth, inducing apoptosis, and increasing radioiodine uptake in thyroid cancer (Russo et al., 2013). Bromodomain containing (Singh et al., 2018) (BRD4), lysine demethylase 1a (KDM1A), and enhancer of zeste homolog 2 (EZH2) are histone modifiers which have all been recently implicated as drivers critical for TC proliferation, invasion, and migration (Borbone et al., 2011; Gao et al., 2016; Zhang et al., 2019). Non-coding RNAs have long been recognized as critical regulators of EMT and CSCs in general. Several miRNAs and lncRNAs have found to be dysregulated in multiple subtypes of TC. Recently, two lncRNAs, lncRNA -LINC00311 and lncRNA-H19 were identified as specific promotors of cancer stem-like properties in PTC (Li et al., 2018; Gao et al., 2020). Similarly, two miRNAs: miR-148a and antisense-miR-21, were both found to significantly inhibit TCSCs in ATC cells (Haghpanah et al., 2016; Sheng et al., 2016).

Data regarding the specific relationship between autophagy and thyroid CSCs is limited and largely inferred from experiments seeking to investigate mediators of thyroid CSC viability. For example, high-mobility group box 1 (HMGB1), a highly conserved DNA-binding protein known to influence autophagy, CSC survival, and EMT was recently found to play a role in thyroid cancer (Kang et al., 2013; Chai et al., 2019). HMGB1 was identified as a critical regulator of autophagy-mediated NIS degradation in differentiated thyroid cancer. Knockdown of HMGB1 suppressed autophagy and sodium iodide symporter (NIS) degradation both *in vitro* and in xenograft mouse models (Chai et al., 2019). As NIS expression is a marker of differentiation in thyroid cells, it is inferred that HMGB1 has a role in TCSC survival. One recent study evaluated the direct effect of autophagy inhibition on CSC maintenance (Holm et al., 2021). They found that direct inhibition of autophagy with Lys05 or chloroquine led to almost complete abrogation of thyrosphere formation in PTC, FTC, and ATC cells *in vitro*. Further, knockdown of FIP200 significantly reduced ALDH activity and CD44 expression. Interestingly, autophagy inhibition with Lys05 mitigated cell migration, invasion, and proliferation. Vimentin expression, a marker of EMT, was also reduced. Taken together, these data would suggest that TCSCs are indeed drivers of a more aggressive phenotype (upregulated EMT, migration, invasion) which is, at least in part, supported by autophagy.

CONCLUSION AND FUTURE DIRECTIONS

Thyroid cancer continues to be a major public health issue with almost one million people living with the disease in the United States alone. Autophagy clearly plays a role in the pathogenesis of disease and remarkable progress has been made regarding the relationship between modulation of autophagy and progression of thyroid cancer (**Table 1**). Emerging evidence supports a possible role for autophagy inhibition either alone or in concert with targeted therapies (e.g., sorafenib, apatinib) as a means to induce cell death and/or apoptosis while also inhibiting proliferation, invasion, and migration (Feng et al., 2018; Yi et al., 2018; Meng et al., 2020). Clarifying the role of autophagy in TCSC viability and maintenance is particularly attractive as their prolonged quiescent state and resistance to conventional therapies likely contributes to both recurrence and persistence in thyroid cancer. Seemingly contradictory observations regarding the implications of autophagy modulation underscore our incomplete understanding of the complex cross-talk between the various molecular pathways involved and how cellular context regarding timing and autophagy stimulus (starvation vs. chemical) affect outcomes. Future studies which elucidate the molecular mechanisms underlying the crosstalk between autophagy, EMT, apoptosis, and TCSC viability will allow for the development of targeted, selective and individualized treatment strategies to eradicate thyroid cancer.

AUTHOR CONTRIBUTIONS

TH and J-LG conceived the concept and design of the review. TH drafted the manuscript and all authors participated in extensive revisions of the manuscript and provided critical intellectual content.

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EDITED BY
Zvulun Elazar,
Weizmann Institute of Science, Israel

REVIEWED BY
Jordi Muntané,
Institute of Biomedicine of Seville (CSIC), Spain

*CORRESPONDENCE
Yuting Zhao,
yuting_zhao@nwafu.edu.cn

†These authors have contributed equally to this work

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Development of an autophagy activator from Class III PI3K complexes, Tat-BECN1 peptide: Mechanisms and applications

Yanfei He†, Huaqing Lu† and Yuting Zhao*

Institute of Future Agriculture, Northwest A&F University, Yangling, China

Impairment or dysregulation of autophagy has been implicated in many human pathologies ranging from neurodegenerative diseases, infectious diseases, cardiovascular diseases, metabolic diseases, to malignancies. Efforts have been made to explore the therapeutic potential of pharmacological autophagy activators, as beneficial health effects from caloric restriction or physical exercise are linked to autophagy activation. However, the lack of specificity remains the major challenge to the development and clinical use of autophagy activators. One candidate of specific autophagy activators is Tat-BECN1 peptide, derived from Beclin 1 subunit of Class III PI3K complexes. Here, we summarize the molecular mechanisms by which Tat-BECN1 peptide activates autophagy, the strategies for optimization and development, and the applications of Tat-BECN1 peptide in cellular and organismal models of physiology and pathology.

KEYWORDS

autophagy, drug development, Beclin 1, Class III PI3K complexes, Tat-BECN1 peptide, cell-penetrating peptides

Introduction

Autophagy, the evolutionarily conserved pathway to target unwanted or damaged cellular contents to lysosomes for degradation, is linked to numerous human diseases (Levine and Kroemer, 2019; Mizushima and Levine, 2020) and considered a therapeutic target (Galluzzi et al., 2017).

Autophagy can be activated by many approaches, ranging from amino acid starvation, caloric restriction, physical exercises, to treatment with chemicals by known or unknown mechanisms (Galluzzi et al., 2017). Some chemicals that induce autophagy are under clinical trials for neurodegenerative diseases, cancers, autoimmune disorders or metabolic diseases, including (but not limited to) rapamycin, idalopirdine, SB-742457, metformin, resveratrol, lithium, spermidine, and trehalose (Galluzzi et al., 2017; Mizushima and Levine, 2020; Suresh et al., 2020). Among these autophagy activators, rapamycin is widely used in basic and translational research; it inactivates mechanistic target of rapamycin complex 1 (mTORC1), the nutrient-sensing kinase complex that promotes anabolism and inhibits catabolism, mainly autophagy (Saxton and Sabatini, 2017). However, specific

TABLE 1 Tat-BECN1 peptide-responsive cells and tissues

Species	Origins	Cells	References
Rat	Embryonic cardiac myoblast	H9c2	(Misaka et al., 2018)
Human	Cardiomyocyte	AC16	(Sun et al., 2021)
Rat, mouse, human iPSC-derived	Cardiomyocytes	Primary culture	(Nah et al., 2020)
Rat	Cortical neurons	Primary culture	(He et al., 2016)
Rat	Cerebral endothelial cells	Primary culture	(Forte et al., 2020)
Mouse	Cranial neural crest cells	Primary culture	(Yang et al., 2021)
Rat	Retinal neuron	R28	(Mathew et al., 2021)
Human	trabecular meshwork cells	Primary culture	(Kasetti et al., 2021)
Human	Kidney proximal tubule epithelial cell	HK-2	(Wang S. et al., 2015; Iaconis et al., 2020; Wang S. et al., 2021)
Opossum	Kidney proximal tubule epithelial cell	OKP	(Shi et al., 2020)
Mouse	Kidney proximal tubular cells	Primary culture	(Livingston et al., 2016)
Rat	Chondrosarcoma	RCS	(Bartolomeo et al., 2017)
Human	Chondrocytes	Primary culture	(Wu et al., 2020)
Mouse	Bone marrow stromal cells	Primary culture	(Choi et al., 2018)
Mouse	Pancreatic α cell	αTC9	(Rajak et al., 2021)
Rat	Insulinoma	INS-1E	(Riahi et al., 2016;Israeli et al., 2018)
Human	Breast cancer (Her2-positive)	BT-474, SK-BR3, MDA-MB-361	(Vega-Rubín-de-Celis et al., 2018)
Mouse	Breast cancer	4T1	(Wang et al., 2015b)
Human	Breast cancer (triple negative)	MDA-MB-231	(Zhou et al., 2019)
Mouse	Neuroblastoma	Neuro-2A	(Luo et al., 2018)
Human	Neuroblastoma	SK-N-SH	(Kobayashi et al., 2014;Kobayashi et al., 2020)
Human	Hepatocellular carcinoma	HepG2	(Wang et al., 2015b)
Human	Melanoma	WM793	(Kraya et al., 2015)
Human	Teratocarcinoma	NTERA-2/D1	(Sharif et al., 2017)
Human	Ovarian cancer	SKOV3	(Ding et al., 2018)
Human	Colon cancer	HCT116	(Andrejeva et al., 2020)
Human	Myeloid leukemia	U937	(Sharma et al., 2021)
Human	CD4+ T cells	Primary culture	(Zhang et al., 2019)
Mouse	CD8+ T cells	Primary culture	(Ko et al., 2021)
Human	Endothelial progenitor cells	Primary culture	(Forte et al., 2020)
Mouse	Bone marrow-endothelial progenitor cells	Primary culture	(Jiang et al., 2020)
Human	Embryonic kidney	HEK293	(Frudd et al., 2018)
Human	Bone osteogenic sarcoma	U2OS	(Wang et al., 2018)
African green monkey	Kidney	Vero-B4	(Gassen et al., 2019)
Chinese hamster	Ovary	CHO	(Braasch et al., 2021)

Tissues in which Tat-BECN1-induced autophagy is experimentally validated

Species	Tissues	References
Mouse	Heart	(Shirakabe et al., 2016;An et al., 2017;Sun et al., 2018;Tong et al., 2019;Nah et al., 2020)
Rat, mouse	Brain	(Li et al., 2016;He et al., 2017;Zhang et al., 2017;Luo et al., 2018; Shehata et al., 2018;Glatigny et al., 2019;He et al., 2019;De Risi et al., 2020;Forte et al., 2020;Kim et al., 2021)
Mouse	Spinal cord	(He et al., 2016)
Mouse	Eye	(Kasetti et al., 2021)

(Continued on following page)

TABLE 1 (*Continued*) Tat-BECN1 peptide-responsive cells and tissues

Species	Origins	Cells	References
Mouse	Kidney	(Livingston et al., 2019;Shi et al., 2020)	
Mouse	Liver	(Soria et al., 2018;Soria et al., 2021)	
Mouse	Lung	(Nikouee et al., 2021)	
Mouse	Bone	(Cinque et al., 2015;Bartolomeo et al., 2017)	
Rat	Articular cartilage	(Wang F. et al., 2019)	
Mouse	Cartilage and synovium	(Rockel et al., 2020)	
Mouse	Ovary	(Watanabe et al., 2020)	
Mouse	Tumor xenografts	(Wang et al., 2015a; Wang et al., 2015b;Pietrocola et al., 2016;Ding et al., 2018;Vega-Rubín-de-Celis et al., 2018;Zhou et al., 2019)	
Zebrafish	Embryo	(Zhu et al., 2017)	

Cells in which Tat-BECN1 induces certain effects (autophagy induction not experimentally validated)

Species	Origins	Cells	References
Mouse	Sinus nodal cells	Primary culture	(Woo and Kim, 2021)
Rat	Brain microvascular endothelial cells	Primary culture	(Forte et al., 2021)
Rat	Renal proximal tubular epithelial cells	Primary culture	(Forte et al., 2021)
Mouse	Embryonic carcinoma	P19	(Sharif et al., 2019)
Mouse	Macrophage-like	RAW 264.7	(Hadadi-Bechor et al., 2019)
Human	Acute myeloid leukemia	OCI-AML3	(Wang L. et al., 2019)
Mouse	Pancreatic islets	Primary culture	(Goginashvili et al., 2015)
Human	Lung fibroblast	Normal human lung fibroblasts (NHLFs)	(Sosulski et al., 2015)

Tissues in which Tat-BECN1 induces certain effects (autophagy induction not experimentally validated)

Species	Tissues	References
Mouse	Bladder	(Miao et al., 2015)
Mouse	Gastrocnemius and Flexor Digitorum Brevis muscles	(Baraldo et al., 2020)
Mouse	Orthotopic pancreas cancer of mouse PDAC cell line KPC	(Song et al., 2018)

Cells and tissues in which Tat-BECN1-induced autophagy is reported in (Shoji-Kawata et al., 2013).
Cell lines: Human cervical cancer cell line HeLa, breast cancer cell line MCF-7, leukemia monocytic cell line THP-1, lung adenocarcinoma cell line HCC827 and A549, bronchial epithelial cell line HBEC30-KT, African green monkey kidney fibroblast-like cell line COS-7.
Primary cultures: human monocyte-derived macrophages (MDMs), murine bone marrow-derived macrophages (BMDMs), murine embryonic fibroblasts (MEFs).
Tissues: heart, skeletal muscle (vastus lateralis) and pancreas.
Cells in which Tat-BECN1-induced autophagy is experimentally validated.

autophagy activators that have no or limited impact on other pathways are lacking for research needs and for potential clinal use.

In 2013, Beth Levine lab reported an autophagy-inducing Tat-BECN1 peptide (hereinafter referred as to Tat-BECN1), derived from Beclin 1 region that is required for HIV-1 Nef binding (Shoji-Kawata et al., 2013). Beclin 1 is a subunit of both Class III PI3K complexes, where Class III PI3K-C1 is regarded as autophagy initiation complex for autophagosome nucleation, and Class III PI3K-C2 mediates autophagosome maturation, endocytosis and LC3-associated phagocytosis (LAP) (Levine et al., 2015; Hurley and Young, 2017). Upon Tat-BECN1 treatment, autophagy induction was observed in 10 cell lines and primary cultures (Table 1); when Tat-BECN1 was administrated to mice intraperitoneally, autophagy induction was observed in heart, skeletal muscle (vastus lateralis) and pancreas (Shoji-Kawata et al., 2013). Tat-BECN1 showed protective effects in several *in vitro* and *in vivo* infection models of Sindbis virus, chikungunya virus, West Nile virus (WNV), HIV-1, *L. monocytogenes* (Shoji-Kawata et al., 2013). It also enhanced the degradation of small mutant huntintin protein aggregates in cells, indicating a therapeutic potential for neurodegenerative diseases (Shoji-Kawata et al., 2013).

In this review, we analyze the applications of Tat-BECN1 in around 100 studies since its discovery, discuss the current understanding of how it functions, the strategies to improve its activity, and the impact of Tat-BECN1 on pathophysiology. The modifications of Tat-BECN1 are discussed in details in OPTIMIZATION AND DEVELOPMENT session; in other sessions, Tat-BECN1 is used to refer to the agent, regardless of being the original or modified version.

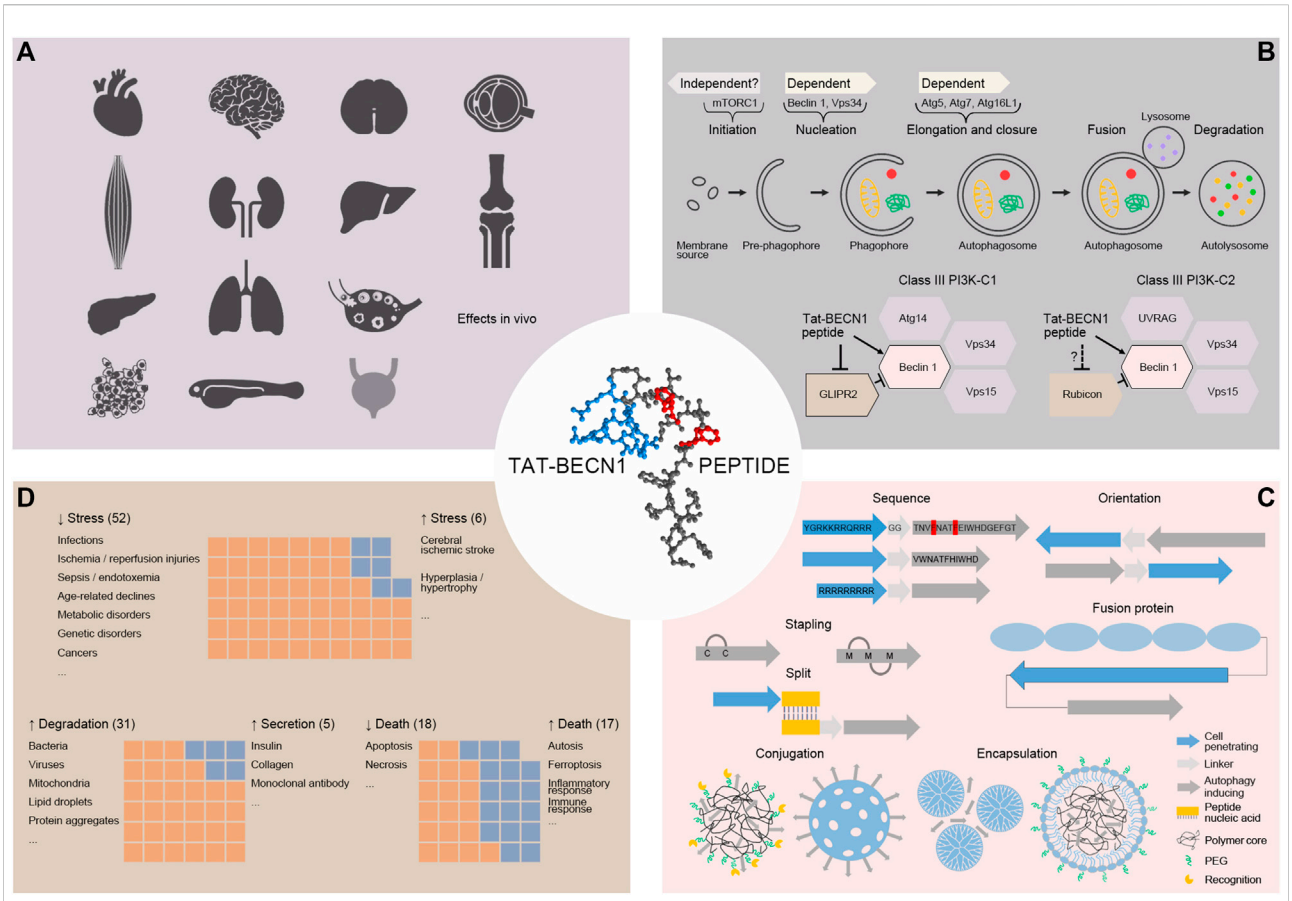

FIGURE 1
(A). Autophagy induction by Tat-BECN1 peptide *in vivo*. In rodent heart, skeletal muscle, pancreas, brain, spinal cord, eye, kidney, liver, lung, bone, articular cartilage, ovary, cancer, as well as zebrafish embryo, Tat-BECN1 is reported to induce autophagy with experimental validation. For bladder (light grey), Tat-BECN1 induces effects but autophagy induction validation is not available. **(B)**. Molecular mechanisms of Tat-BECN1-induced autophagy. Tat-BECN1 functions in an autophagy gene-dependent and likely mTORC1-independent manner. Known protein targets include Beclin 1 in both Class III PI3K-C1 and -C2 complex and GLIPR2, a negative regulator of autophagy, binding to Beclin 1. **(C)**. Optimization of Tat-BECN1. Autophagy inducing element is from Beclin 1 BARA domain β-sheet 1 (18mer or 11mer) and hydrophobic residues in red are required for activity. To achieve cellular delivery, autophagy inducing element is fused or hybridized with cell penetrating element (in blue, Tat, oligoarginines, fusion protein), self-stapled, conjugated (polymer or silica based) or encapsulated (dendrimer based or lipid coated) with nanoparticles. **(D)**. The effects induced by Tat-BECN1 that impact pathophysiology. Graphs represent approximal numbers of studies showing indicated effects, i.e., alleviating stress (52) vs. augmenting stress (6), promoting degradation (31) vs. promoting secretion (5), inhibiting cell death (18) vs. promoting cell death (17).

TAT-BECN1 and mechanisms of action

Tat-BECN1 and modified versions have been employed by around 100 studies and shown robust induction of autophagy in a variety of cellular and organismal models. Tat-BECN1 can be directly applied to cultured cells, isolated tissues and embryos, injected intraperitoneally, intravenously, hippocampally, or infused directly to rodent animals. It is soluble in aqueous solution. Depending on cell or tissue type, the concentration and duration of treatment may vary. Other than the cells reported in the original study (Shoji-Kawata et al., 2013), around 40 cell lines or primary cell cultures respond to Tat-BECN1 for autophagy activation by experimental validation (Table 1). In addition to cardiac muscle, skeletal muscle and pancreas tissues reported in the original study (Shoji-Kawata et al., 2013), rodent heart (Shirakabe et al., 2016; An et al., 2017; Sun et al., 2018; Tong et al., 2019; Nah et al., 2020), brain (intraperitoneal injection (Li et al., 2016; He et al., 2017; Zhang et al., 2017; Forte et al., 2020; Kim et al., 2021), hippocampal injection (Glatigny et al., 2019; De Risi et al., 2020), intravenous injection (Luo et al., 2018), direct infusion (Shehata et al., 2018), intracerebroventricular injection (He et al., 2019)), spinal cord (He et al., 2016), eye (eye drop) (Kasetti et al., 2021), kidney (Livingston et al., 2019; Shi et al., 2020), liver (Soria et al., 2018; Soria et al., 2021), lung (Nikouee et al., 2021), bone

(Cinque et al., 2015; Bartolomeo et al., 2017), articular cartilage (intra-articular injection) (Wang F. et al., 2019; Rockel et al., 2020), ovary (Watanabe et al., 2020), tumor xenografts of breast cancer cells (Wang et al., 2015a; Wang et al., 2015b; Vega-Rubín-De-Celis et al., 2018; Zhou et al., 2019), fibrosarcoma cells (Pietrocola et al., 2016) or ovarian cancer cells (Ding et al., 2018), as well as zebrafish embryos (Zhu et al., 2017), show increased autophagy level upon Tat-BECN1 treatment (Table 1; Figure 1A). Table 1 also summarizes cells and tissues that show responses to Tat-BECN1 while autophagy induction is not tested.

To understand the molecular mechanisms by which Tat-BECN1 exerts its effects will not only help elucidate the functionality of autophagy activation in physiology and pathology, but also shed light on how autophagy activators can be further optimized and designed. Here we discuss the progress of mechanistic studies of Tat-BECN1 (Figure 1B).

Dependency of autophagy genes

Tat-BECN1-induced autophagy requires essential autophagy genes. Depletion of Beclin 1, Atg5 or Atg7, or inhibition of Class III PI3K complexes Vps34 lipid kinase activity by 3-methyladenine (3-MA) reduces Tat-BECN1-induced autophagy (Shoji-Kawata et al., 2013).

Essential autophagy genes are also required for Tat-BECN1-induced effects. Deletion of Atg5 abolishes the beneficial effects of Tat-BECN1, for instance, antiviral effect in human MDMs and the antibacterial effect in murine BMDMs (Shoji-Kawata et al., 2013), chronological lifespan extension in budding yeast cells (Plummer and Johnson, 2019), suppression of neutrophil infiltration into acutely inflamed mouse tissues (Reglero-Real et al., 2021). Tat-BECN1 mitigates myocardial reperfusion injury in wild type but not in cardiomyocyte-specific Atg7 knockout mice (Xie et al., 2021). Tat-BECN1 selectively eliminates memory $CD4^+$ T cells with latent HIV infection, in an Atg5 and Atg7-dependent manner (Zhang et al., 2019). Tat-BECN1 differentially regulates lipid droplets degradation in macrophages depending on Atg7 and Atg12 (Hadadi-Bechor et al., 2019). Deletion of Atg16L1 decreases Tat-BECN1-induced degradation of mutant huntingtin aggregate in HeLa cells (Pavel et al., 2016). Inhibition of Class III PI3K complexes reverses Tat-BECN1-induced endothelial progenitor cells survival in oxygen and glucose deprivation conditions (Jiang et al., 2020).

Dependency of mTORC1 pathway

Tat-BECN1 is widely regarded as an mTORC1-independent autophagy activator; however, experimental validation is limited. In HCT116 cells, Tat-BECN1 treatment does not affect the phosphorylation status of RPS6, a downstream target of mTORC1 (Andrejeva et al., 2020); in mouse pancreatic α cell line, Tat-BECN1 treatment does not affect the phosphorylation status of mTORC1 substrate RPS6KB1 (Rajak et al., 2021). Compared to inhibition of mTORC1 by rapamycin or Torin, Tat-BECN1 shows distinct effects on endothelial progenitor cells survival (Jiang et al., 2020), on glucagon degradation in pancreatic α cells (Rajak et al., 2021) and on blood glucose level in aged food-restricted mice (Nahata et al., 2021). Moreover, Tat-BECN1 prevents mitochondrial dysfunction and the appearance of markers of muscle fiber denervation caused by prolonged mTORC1 loss (rapamycin-treated Raptor-deleted muscles) (Baraldo et al., 2020), suggesting that Tat-BECN1 is likely to function independent of mTORC1. Extensive examination of the relationship between Tat-BECN1 and mTORC1 pathway is urgently needed.

Protein targets

The cellular targets of Tat-BECN1 remain elusive. Efforts were made to identify proteins interacting with Tat-BECN1 by proteomic screen (Shoji-Kawata et al., 2013). On such list, GLIPR2, an evolutionarily conserved CAP protein superfamily member (Eberle et al., 2002), interacts with Tat-BECN1 and Beclin 1 via the peptide region (amino acids 267-284) (Shoji-Kawata et al., 2013; Li et al., 2017). GLIPR2 sequesters Beclin 1 in the Golgi apparatus; in the presence of Tat-BECN1, Beclin 1 is released from the Golgi pool for activation (Shoji-Kawata et al., 2013). However, GLIPR2 is not the sole target of Tat-BECN1 which can induce autophagy in GLIPR2 KO HeLa cells (Zhao et al., 2021). Other proteins that interact with both Tat-BECN1 and Beclin 1 include endosomal toll-like receptors TLR9 and TLR7 (Liu et al., 2020). Whether they play a role in Tat-BECN1-induced autophagy is yet to be examined.

Structural analyses of Class III PI3K complexes revealed that the first β-sheet of the Beclin 1 BARA (β-α repeated, autophagy-specific) domain is critical for membrane targeting of the complexes because this region (amino acids 265-287), which Tat-BECN1 falls into, is more accessible when the complexes are associated with liposomes in hydrogen deuterium exchange (HDX) assays (Rostislavleva et al., 2015; Ohashi et al., 2020). Two separate studies demonstrated that Tat-BECN1 increases Class III PI3K complexes kinase activity as well as membrane association on giant liposomes by fluorescence microscopy (Chang et al., 2019b) or on small liposomes by mass spectrometry and biochemical analyses (Zhao et al., 2021). The effects of Tat-BECN1 on Class III PI3K complexes require the hydrophobic residues (F270 and F274) (Chang et al., 2019b; Zhao et al., 2021), which are also required for autophagy induction (Shoji-Kawata et al., 2013; Peraro et al., 2017). Thus, Beclin 1 in Class III PI3K complexes is a direct target of Tat-BECN1. A domain-swapping model speculates that Tat-BECN1 competes with Beclin 1 BARA β-sheet 1 in binding with the rest of BARA domain and promotes β-sheet 1 for membrane association (Chang et al., 2019a).

Of note, as Tat-BECN1 targets Beclin 1, it may affect autophagy-independent Beclin 1 and/or Class III PI3K complexes functions, such as endocytosis. At a concentration which is not sufficient to induce autophagy, Tat-BECN1 promotes the endocytosis and degradation of tight junction protein occludin in an Atg16L1-indepentdent manner, and increases tight junction barrier permeability in human intestinal epithelial cells or mouse colons (Wong et al., 2019). At low dose, Tat-BECN1 increases the transduction of HIV-1-derived lentiviral vectors to human colorectal carcinoma cell line HCT116, and human hematopoietic stem and progenitor cells hCD34+ by enhancing viral fusion, in a Class III PI3K complex activity-independent manner (Majdoul et al., 2017). Therefore, before using Tat-BECN1 as an autophagy activator to assess the effect of autophagy induction on certain biological process, the treatment conditions shall be carefully evaluated and the dependency of autophagy genes shall be tested. Tat-BECN1 can also be further optimized to trigger autophagy-independent activities of Beclin 1 or Class III PI3K complexes.

Optimization and development

On-going optimization and development of Tat-BECN1 aims to improve activity, solubility, membrane permeability and stability, as well as targeted delivery (Figure 1C).

Optimization on Tat-BECN1

The original version of Tat-BECN1 consists of an HIV-1 Tat protein transduction domain amino acids 47–57 (YGRKKRRQRRR), a diglycine linker (GG) and a modified human Beclin 1 BARA β-sheet 1 (TNVFNATFEIWHDGEFGT); the H275E, S279D and Q281E substitutions from Beclin 1 amino acids 267–284 (TNVFNATFHIWHSGQFGT) are designed to enhance solubility (Shoji-Kawata et al., 2013). A later version of Tat-BECN1 is 7 amino acids shorter (also referred to as Tat-11mer, YGRKKRRQRRR-GG-VWNATFHIWHD) and two-fold more potent than the original version in cultured cells (Peraro et al., 2017). An alanine scan revealed that three residues corresponding to human Beclin 1 F270, F274 and I276 are essential for Tat-BECN1 activity, which are also evolutionarily conserved from yeast, fruit fly to mammals (Shoji-Kawata et al., 2013; Peraro et al., 2017).

Analogs of Tat-BECN1 are active with improved features. The retro-inverso Tat-BECN1 D-amino acid sequence is RRRQRRKKRGY-GG-TGFEGDHWIEFTANFVNT, with higher activity and resistance to proteolytic degradation *in vivo* compared to L-amino acids peptide (Shoji-Kawata et al., 2013). The retro-inverso version of Tat-11mer RRRQRRKKRGY-GG-DHWIHFTANWV, is active *in vivo*, however, less potent than its L-amino acid counterpart (Peraro et al., 2017). Moving Tat to C-terminal (11mer-Tat, VWNATFHIWHD-GG-YGRKKRRQRRR) or replacing Tat with nona-Arginine (Arg9-11mer), greatly increases cell penetration and autophagy-inducing activity (Peraro et al., 2018).

Alternatives to cell-penetrating peptides

Tat and oligoarginines cell-penetrating peptides are highly positive-charged and can interact with negative-charged cellular molecules non-specifically. To minimize the off-target and toxic effects associated with cell-penetrating peptides, different approaches are employed to make Tat-BECN1 Tat-free.

The first strategy involves diversity-oriented stapling, where two cysteines are introduced to the Beclin 1 peptide sequence and cross-linked by thiol bis-alkylation to produce a stapled active peptide DD5-o (Peraro et al., 2017), or three methionine are introduced to the sequence and cross-linked by methionine bis-alkylation to produce a bicyclic active peptide 7f (Qin et al., 2019). Interestingly, although Tat-BECN1 shows as a random coil in circular dichroism, the structure of DD5-o and the activity of retro-inverso variants of Tat-BECN1 suggest helical conformations may be adopted upon action (Peraro et al., 2017). The second strategy is to split Tat-BECN1 based on peptide nucleic acids (PNAs), where the cell-penetrating peptide and Beclin 1 peptide are conjugated to two complementary PNAs, respectively; the two PNA-peptides form hybrid to enter the cells and partially dissociate to release Beclin 1 peptide (Hakata et al., 2020). The PNA1-Arg8/PNA2-Beclin 1 peptide hybrid is more active in autophagy induction compared to Arg8-Beclin 1, without causing cytotoxicity (Hakata et al., 2020). The third strategy takes advantage of the formation of nanoparticles with Beclin 1 peptide conjugated or encapsulated. For instance, Beclin 1 peptide can be fused to amphiphilic poly (β-amino ester) (Wang et al., 2015a), cationic chitosan (Luo et al., 2018), or photothermal agent polydopamine (Zhou et al., 2019) to assemble into nanoparticles. Other elements can be introduced simultaneously to facilitate targeted delivery, which will be discussed in the next session. Beclin 1 peptide can be directly packaged into metal (Mn^{2+})-terpyridine based coordinative dendrimer and released after cellular internalization with high efficiency compared to 11mer-Tat (Ren et al., 2022). The last strategy develops soluble Beclin 1 peptide-containing fusion proteins, where thioredoxin (Trx) confers solubility and thermal stability, pH low insertion peptide (pHLIP) triggers the fusion protein translocation across the plasma membrane in a tumor acidic environment (Ding et al., 2018).

Targeted delivery of beclin 1 peptide

Tat-BECN1 shows punctate subcellular localization, which is not seen for F270 mutant (Shoji-Kawata et al., 2013). However, it is not fully characterized what organelles Tat-BECN1 is localized to. Engineering on Tat-BECN1 may facilitate specific organelle-targeting. Tat-BECN1 is grafted onto mesoporous silica

nanoparticles (MSNs, around 72 nm in diameter) for perinuclear ER-targeted delivery; when such MSNs are loaded with brefeldin A, ER-phagy is induced (Wang et al., 2018). The pH-sensitive poly (β-amino ester) conjugated Beclin 1 peptides self-assemble into micelle-like nanoparticles (P-Bec1) and accumulate in lysosomes after cellular uptake; P-Bec1 causes lysosome damage and cell death in breast cancer cells (Wang et al., 2015a). Whether the observed cell death is due to autophagy induction or lysosome impairment is yet to be determined. Injected P-Bec1 effectively accumulates in MCF-7 tumor xenografts and inhibits tumor growth, without affecting normal tissues (Wang et al., 2015a).

Cell type-specific or preferential delivery of Beclin 1 peptide can be achieved. As tumor tissues are weakly acidic, a fusion protein Trx-pHLIP-Beclin 1 is designed to specifically accumulate in weakly acidic conditions (pH 6.5); Trx-pHLIP-Beclin 1 induces autophagic cell death in breast and ovarian cancer cells and suppresses growth of ovarian cancer xenografts, without causing systemic toxicity (Ding et al., 2018). As integrin αvβ3-overexpressing cancer cells recognize RGD sequence, PPBR nanoparticles, composed of polydopamine nanoparticle conjugated with Beclin 1 peptide, polyethylene glycol (PEG) and cyclic RGD, improves photothermal killing efficacy in breast cancer cells in an autophagy-dependent manner; PPBR nanoparticles suppresses the growth of near-infrared irradiated breast cancer xenografts more efficiently than nanoparticles without RGD (Zhou et al., 2019). To capture extracellular amyloid β-peptide (Aβ) for autophagic clearance, a self-destructive nanosweeper is designed with cationic chitosan core, Beclin 1 peptide and PEG conjugated KLVFF sequence that recognizes and co-assembles with Aβ; the nanosweeper reduces Aβ-induced cytotoxicity in mouse neuroblastoma cells, clears Aβ in the brain of Alzheimer's disease mouse model and rescues memory deficits (Luo et al., 2018). Tat-BECN1 encapsulated and lipid-coated hybrid PLGA (poly lactic-co-glycolic acid) nanoparticles are found to preferentially induce autosis in memory $CD4^+$ T cells with latent HIV infection but not in uninfected cells (Zhang et al., 2019); the molecular mechanisms underlying the specificity of such nanoparticles are yet to be explored.

Impact of TAT-BECN1 on pathophysiology

As discussed above, Tat-BECN1 is widely tested as an autophagy activator for basic and translational research (Table 1), and shows great therapeutic potential in cancers, neurodegenerative diseases, infectious diseases, injury recoveries, aging and so on. Here we try to compare the effects of Tat-BECN1 that impact pathophysiology from three aspects, through mediating autophagic degradation or autophagy-dependent secretion, through promoting autophagic cell death or preventing apoptotic cell death, and through alleviating stress or augmenting stress (Figure 1D), in order to offer a comprehensive view of Tat-BECN1 application across different pathophysiological models.

Effects of Tat-BECN1 on degradation

Tat-BECN1 can exert effects by inducing autophagic degradation, including general autophagy and selective autophagy (i.e., xenophagy, virophagy, mitophagy, lipophagy, ER-phagy and aggrephagy). Tat-BECN1 enhances bacterial clearance, during uropathogenic *E. coli* infection in mouse bladder (Miao et al., 2015), *M. tuberculosis* infection in mouse bone marrow-derived macrophages (BMDMs) (Franco et al., 2017), *K. pneumoniae* infection in mouse lungs (Nikouee et al., 2021) and opportunistic infection of *M. tuberculosis* or *M. avium* in HIV-1-infected human macrophages (Sharma et al., 2021). Tat-BECN1 protects against viral infections, by inhibiting HIV replication in human monocyte-derived macrophages (MDMs) (Zhang et al., 2018) and memory $CD4^+$ T cells (Zhang et al., 2019), and by inhibiting Middle East respiratory syndrome coronavirus (MERS-CoV) replication in VeroB4 cells (Gassen et al., 2019). Tat-BECN1 activates mitophagy in rodent heart and protects against pressure-overload-induced heart failure (Shirakabe et al., 2016) and high fat diet-induced diabetic cardiomyopathy (Tong et al., 2019), in kidney and protects against renal ischemia/reperfusion injury (Livingston et al., 2016; Livingston et al., 2019), in brain and kidney and prevents high salt diet-induced hypertension-related stroke occurrence (Forte et al., 2020); it prevents mitochondrial dysfunction in skeletal muscles and the appearance of markers of muscle fiber denervation caused by prolonged mTORC1 loss (Baraldo et al., 2020). Tat-BECN1 induces lipophagy of acetylated low density lipoprotein-induced lipid droplets in macrophages; interestingly, it increases the biogenesis of oleic acid-induced lipid droplets (Hadadi-Bechor et al., 2019). Tat-BECN1 triggers degradation of amyloid fibrils in Alzheimer's disease (Luo et al., 2018) and middle-aged (De Risi et al., 2020) brains and rescues memory deficits, removes ubiquitinated protein aggregates induced by WNV infection (Kobayashi et al., 2020), relieves abnormal cytosolic and nuclear glycogen storage in liver with distal urea cycle disorders (Soria et al., 2021), clears glaucoma-causing mutant myocilin and reduces elevated intraocular pressure (Kasetti et al., 2021), enhances FAM134B-mediated ER-phagy of misfolded procollagen in chondrocytes (Forrester et al., 2019). Other than protein quality control, Tat-BECN1 promotes degradation of synaptic proteins and erasure of reconsolidation-resistant fear memories (Shehata et al., 2018), stabilizes microtubules in neurons by targeting microtubule-destabilizing protein and accelerates axon regeneration after spinal cord injury (He et al., 2016), lowers cardiac lipoprotein lipase and triglyceride accumulation and restores cardiac function in obese mice (An et al., 2017), increases ureagenesis in liver by providing key urea-cycle intermediates and improves ammonia clearance in hyperammonemia model (Soria et al., 2018), reverts the accumulation of oxidized proteins like CaMKII and sinus node dysfunction induced by insulin sensitizers (Woo and Kim, 2021), downregulates β-catenin pathway and regulates

chondrogenic fate specification of cranial neural crest cells (Yang et al., 2021) while downregulates Notch1 which counteracts β-catenin in bone marrow stromal cells (Choi et al., 2018), decreases the level of pluripotency factors POU5F1, NANOG and SOX2 in cancer stem cells (Sharif et al., 2017), decreases fibrotic markers and inhibits lung fibroblast to myofibroblast differentiation (Sosulski et al., 2015).

Emerging evidence indicates that autophagy pathway regulates secretion of specific cargos instead of lysosomal degradation (New and Thomas, 2019). Tat-BECN1 increases the release of insulin in murine and human primary islets (Goginashvili et al., 2015) (Tat-BECN1 is also reported to reduce adenosine-stimulated insulin secretion in islets (Israeli et al., 2018)), the secretion of candidate protein biomarkers of tumor cell autophagy (Kraya et al., 2015), and the production of monoclonal antibody in CHO cells (Braasch et al., 2021). Tat-BECN1 restores collagen secretion from chondrocytes to extracellular matrix and rescues bone growth in mice with lysosomal storage disorders (Bartolomeo et al., 2017) or mice with FGF signaling defects (Cinque et al., 2015). Tat-BECN1 attenuates extracellular matrix degradation in osteoarthritis chondrocytes (Wang F. et al., 2019; Wu et al., 2020) and ameliorates cartilage degeneration in rodent osteoarthritis model (Wang F. et al., 2019). Future work is needed to elucidate what mechanisms determine the destination of such cargos.

Effects of Tat-BECN1 on cell death

Prolonged Tat-BECN1 treatment leads to autosis, an autophagy-dependent, apoptosis and necrosis-independent cell death, mediated by Na^+/K^+ ATPase pump (Liu et al., 2013; Fernández et al., 2020). Tat-BECN1 induces autosis in HIV-infected human MDMs (Zhang et al., 2018) and memory $CD4^+$ T cells (Zhang et al., 2019), but not in uninfected counterparts; it sensitizes primary renal tubular cells to TGFβ1-induced non-apoptotic cell death (Livingston et al., 2016). Tat-BECN1 has been assessed for systemic toxicity *in vivo*, which is negligible (Shoji-Kawata et al., 2013; Wang et al., 2015a; Ding et al., 2018). However, it exhibits cytotoxicity in cancer cells (Wang et al., 2015a; Ding et al., 2018; Song et al., 2018; Zhou et al., 2019) and cancer stem-like cells (Sharif et al., 2017; Sharif et al., 2019), reduces the growth of tumor xenografts *in vivo* (Wang et al., 2015a; Ding et al., 2018; Song et al., 2018; Vega-Rubín-De-Celis et al., 2018; Zhou et al., 2019). Tat-BECN1 worsens the brain damage after cerebral ischemic stroke (He et al., 2017; Zhang et al., 2017; He et al., 2019) and abolishes the neuroprotective effects of such agents as ganglioside GM1 (Li et al., 2016), puerarin (He et al., 2017) and breviscapine (Zhang et al., 2017), likely due to aggravating inflammatory responses (He et al., 2019). What confer the sensitivity of HIV-infected cells, cancer cells and ischemic neurons to Tat-BECN1-induced cytotoxicity? How does Tat-BECN1 affect tumor growth, by autosis, ferroptosis (Song et al., 2018) or T cell-mediated immune response (Pietrocola et al., 2016)? Can Tat-BECN1 be modified for targeted delivery? Future research will be required to address these questions.

When autosis is already induced in cardiomyocytes during ischemia/reperfusion, Tat-BECN1 exacerbates myocardial injury (Nah et al., 2020); while administration of Tat-BECN1 prior to ischemia/reperfusion (Nah et al., 2020) or at the time of reperfusion (Xie et al., 2021) reduces cell death and protects cardiac function. This suggests that the timing of intervention is important; autophagy activation at early stage of a developing condition may be beneficial. Tat-BECN1 inhibits apoptotic cell death in many *in vitro* or *in vivo* models: during renal ischemia/reperfusion, pretreatment with Tat-BECN1 inhibits apoptosis and prevents renal injury (Livingston et al., 2019); under oxygen glucose deprivation, the *in vitro* model of ischemia, Tat-BECN1 decreases apoptosis of endothelial progenitor cells (Jiang et al., 2020) and retinal neurons (Mathew et al., 2021); Tat-BECN1 blocks cardiomyocytes apoptosis under pressure overload (Shirakabe et al., 2016); it also alleviates cisplatin (a cancer drug known for nephrotoxicity)-induced cell death in renal proximal tubule cells (Wang S. et al., 2021) or PKM2 silence-induced apoptosis in acute myeloid leukemia cell line with mutated nucleophosmin (Wang L. et al., 2019). Tat-BECN1 not only reduces apoptosis but enhances proliferation in synovial intima cells leading to synovial hyperplasia in mouse knee joints (Rockel et al., 2020); it augments cardiac hypertrophy in autosomal dominant polycystic kidney disease mouse model (Atwood et al., 2020). Tat-BECN1 attenuates apoptosis and necrosis, in brain endothelial cells and renal epithelial cells exposed to high salt (Forte et al., 2021), in human neuroblastoma cells infected with WNV (Kobayashi et al., 2020). Tat-BECN1 preserves brain structures and sensorimotor functions after neonatal hypoxic-ischemic injury (Kim et al., 2021), protects renal proximal tubule cells against phosphotoxicity induced by high phosphate and increases urinary phosphate excretion in mice (Shi et al., 2020), rescues Aldehyde dehydrogenase 5a1-deficient mice from premature lethality (Vogel et al., 2016), increases survival rate of zebrafish embryos with anthracycline-induced cardiotoxicity (Wang Y. et al., 2021). Administration of Tat-BECN1 at neonatal stage upregulates the number of primordial follicles even at middle-age and extends the fertility and reproductive lifespan of female mice (Watanabe et al., 2020).

Effects of Tat-BECN1 on stress

Autophagy is a major pathway to respond to stress and maintain homeostasis. In most examples discussed in the sessions above, Tat-BECN1 exhibits beneficial effects to cells

or organisms under stress conditions, like protections against bacterial and viral infections, recoveries from injuries, detoxication of aberrant metabolism. A few exceptions include that Tat-BECN1 increases unexpected synovial hyperplasia (Rockel et al., 2020) and cardiac hypertrophy (Atwood et al., 2020); it intensifies brain damage after cerebral ischemic stroke (Li et al., 2016; He et al., 2017; Zhang et al., 2017; He et al., 2019). On the contrary, in neonatal hypoxia-ischemia model (Kim et al., 2021) or high salt diet-induced hypertension-related stroke model (Forte et al., 2020), Tat-BECN1 prevents brain damage; it also improves the formation of long-term spatial memory (Hylin et al., 2018) or novel memory (Glatigny et al., 2019) in young rodents, rescues age-related memory decline in middle-aged mice (Glatigny et al., 2019; De Risi et al., 2020). How brain tissues respond to Tat-BECN1 treatment in different models shall be further investigated.

In addition to the abovementioned scenarios, during endotoxemia induced by lipopolysaccharide (LPS) or sepsis induced by pneumonia, Tat-BECN1 alleviates inflammation and reduces injuries in heart (Sun et al., 2018; Sun et al., 2021) and lung (Nikouee et al., 2021); it suppresses neutrophil infiltration into acutely inflamed mouse tissues (Reglero-Real et al., 2021). In undernourished aged mice, Tat-BECN1 decreases the plasma levels of the glucogenic amino acid and restores the blood glucose levels to maintain energy homeostasis (Nahata et al., 2021), in line with the beneficial effects of Tat-BECN1 in age-related decline in memory (Glatigny et al., 2019; De Risi et al., 2020) or fertility (Watanabe et al., 2020). Tat-BECN1 mediates cell differentiation and fate determination (Sosulski et al., 2015; Sharif et al., 2017; Choi et al., 2018; Yang et al., 2021). It is reported to affect the length of primary cilia; however, the effect is controversial (Wang S. et al., 2015; Iaconis et al., 2020; Wang S. et al., 2021).

Discussion

As discussed above, Tat-BECN1 is a potent autophagy activator and a powerful tool to investigate the role of autophagy in various cells and tissues; optimal treatment conditions, especially timing, concentration/dosage and duration, shall be tested in cells or tissues of interest beforehand. Tat-BECN1 is likely to function independent of mTORC1 and a side-by-side comparison of Tat-BECN1 and mTORC1 inhibitors is recommended.

A thorough understanding of the molecular mechanisms underlying Tat-BECN1-induced autophagy is of importance. So far, GLIPR2 (Shoji-Kawata et al., 2013; Zhao et al., 2021) and Beclin 1 in Class III PI3K complexes (Chang et al., 2019b; Zhao et al., 2021) are experimentally confirmed Tat-BECN1 targets. GLIPR2 is a negative regulator of autophagy interacting with Tat-BECN1 and Beclin 1 via BARA domain β-sheet 1 (Shoji-Kawata et al., 2013; Li et al., 2017; Zhao et al., 2021); it will be interesting to test whether Rubicon, a well-known negative regulator of autophagy, which also interacts with Beclin 1 via BARA domain β-sheet 1 (Chang et al., 2019b), is another target of Tat-BECN1. Class III PI3K-C1 (autophagy initiation) and -C2 (autophagosome maturation, endocytosis, LAP) complexes (Levine et al., 2015; Hurley and Young, 2017) are not discerned by Tat-BECN1 for activation, at least *in vitro* (Chang et al., 2019b), which may explain why Tat-BECN1 triggers autophagy-independent effect such as endocytosis (Wong et al., 2019). Further investigation on Class III PI3K complexes and regulators may facilitate the design of Class III PI3K-C1 or -C2-specific Tat-BECN1.

Advancement in chemical biology and materials science will accelerate the optimization and development of Tat-BECN1 with better bioactivity and efficacy. Additional work is required to determine long-term and systemic effects of Tat-BECN1 treatment *in vivo*, before moving forward to test Tat-BECN1 as novel clinical therapeutics.

Author contributions

YH and HL consulted the literature and drafted the table and manuscript. YZ conceived the review, consulted the literature and wrote the final paper with the feedback from all authors.

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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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