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Cardiac ubiquitin ligases: Their role in cardiac metabolism, autophagy, cardioprotection and therapeutic potential☆

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ABSTRACT

Both the ubiquitin-proteasome system (UPS) and the lysosomal autophagy system have emerged as complementary key players responsible for the turnover of cellular proteins. The regulation of protein turnover is critical to cardiomyocytes as post-mitotic cells with very limited regenerative capacity. In this focused review, we describe the emerging interface between the UPS and autophagy, with E3's regulating autophagy at two critical points through multiple mechanisms. Moreover, we discuss recent insights in how both the UPS and autophagy can alter metabolism at various levels, to present new ways to think about therapeutically regulating autophagy in a focused manner to optimize disease-specific cardioprotection, without harming the overall homeostasis of protein quality control. This article is part of a Special Issue entitled: The role of post-translational protein modifications on heart and vascular metabolism edited by Jason R.B. Dyck & Jan F.C. Glatz.

1. Introduction

There is growing recognition of the role that protein quality control systems play in the maintenance of the heart during health and disease. These include the ubiquitin-proteasome system (UPS), which is responsible for the turnover of many cellular proteins at the molecular level, with the complementary lysosomal autophagy system clearing dysfunctional organelles (e.g. mitochondria), damaged macromolecules, and larger aggregate-prone proteins (pre-amyloid oligomers) [1,2]. With a greater understanding of the diverse role that the UPS and autophagy play in the heart, we have identified new and novel links with metabolism that build upon simpler first constructs. These findings provide additional ways in which cardiac metabolism may be regulated therapeutically through the manipulation of specific ubiquitin ligase activities, or more broadly in disease context-specific short-term regulation of autophagy.

1.1. Why does protein quality control matter?

Mechanisms that regulate protein turnover and prevent protein aggregation either through refolding and/or degradation are critical to the heart in ways that differ from most cells in the body. This is because the cardiomyocytes, like neurons, are post-mitotic cells with very limited regenerative capacity, in contrast to skin or gut epithelial cells [3]. Evolutionarily, the autophagic removal of damaged organelles [4,5] and misfolded proteins by the ubiquitin-proteasome system [6] allows maintenance of cardiac function. Recent studies illustrate that the regulation of these two systems additionally controls cellular metabolism [7–10]. As the mechanisms that link these two systems with metabolism become clearer, opportunities to intervene and protect the heart in disease may more obvious.

2. Metabolism and autophagy

Due to the high energy demands of the heart, the ability to extract energy (i.e. produce ATP) from variable sources is critical. One contributing factor may be the limited energy reserves available to the heart [11]. The

Abbreviations: ATG, autophagy-related gene; AMPK, AMP activated protein kinase; BECN1, beclin1; b-TrCP, beta-transducin repeat-containing protein; Cbl, Casitas B-lineage lymphoma; CHIP, Carboxyl terminus of Hsc70 interacting protein; CHMP2B, charged multivesicular body protein 2B; DEPTOR, DEP-domain-containing mTOR-interacting protein; DFCP1, double FYVE-containing protein 1; HDAC, histone deacetylases; I/R, ischemia/reperfusion; mTOR, mammalian target of rapamycin; MuRF1, muscle ring finger-1; NEDD4, neural precursor cell-expressed developmentally; RNF, RING finger protein; SILAC, stable isotope labeling of amino acids in cell culture; TRAF6, TNF-receptor-associated factor 6; TAC, trans-aortic constriction; TRIM, Tripartite motif-containing protein; UPS, ubiquitin proteasome system; VSP34, vacuolar sorting protein 34.

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links between autophagy and metabolism are most evident in time of crisis and/or adaptation as seen during mammalian fetal development. For example, at birth, the maternal energy source is interrupted [12], leaving the newborn heart to function without nutrition until milk arrives. The activation of autophagy has been reported in the mouse heart within 30 min after birth, staying elevated for at least 24 h [13]. With maternal delivery of nutrition, autophagy is inhibited by the insulin-mediated downregulation of protein degradation [14].

2.1. Autophagy, amino acids, and ATP in starvation

In the adult heart, starvation-induced autophagy can fuel the heart in multiple ways, including the degradation of nucleic acids, the breakdown of proteins, and the lysosomal digestion of lipids and sugars [15]. In as little as 12 h without nutrition, cardiac autophagy is upregulated; however, the significance of this metabolic source can best be seen when it is blocked [16]. Blocking autophagic activity has been reported to accelerate cell death and reduce cardiac performance while decreasing available amino acids and ATP in the heart [16]. Conversely, stimulating autophagy has been reported to protect against starvation by limiting ATP loss and attenuating ER stress [17].

2.2. Autophagy regulation of glucose and lipid metabolism in the heart

While fatty acid is the substrate utilized most in the adult heart (cardiomyocytes), glucose metabolism has important roles in the heart. For example, the fetal heart primarily utilizes glucose as a substrate for ATP production. Similarly, cardiac stress in the adult heart induces a shift away from fatty acid utilization toward glucose as a source of energy. [11], as is seen in human heart failure [18,19], with regulation attributed to inhibition of PPARα activity [20], HIF-1α, and other transcription factors (discussed in more detail next). In parallel, the mammalian target of rapamycin (mTOR) regulates autophagy in the failing heart [21]. During nutrient deprivation, autophagy activity is increased by at least two mechanisms, including AMP activated protein kinase (AMPK) mediated activation of autophagy-initiating kinase Ulk1 [22] and by upregulation of the hexokinase-II enzyme, a glycolytic enzyme that protects against starvation by inhibiting mTORC1 [23]. Interestingly, impaired lipid degradation by autophagy (a process termed lipophagy) has been reported to contribute to the accumulation of toxic lipids [24]. In the context of high-fat diet induced accumulation of lipids, the proper autophagic clearance of lipids has proven important in attenuating reactive oxygen species [25–27]. The attenuation of the lipotoxic cardiomyopathy in pressure overload-induced heart failure [28] similarly demonstrates the importance of autophagy in clearing lipids in cardiac stress, a process regulated by oxidized phospholipids [29].

3. Autophagy in the heart

Autophagy is a highly conserved protein quality control system that catabolizes misfolded and aggregated proteins as well as damaged, worn organelles for recycling and energy homeostasis. Autophagosome nucleation begins with the activation of several autophagy-related gene (ATG) proteins and with the help of VPS34 (class III phosphoinositide 3 kinase vacuolar sorting protein 34) and BECLIN1 (Fig. 1A). A double-membraned isolation membrane elongates and forms around the ubiquitinated substrate of interest (Fig. 1B). Adaptor proteins, like p62 (also known as sequestosome 1), bind ubiquitin and assist with docking substrate cargo inside the phagophore, to later be degraded. Closing and sealing of the membrane completes autophagosome formation (Fig. 1C). Finally, lysosome and autophagosome fuse to form the autolysosome. Acidic hydrolases from the lysosome degrade the contents of the autolysosome (Fig. 1D). The resulting molecular components (carbohydrates, lipids, amino acids, and nucleic acids) are released to support cellular metabolism and homeostasis [30–32]. Autophagic flux refers to the entire process of autophagy, beginning with the formation of an autophagosome around the cargo to be degraded and ending with the release of degraded macromolecules into the cytosol [33].

Under basal conditions or low stress, autophagy occurs at low levels to maintain homeostasis. These low levels of autophagy are critical for cell survival. Cellular homeostasis requires a minimal amount of autophagic flux. Inhibiting autophagy disrupts a cell's homeostasis and can lead to cell death [30]. Alternatively, autophagy responds rapidly to stress, particularly nutrient starvation, which elicits a robust enhancement of autophagic flux to meet the energy needs of the cell [30]. Specifically, cardiac autophagy is required for metabolic adaptation by providing amino acids, glucose, and lipids, as described above.

4. Role of autophagy in common heart diseases

Our understanding of the role of autophagy in cardiac injury has grown tremendously in recent years. It's significance, and context-dependent cardioprotection has recently been reviewed in depth (see Schiattarella and Hill [34]). We briefly summarize the role of autophagy in ischemic heart disease, pathological cardiac hypertrophy, and chemotherapy-induced cardiotoxicity.

4.1. Ischemic heart disease

The number of people living with ischemic heart disease continues to increase; in fact, it was the worldwide leading cause of death in 2010 [35]. During ischemic heart disease, narrowed coronary arteries cause a reduction of blood flow to the heart resulting in ischemia. Injury results from both cardiac ischemia and cardiac reperfusion, each triggering stress in distinct ways, however cardiac autophagy has been shown to be cardioprotective during both phases [36–38]. The energy depletion observed during ischemia triggers autophagic mechanisms to replenish metabolic substrates and to remove damaged organelles [39]. Nutrient depletion results in the activation of AMPK (via increased AMP resulting from utilization of ATP), which then phosphorylates (inactivates) mTOR and thus disinhibits autophagy to enhance flux [40,41]. During reperfusion, restoration of oxygen and nutrients lead to the massive production of reactive oxygen species (ROS). As the electron transport chain becomes active in the presence of reestablished oxygen, mitochondrial ROS amplification results from ROS-induced ROS release [42]. Damaged proteins and organelles from lipid peroxidation-driven ROS enhances autophagy to clear damaged organelles that would further increase oxidative stress and cellular dysfunction [42].

Interestingly, there have been reports for and against the benefits of autophagy during ischemia and reperfusion. Some reports show that enhances in autophagy are cardioprotective during ischemia/reperfusion (I/R) and serve to salvage the myocardium [43]. Similarly, chronic ischemia in a porcine model demonstrated an association between elevated autophagy and reduced apoptosis [44]. Yet, other reports show that autophagy and cell death correlate and that inhibition of autophagy reduces cell death [45,46]. Interestingly, work by Matsui et al. demonstrates that autophagy may have dual roles and may be protective during ischemia but contributes to cell death during reperfusion [40]. In this study, mouse hearts subjected to ischemia resulted in the induction of enhanced autophagy in an AMPK-dependent and cardioprotective manner, but mouse hearts subjected to I/R showed beclin1-dependent upregulation of autophagy that was not beneficial to the heart [40]. These data are confusing, making inferences of the benefits of autophagy during ischemia and reperfusion difficult. The field of autophagy research is young and differing results are likely a result of different models, mechanisms, and tools used to measure and induce or inhibit autophagy at different times during the I/R continuum. At this time, it appears that autophagy may provide differing levels of cardioprotection during the ischemia and reperfusion phases: autophagy during I/R can afford cardioprotective effects by providing ATP during the ischemia

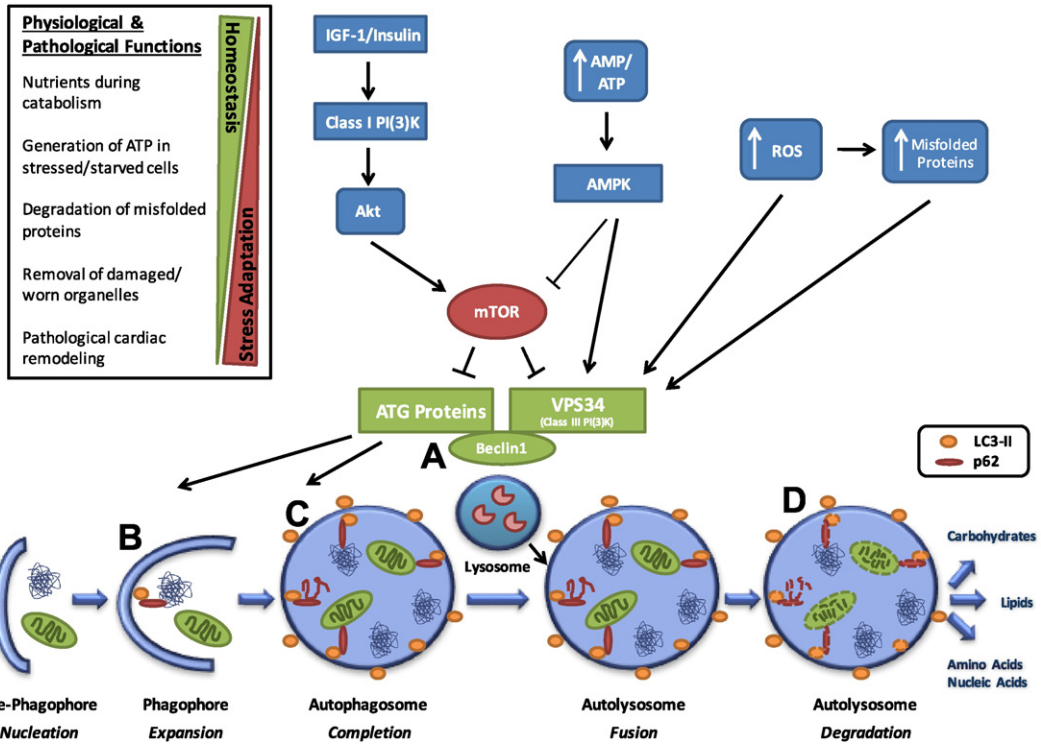

Fig. 1. Overview of autophagy regulation and relationship with metabolic adaptation. Autophagy is a protein quality control system that catabolizes misfolded and aggregated proteins as well as damaged, worn organelles for recycling and energy homeostasis. Growth signaling through IGF-1/Akt/mTOR and high levels of ATP inhibit autophagy. Levels of low energy (increased AMP/ATP), high levels of ROS, and misfolded proteins increase autophagy through direct activation of VPS34, beclin1, or ATG proteins, or through the disinhibition of mTOR. Autophagosome nucleation begins with the activation of several autophagy-related gene (ATG) proteins, with the help of VPS34 and BECLIN1. A double-membraned isolation membrane elongates and forms around the ubiquitinated substrate of interest. Adaptor proteins, like p62 (also known as sequestosome 1), bind ubiquitin and interact with LC3-II to assist with docking substrate cargo inside the phagophore to be degraded. Closing and sealing of the membrane completes autophagosome formation. Finally, lysosome and autophagosome fuse to form the autolysosome. Acidic hydrolases from the lysosome degrade the contents of the autolysosome. The resulting molecular components (carbohydrates, lipids, amino acids, and nucleic acids) are released to support cellular metabolism and homeostasis. ***INSERT: Basal levels of autophagy are necessary to maintain homeostasis.*** As autophagy increases in response to stress, autophagic flux may reach maladaptive levels, assisting in pathological cardiac remodeling. Abbreviations: IGF-1, insulin like growth factor-1; PI(3)K, phosphoinositol-2-kinase; Akt, protein kinase A; AMP, adenosine monophosphate; ATP, adenosine triphosphate; ROS, reactive oxygen species; AMPK, AMP activated kinase; mTOR, mammalian target of rapamycin; ATG, autophagy related genes; VPS34, class III phosphoinositide 3 kinase vacuolar sorting protein 34.

phase and clearing ROS-induced damage during reperfusion. The current consensus is that disease, such as ischemic heart disease can result in maladaptive autophagy (too much or too little, Fig. 2A) and that this may be directed by differing signaling pathways during ischemia (AMPK-mediated) and reperfusion (beclin1-mediated). Research should focus on modulating the delicate balance of autophagy during the transient nature of this disease aims to harness the benefits of autophagy during I/R.

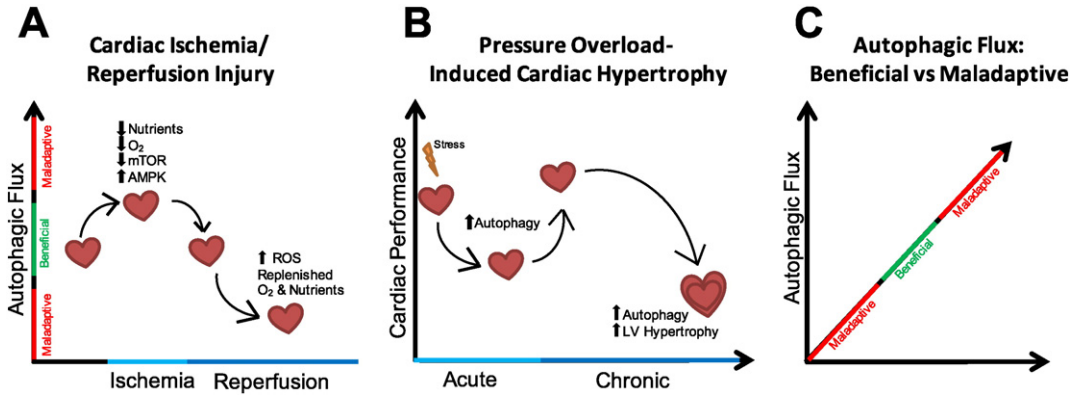

Fig. 2. The role beneficial versus maladaptive role of autophagy during cardiac I/R and pressure overload-induced hypertrophy. A) During ischemia, cardiac autophagy is increased in response to low nutrients and oxygen in order to provide ATP during "starvation". During reperfusion, cardiac autophagy may decrease in response to the reestablishment of oxygen and nutrients. This reestablishment of myocardial blood flow results in increased ROS, leading to lipid peroxidation and damaged organelles and proteins. B) During acute cardiac pressure overload, cardiac performance decreases. Acute increases in autophagy improve cardiac performance. But over time, with chronic pressure overload and chronically increased autophagy, autophagic flux becomes maladaptive, assisting in pathological cardiac hypertrophy and remodeling, resulting in a decline in cardiac performance and heart failure. C) Basal levels of autophagy are critical to maintain cardiomyocyte homeostasis. If autophagic flux drops too low, worn and damaged proteins fail to be cleared and cellular functions suffer. If autophagic flux increases too much, the cell suffers excessive protein and organelle breakdown. Both cases (too low or too high) can lead to cell death and cardiac dysfunction. Adapted from Schiattarella and Hill [30]. Abbreviations: LV, left ventricular.

4.2. Autophagy in pathological cardiac hypertrophy

Hypertension, aortic stenosis, and other disease-related stresses induce pathological cardiac hypertrophy, which in time progresses to heart failure. Pre-clinical models of pressure overload-induced cardiac hypertrophy demonstrate that autophagic flux increases correlate with the degree of hypertrophy [47]. Repression of autophagy accelerated the progression of cardiac hypertrophy [48], while therapeutic inhibition of autophagy reduced the amount of fibrosis seen [49]. Genetically increasing autophagic flux (e.g. by cardiac-specific increased beclin-1 expression) resulted in an amplified hypertrophy, while decreased autophagy (via beclin1 ±) and inducing pressure-overload induced hypertrophy attenuated heart failure progression [47]. Recent studies have demonstrated the importance of protein acetylation in regulating autophagy and shown that inhibiting the additional autophagy induced by pressure-overload prevents an acceleration of maladaptive responses and progression to heart failure (Fig. 2B) [47,50].

The role of autophagy in protecting the heart against stress is complex, as illustrated in pressure overload-induced cardiac stress. Adaptive responses in the heart require a minimum amount of autophagy [4], but induction of autophagy above this baseline level can be deleterious [51]. Too little autophagy may infer nutrient stress of failure to clear harmful damaged or misfolded proteins and worn organelles. Too much may needlessly catabolize proteins and organelles and thus stress the cell. This obviously depends on the situation of the cell's timely environment. Therefore, therapeutics that aim to harness the vast benefits of autophagy in the heart must modulate autophagy in a time and disease-specific manner (Fig. 2C).

4.3. Autophagy in chemotherapy-induced cardiotoxicity

A common side effect of chemotherapies is cardiac dysfunction, with doxorubicin being the typical offender in patients. Its dose-dependent cardiotoxicity [52,53] may be due to DNA damage, mitochondrial damage, and the accumulation of ROS. Treatment with anthracyclines (e.g. doxorubicin) results in marked enhancement in autophagy contributing to the resulting heart failure [54]. Interestingly, pre-clinical models show that doxorubicin treatment results in an accumulation of late-phase autolysosomes; moreover, reducing autophagy during doxorubicin treatment protects against doxorubicin cardiotoxicity [55], offering a potential therapeutic target for reducing chemotherapy-induced cardiac dysfunction.

5. Therapies targeting cardiac autophagy to promote cardioprotection

The challenge of thinking about autophagy as a therapeutic target are the dual roles autophagy plays in the heart. In ischemia/reperfusion injury, increasing autophagy is cardioprotective. In other contexts, increases in autophagy are beneficial in the initial phases (e.g. pressure overload), but then become detrimental over time. Additionally, the complete inhibition of autophagy is not tolerated well, making the approach to targeting autophagy challenging, and may illustrate both our limited understanding of the underlying molecular regulation of autophagy and also limitations in our therapeutic options currently targeting primarily mTOR.

Several drugs act on the autophagy cascade to regulate its activity, with the cornerstone of control being on the mTOR complex. Rapamycin, an inhibitor of mTORC1, increases autophagic flux in the heart, as do other mTOR-targeted drugs such as Torin1, which inhibits both mTORC1 and mTORC2 to induce autophagy [56]. As a drug used for years to prevent transplant rejection and suppressing smooth muscle proliferation and cell migration (in drug eluting devices for angioplasty) [57,58], rapamycin may have application to ischemic heart disease, although only pre-clinical studies are currently published [59] (see Table 1).

Table 1
Drugs that induce autophagy by inhibiting mTOR.

Drug	Effect on autophagy	Cardiovascular therapeutic action	Reference
Rapamycin	Activate	Suppresses pressure overload-induced cardiac hypertrophy	[58]
		Protects myocardium from I/R injury	[59]
		Improves cardiac function in diabetic mice	[61]
SAHA[a]	Activate	Reduces I/R mediated infarct size	[60]

[a] SAHA, suberoylanilide hydroxamic acid.

5.1. Increasing autophagy for cardioprotection

Mice subjected to one week of trans-aortic constriction (TAC) to induce pressure overload-induced hypertrophy, then given rapamycin for one week (2 mg/kg/day) showed suppressed cardiac pressure overload-induced cardiac hypertrophy and improved left ventricular ejection fraction compared to control mice [60]. Mouse hearts subjected to left coronary artery ligation to simulate I/R injury followed by rapamycin treatment (0.25 mg/kg) exhibited reduced infarct size and improved left ventricular function [61]. In a rabbit I/R model (30 min coronary ligation, 24 h reperfusion), suberoylanilide hydroxamic acid (SAHA) treatment one day before the procedure or SAHA treatment at the time of reperfusion reduced infarct size and partially rescued systolic function [62]. Mechanistically, SAHA's induction of autophagy in the infarct region was found to mediate these cardioprotective effects in vivo [62].

Recent studies investigating the ability of FDA-approved drugs to modulate mTOR-independent autophagy have identified the K + ATP channel opener minoxidil and the G(i) signaling activator clonidine as small molecules that induce autophagy [63]. These drugs revealed an mTOR-independent pathway regulating autophagy, in which cAMP regulates IP3 levels [63]. Other FDA-approved drugs that happen to induce autophagy include statins [64], the L-type Ca^{2+} channel blockers verapamil, nicardipine, nimodipine [65], the anti-diabetic agent metformin [66] and the Class III anti-arrhythmic amiodarone [67].

To date, four drugs developed as histone deacetylases (HDAC) inhibitors have been developed for hematologic cancers (e.g. advanced primary cutaneous T-cell lymphoma [68]), including Vorinostat (aka suberoylanilide hydroxamic acid or SAHA), Romidepsin, Panobinostat, and Belinostat [69]. Pre-clinical studies suggest that atorvastatin reduces pathological left ventricular hypertrophy and remodeling and reduce cardiomyocyte size in spontaneously hypertensive rats compared wildtype controls due in part to statin-mediated increases in autophagy through the Akt/mTOR pathway [70]. Simvastatin treated I/R hearts exhibited reduced infarct size accompanied by increases in autophagy evidenced by suppressed mTOR signaling, increased ULK-1, and increased Parkin-mediated mitophagy [64].

A host of additional therapies have been shown to alter autophagy and protect the heart from I/R injury and to reduce infarct size by upregulating autophagy (Table 2), including anti-microbial agents like sulfaphenazole and chloramphenicol [71,72]. Statins, metformin, resveratrol, minoxidil, clonidine, amiodarone, intermedin similarly demonstrate protection in cardiac ischemia-reperfusion injury, diabetic cardiomyopathy, and doxorubicin-induced cardiotoxicity, as summarized in Table 2.

5.2. Inhibiting autophagy for cardioprotection

In pre-clinical studies, inhibiting autophagy in doxorubicin-induced cardiomyopathy, pathological cardiac hypertrophy, ischemia, and in dilated cardiomyopathy is cardioprotective (Table 3). The PI3K inhibitor 3-methyladenine has a dual role of inhibiting class I and class III PI3K, which inhibits autophagy [73]. In doxorubicin-

Table 2
mTOR-independent drugs that induce autophagy and their cardiovascular therapeutic actions.

Drug	Effect on autophagy	Cardiovascular therapeutic action	Reference
Sulfaphenazole	Activate	Protects myocardium from I/R injury and reduces infarct size	[70]
Chloramphenicol	Activate	Attenuates myocardial I/R injury and reduces infarct size	[71]
Statins	Activate	Reduce infarct size	[63]
		Improves function and reverses pathological remodeling due to hemodynamic stress	[69]
Metformin	Activate	Improves cardiac function in diabetic mice	[72]
Resveratrol	Activate	Reverses pathological remodeling due to myocardial infarction	[73]
		Protects against doxorubicin-induced cardiotoxicity	[74]
		Improves cardiac function in diabetic mice	[75]
Minoxidil	Activate	Vasodilator	[62]
Clonidine	Activate	Antihypertensive	[62]
Amiodarone	Activate	Class III antiarrhythmic	[66]
Intermedin	Activate	Suppresses pressure overload-induced cardiac hypertrophy	[76]

induced cardiomyopathy, 3-methyladenine treatment significantly improved cardiac function and reduced myocardial injury [74]. Preserved cardiac function was attributed to reduced autophagy as measured by beclin1 protein expression and formation of autophagic vacuoles [74]. In pressure overload induced pathological hypertrophy, treatment with the antifungal antibiotic trichostatin A blunted both autophagy and the development of cardiac hypertrophy [75]. The general anesthetic propofol has been shown to down-regulate autophagy, evidenced by reduced LC3-II and beclin1 protein levels as well as increased levels of phosphorylated mTOR [76]. Using propofol to treat myocardial infarction significantly reduced infarct size in response to I/R injury [76]. Lastly, treatment with granulocyte colony-stimulating factor (G-CSF) improved cardiac function and remodeling and reduced myocardial fibrosis in a hamster model of human dilated cardiomyopathy (UM-X7.1), which may be attributed to its ability to reduce autophagy in an Akt-dependent manner [77].

As can be seen by these studies, modulating autophagy in a diseased organ is difficult and likely to have off-target effects that are likely to be harmful. Additionally, it is important to modulate autophagy – not abolish autophagy – since the latter is likely to be toxic to the tissue. Furthermore, autophagy is a dynamic system, and its beneficial effects have been shown to be time-dependent. For these reasons, it is crucial that therapies are created and implemented with these requirements in mind, and it is necessary to target therapies more narrowly in the future.

Table 3
Drugs that inhibit autophagy and their cardiovascular therapeutic actions.

Drug	Effect on autophagy	Cardiovascular therapeutic action	Reference
3-Methyladenine	Inhibit	Inhibits doxorubicin-mediated cell death and autophagy, improves cardiac function	[77]
Trichostatin A	Inhibit	Reverses pathological remodeling induced by pressure-overload (TAC)[a]	[78]
Propofol	Inhibit	Reduces infarct size	[79]
G-CSF[b]	Inhibit	Reduces cardiomyocyte death and autophagy in dilated cardiomyopathy model	[80]

[a] TAC, trans-aortic construction.
[b] G-CSF, granulocyte colony-stimulating factor.

6. Beyond mTOR phosphorylation: taking a mechanistic look at post-translational regulation of autophagy by ubiquitin

Discussion of the regulation of autophagy centers primarily on the global process, with the molecular regulation centering on the mTOR signaling pathway. The availability of therapeutics that act either directly or indirectly on mTOR has made this focus in cardiovascular disease the obvious first choice to move forward as quickly as possible in both pre-clinical and clinical trials [69]. However, the post-translational regulation of autophagy by phosphorylation has been described to both inhibit and promote autophagy and has been recently reviewed [78]. Phosphorylation of the ATG subunits, ULK1, BECLIN1, and LC3 has been shown to inhibit or promote autophagy, dependent upon which phosphorylation site kinases add the phosphate [78]. Briefly, phosphorylation of Atg13 (via PKA/TORC1/Atg1), Atg1 (via PKA/TORC1), Atg9 (via Atg1), the Atg13/ULK1 complex (via ULK1, ATG101, mTOR, Akt, PRKAC, AMPK, RB1CC1), Belcin1 (via AKT, EGFR, DAPK, ROCK1, ULK1, AMPK), LC3 (via PRKAC), and ATG5 (via MAPK14) have been described in yeast and mammalian systems (for more detail, see Xie, et al. [78]). A notable number of additional kinases beyond mTOR can be seen on this list, and may represent other ways to regulate autophagy as indicated in the previous section. We now turn our attention to ubiquitination as another post-translational modification that regulates autophagy.

6.1. The E3 ligases and the UPS

The E1, E2, E3 (ubiquitin ligase), and deubiquitinating enzymes (DUBs) are primary components of the ubiquitin-proteasome system (UPS) that bind specific substrates with ubiquitin moieties to target their fate. The specificity of the placement of a ubiquitin tag on protein substrates comes from the ubiquitin ligases (aka E3), of which there are up to 800+ estimated in the human genome [79]. The UPS is responsible for the turnover of many proteins substrates, both during routine protein turnover or during the clearance of damaged/misfolded protein (see recent reviews [80,81] for more detail). In addition to targeting the degradation of protein substrates, ubiquitination commonly regulates critical cellular processes in non-degradative ways, such as mono-ubiquitination to alter cellular localization or activity [8,82] or by modifying activity (e.g. signaling and transcriptional activity) [83,84]. The mechanistic regulation of autophagy by ubiquitination occurs at two primary points: 1) at the autophagy machinery itself (i.e. ATG subunits); and 2) points involved in facilitating the recruitment of autophagy adapters (e.g. p62, NBR1, HDAC6, Hdp52) [85–88]. In addition to these specific molecular mechanisms acting at these two points, ubiquitination can also regulate autophagy in an indirect manner whereby poly-ubiquitinated protein aggregates are taken away by selective autophagy. In neurodegenerative diseases, such as Parkinson's disease or Alzheimer's disease, poly-ubiquitinated protein aggregates and misfolded soluble amyloid precursor proteins recruit autophagy machinery and activate the process of selective autophagy [89,90].

7. Direct regulation of autophagy by ubiquitination

7.1. Ubiquitin-regulated mTOR activity (via DEPTOR regulation)

The β-TrCP (beta-transducin repeat-containing protein) ubiquitin ligase (E3) specifically ubiquitinates DEPTOR (DEP-domain-containing mTOR-interacting protein), resulting in the disinhibition of mTORC1 (Fig. 3A) [91,92]. β-TrCP apparently recognizes phosphorylated substrates (e.g. phosphorylated DEPTOR) and targets them for degradation thereby acting as a negative feedback loop to counterbalance kinase activity [93]. When HeLa cells (cervical cancer) or a glioblastoma cell line (T98G) are serum refed to induce phosphorylation, β-TrCP has been reported to mediate the ubiquitin-dependent degradation of DEPTOR [94–96]. In this way, the β-TrCP increases mTOR activity resulting in an inhibition of autophagy (Fig. 3A)

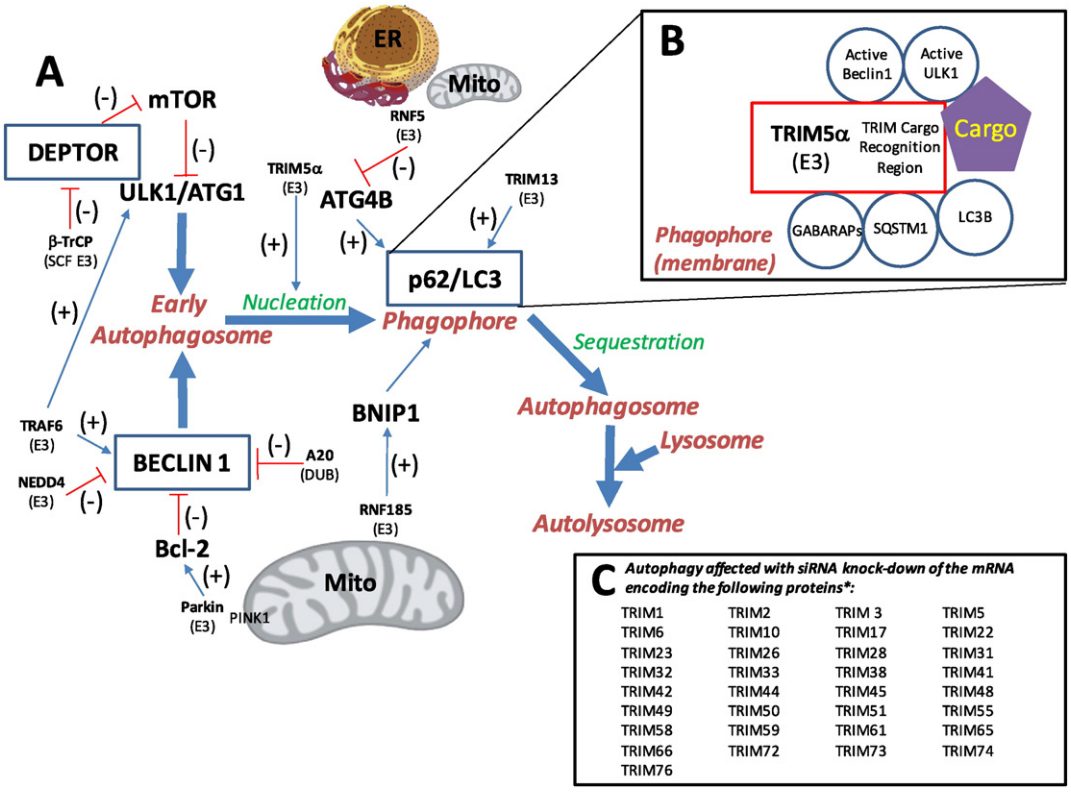

Fig. 3. Over 35 ubiquitin ligases regulate autophagy and fine tune the process by acting at three distinct points, based on mechanisms published to date. Our understanding of their specific targets is just beginning, with the A. E3 designation noting the E3 substrate targeting the A.1) mTOR, 2) BECLIN1, and 3) P62/LC3 (shown in boxes). B. TRIM5 acts as a pattern receongition receptor (PRR) to identify substrates that are then cleared by autophagy (e.g. HIV). C. In siRNA screening of regulators of autophagy, 33 TRIM family proteins were identified that regulate autophagy whose specific molecular regulation of autophagy is currently unknown. Panel A: Adapted from Kuang, et al. [106] and Yamano, et al. [123]. Panels B and C: Data compiled from Kimura et al. [168] and Mandell, et al. [169]. Public domain mitochondria (Mito) and endoplasmic reticulum (ER) from http://www.clker.com/clipart-77,218.html and http://www.earth-site.co.uk/Education/cells-and-cell-structure/#Endoplasmic-Reticulum, respectively. (+) = positive affect on downstream substrate activity; (−) inhibitory affect on downstream substrate activity. **NOTE:** Net effect is the additive regulation of these (+) and (−) activities on the formation of the Early Autophagosome, Phagophore, Autophagosome, and Autolysosome. *Abbreviations*: β-TrCP, beta-transducin repeat-containing protein; MDM2?, mouse double minute 2 homolog; RNF5, RING finger protein 5; RNF185, RING finger protein 185; SCF complex, Skp, Cullin, F-box containing complex; TRIM5, Tripartite motif-containing protein 5; TRIM13, Tripartite motif-containing protein 13; TRAF6, TNF receptor associated factor 6.

[95,96]. Multiple studies have identified DEPTOR in cardiomyocytes, implicating p38 activity in its regulation [97].

7.2. Ubiquitin-regulated BECLIN1 in nucleation

The ubiquitin ligases TRAF6 (TNF-receptor-associated factor 6) and NEDD4 (neural precursor cell-expressed developmentally downregulated 4) are ubiquitin ligases that have been implicated in regulating autophagy nucleation by their regulation of BECLIN1 (Fig. 3A). TRAF6 is associated with toll-like receptors (TLRs) and the IL-1 receptor in activating NF-kB [98,99]. Activation of TLR4, IL-1, or IFN-gamma induce TRAF6-mediated K63 (non-degradative) poly-ubiquitination to inhibit NF-kB activity [100]. In the context of autophagy, TRAF6 similarly poly-ubiquitinates BECLIN1with K63-linked ubiquitination in macrophage [101,102]. The deubiquitinating enzyme (DUB) A20 reported counteracts TRAF6 activity by limiting beclin1 ubiquitination [102]. BECLIN1 interacts with ULK1 in a complex with the AMBRA1 protein and TRAF6 [103]. The activation of autophagy induces ULK1 ubiquitination with K63-linked chains, stabilizing the ULK1 complex in a TRAF6-dependent manner [103]. TRAF6 is expressed in the heart, so TRAF6-mediated alterations in myocardial autophagy may be present.

7.3. Ubiquitin-regulated LC3/p62 in sequestration

The ubiquitin ligase RNF5 (RING finger protein 5) is located in the ER and mitochondrial membranes (Fig. 3A) [104]. The RNF5 E3 associates with and ubiquitinates the membrane-associated ATG4B protease, an enzyme that degrades LC3, to inhibit autophagy (Fig. 3A) [105]. Altering RNF5 expression affects LC3 turnover and the formation of the autophagosome, thus controlling autophagic flux [106]. Mice lacking RNF5 (RNF5 −/−) exhibit enhanced autophagy-mediated clearance of bacteria in macrophage [105]. RNF5 control of ATG4B is mainly seen under basal autophagy conditions, limiting the level of autophagy when not needed [106]. The RNF5 protein is anchored in the ER and has been found in cytoplasmic aggregates in muscle biopsies of patients with sporadic inclusion body myositis (sIBM) [107]. RNF5's role in cardiac autophagy, however, has not been reported.

Like RNF5's indirect regulation of LC3 levels through its interaction with ATG4B, RNF185 (RING Finger protein 185) regulates p62 by ubiquitinating BNIP1 with its critical role in p62 stabilization [108]. While described as a mitochondrial-localized E3, RNF185 mediates the non-degradative K63 ubiquitination of BNIP1 (BCL2/adenovirus E1B 19 kDa interacting protein 1), mediating the recruitment of p62 and LC3 in the formation of the autophagosome (Fig. 3A) [108]. The *RNF185* mRNA is found in most normal tissues in screening assays, including the heart (www.genecards.org), but it's role in the heart has not yet been reported.

The TRIM13 (Tripartite motif-containing protein 13) ubiquitin ligase plays a role in regulating ERAD by ubiquitinating MDM2 (mouse double minute 2 homolog) and Akt to target their proteasome-dependent degradation [109,110]. In the presence of ER Stress, TRIM13 initiates autophagy by interacting with p62 and co-localizing with DFCP1 (double FYVE-containing protein 1) to regulate autophagosome formation (Fig. 3A) [111]. TRIM13 appears to be dispensable in promoting

autophagy, so the details on how it regulates p62 are not known. The *Trim13* mRNA is found in most normal tissues in screening assays, including the heart (www.genecards.org).

Another TRIM protein, TRIM5α, is found in the heart and has demonstrated ubiquitin ligase activity necessary for the HIV-1 replication [112,113]. New studies have identified that TRIM5 directly interacts with p62 and LC3 and play a role in delivering substrates for degradation (Fig. 3B) [114,115]. The hypothesis that the TRIM family of proteins may act as pattern recognition receptors (PRRs) that may play a general role in autophagy has recently been tested [114]. Of the 6 TRIM proteins tested (TRIM5, TRIM6, TRIM17, TRIM22, TRIM49, and TRIM55), all but TRIM55 were in complexes with both ULK1 and BECN1 [114]. TRIM5, TRIM6, TRIM7, and TRIM49 all promoted the formation of multimolecular complexes with ULK1 and BECN1 [114]. Additional screening studies for siRNA regulating autophagy identified that thirty-three (33) TRIM proteins that were involved in autophagy (Fig. 3C).

7.4. Ubiquitin ligases with roles in autophagy independent of their E3 activity (i.e. ubiquitination)

The Cbl (Casitas B-lineage lymphoma) proteins are a highly conserved family of ubiquitin ligases that regulate signaling pathways. In the heart, c-Cbl regulates focal adhesion protein turnover and myofibril degeneration [116]. c-Cbl inhibition improves cardiac function and survival in ischemia [117]. Recent studies in squamous cell carcinoma cell lines have demonstrated Src is degraded by autophagy, using c-Cbl as a cargo receptor for Src (part of the Src-LC3B complex) after the active Src is engulfed in autophagosomes [118,119]. Interestingly, c-Cbl's role in autophagy appears to be independent of its E3 activity; the LIR domain has been found to the be the critical section of c-Cbl necessary for the recruitment of Src to the autophagosomes [120,121]. An image-based genome-wide siRNA screen revealed that Smurf1 (Smad ubiquitylation regulatory factor-1) is a master regulator of viral autophagy and degrades mitochondria (mitophagy) in an E3 activity independent manner [122].

8. Linking ubiquitin, autophagy, and metabolism: selective mitophagy

With the essential role of mitochondria in metabolism to produce ATP, the importance of their damage and function is highlighted in studies; when the strict monitoring of mitochondrial quality control is dampened, a loss of cell homeostasis is seen [123]. The loss of these quality control systems in multiple types of neurodegenerative diseases, including Parkinson's, appears to be part of the disease pathogenesis. Two autosomal recessive forms of Parkinson's disease results from Parkin and PINK1 mutations [123]. These mutations disrupt the essential clearance of damaged mitochondria by autophagic pathways in a process termed "mitophagy". Parkin, a ubiquitin ligase, works in concert with PINK1, a serine/threonine kinase, to identify and remove damaged mitochondria [123].

The Parkin E3 is found primarily in the cytosol [124] and regulates mitochondrial autophagy. When PINK1 protein recognizes damaged mitochondria, Parkin ubiquitinates mitochondrial proteins such as VDAC1, Mfn1, Mfn2, and Bcl-2 to drive their removal via autophagy (Fig. 3A) [106,125,126]. In contrast to Parkin outside of the heart, Parkin-dependent mitophagy in cardiomyocytes does not appear to play a role in the constitutive mitochondrial housekeeping [127]. Increasing cardiomyocyte Parkin activates mitophagy without adverse effects; likewise, deletion of Parkin does not produce a phenotype [128,129]. Instead, PINK1-Parkin mediated mitophagy plays critical roles in the cardiac stress response and has a role in the perinatal transformation of myocardial metabolism [127]. In Parkin −/− mice, normal cardiac function is seen for up to 12 months by echocardiography [130]. However, in response to permanent ligation of the left anterior descending (LAD) coronary artery, Parkin −/− mice had a higher mortality at one week (60% vs. 20% in the wildtype control mice) [130]. Of the surviving mice, severe thinning and dilation were seen histologically, with significantly greater infarct sizes and decreased function 7 days after MI, illustrating the important role of Parkin in the adaptive response to MI [130]. Furthermore, overexpression of Parkin in isolated cardiomyocytes protected against hypoxia-mediated cell death, suggesting that Parkin plays an important role in the heart's ability to adapt to stress through the removal of damaged mitochondria (mitochondrial autophagy, termed mitophagy) [131]. A separate study showed that ablation of Parkin abolished the cardioprotective effects afforded by ischemic preconditioning, a method of cardioprotection that requires enhanced autophagy [132].

9. Muscle-specific ubiquitin ligases: regulation of cardiac mass, metabolism, and autophagy

9.1. Muscle-specific E3s regulating cardiac mass: MuRF1, MuRF2, MuRF3, Atrogin-1

The muscle-specific ubiquitin ligases that have been implicated in the turnover of sarcomere proteins include the muscle ring finger (MuRF) family proteins and Atrogin-1 (as recently reviewed [133]). The importance of these proteins in protein degradation come from studies of both skeletal muscle and cardiac atrophy, whereby MuRF1 −/− mice lack the ability to degrade some or all of the protein lost in models of atrophy [134–137]. Subsequent studies have additionally demonstrated their roles in regulating cardiac metabolism, including their metabolomics profiles at baseline in animal models lacking cardiac MuRF family proteins [138]. Atrogin-1 similarly affects protein degradation when energy metabolism is impaired [139]. Other ubiquitous E3s are found in cardiomyocytes (e.g. CHIP, MDM2, c-Cbl, UBE3A/E6AP, cIAP, etc.) and regulate metabolic responses of the heart (e.g. CHIP via AMPK chaperone activity) [133].

9.2. Protein degradation and anaplerosis in striated muscle and heart

The term anaplerosis with respect to the Tricarboxylic acid (TCA) cycle references the phenomenon of reactions that contribute to the TCA cycle outside of the linear pathway involving citrate synthase. In studies primarily of perfused hearts, the enrichment of TCA intermediates to serve as substrates and prevent contractile dysfunction has supported the concept of anaplerosis in the heart [140,141], but also in skeletal muscle [142]. Importantly, the TCA intermediates that feed citrate synthase include the amino acids aspartate (shuttling to TCA as oxaloacetate) and glutamate (shuttling to TCA as α-ketoglutarate).

So when we consider cardiac atrophy as the breakdown and reduction of sarcomere proteins by the proteasome (via ubiquitin-dependent manner), the resulting increase in the amino acid pool supplies (and specifically aspartate and glutamate) represents energy that can be used to make ATP in place of either glucose or fatty acids [143,144]. Taken together, the ability of muscle-specific E3s to degrade protein to amino acids represents one way in which E3s contribute to energy metabolism in their role degrading sarcomere proteins in health and disease.

10. Muscle-specific E3s regulating cardiac metabolism: MuRF1, MuRF2, and MuRF3

Beyond their hypothesized indirect role in anaplerosis, muscle specific ubiquitin ligases have been reported to regulate metabolism directly. While beyond the scope of this review, it's worth mentioning that MuRF1 interacts with and regulates creatine kinase in vivo, a critical enzyme that shuttles ATP from the mitochondria to the sarcomere M-line [10,145]. Additionally, increasing cardiac MuRF1 results in inhibition of fatty acid oxidation by inhibiting PPARα, but not PPARβ/δ or PPARγ1 through a nuclear export mechanism involving multi-monoubiquitination around its nuclear export sequence [8].

Moreover, recent studies have demonstrated that cardiac MuRF1 inhibits thyroid hormone activity in vivo [7]. MuRF1's interaction with the thyroid receptor alpha and subsequent TRalpha monoubiquitination inhibits cardiac thyroid hormone activities [7], which may impair multiple metabolic processes regulated by thyroid hormone in the heart (e.g. fatty acid metabolism) [146–148]. Complementary to this, cardiac MuRF2 and MuRF3 family members attenuate PPARβ/δ and/or PPARγ1 activities to protect against PPAR-ligand induced cardiomyopathy seen in high fat diet challenges in vivo [149,150].

10.1. E3s regulating cardiac autophagy: CHIP, Atrogin-1, MuRF1

The ubiquitous Carboxyl terminus of Hsc70 interacting protein (CHIP, encoded by the *Stub1* gene) is an interesting protein with unique roles in protein quality control that include both an E3 activity AND a co-chaperone activity, linking misfolded protein recovery (via Hsc70) and protein degradation (E3 activity) directly [151–153]. The role of cardiac CHIP in regulating autophagy was demonstrated recently in mice lacking CHIP (CHIP −/−). In a model of voluntary running-induced physiological hypertrophy, CHIP −/− mice ran faster and longer both before and after training, exhibiting an enhanced cardiac autophagic flux and exaggerated physiological (but surprisingly not pathological) cardiac hypertrophy [154]. While the specific mechanism by which autophagy was upregulated is not clear, transcription upregulation of autophagy genes (*GabarapL1*, *Atg7*, *Atg5*, *Vps34*, and *Bnip3*) was identified after five weeks of voluntary running, paralleling increases in autophagic flux [154]. In vitro studies revealed that one mechanism CHIP may be regulating autophagy is through its support of Akt signaling to reduce autophagy, whereby CHIP knockdown resulted in the inhibition of IGF-1-mediated Akt signaling (and presumably enhanced downstream FOXO1/3 activity) driving increases in the observed autophagic flux [154]. Recent studies have implicated cardiac CHIP in the regulation of AMPK as well [155]. Given the clear link between AMPK and the regulation of autophagy, CHIP may regulate autophagy by multiple mechanisms, including its support of AMPK in a ubiquitin ligases-independent manner [156], linked to AMPK mTOR regulation [157].

Recent studies have linked the Atrogin-1 E3 to the regulation of autophagy in the heart. In Atrogin1 −/− mice, both the UPS and lysosomal system were shown to exhibit reduced efficiency with age [158]. Using a combination of pulsed SILAC (stable isotope labeling of amino acids in cell culture) in vivo analyzed by proteomics and biochemical and cellular analyses, the charged multivesicular body protein 2B (CHMP2B) was identified as an Atrogin-1 substrate [158]. As part of the endosomal sorting complex (ESCRT) necessary for autophagy, mice lacking Atrogin-1 had a buildup of this protein, resulting from failed degradation, leading to autophagy impairment, intracellular protein accumulation, which then activated the unfolded protein response and cardiomyocyte apoptosis [158]. Down-regulation of CHMP2B in Atrogin-1 −/− mice restored autophagy and decreased proteotoxicity, preventing cell death, and implicating the mechanism that Atrogin-1 regulates autophagy in vivo. Subsequently, Atrogin1 deletion in zebrafish similarly led to impaired autophagy, disruption of the cytoarchitecture in the heart and skeletal muscle, leading to a progressive impairment of heart and skeletal muscle [159]. When human dilated cardiomyopathy caused by an Atrogin-1 mutation were studied, they demonstrated evidence of severely impaired autophagy and accumulation of the autophagy proteins CHMP2B (mediator of autophagosome-lysosome fusion), beclin1, and LAMP2 (a marker of end-stage autophagy) [158], consistent with the mechanisms identified in the Atrogin-1 mouse model above [160]. Together these findings suggest a critical role of Atrogin-1 in maintaining autophagy and cardiomyocyte health, which has implications in cardiac susceptibility in disease and linking the cardiac UPS and autophagy systems for the first time convincingly.

Some of MuRF1 (encoded by the *Trim63* gene) and MuRF2 (encoded by the *Trim55* gene) activities are redundant, evidenced by the spontaneous developmental hypertrophy that occurs with deletion of all four MuRF1/MuRF2 alleles and attenuated with the expression of just one allele (of 4) in vivo [161]. MuRF1 and MuRF2 both interact with titin, troponin I and troponin T proteins, and with myosin light chain kinase-2 [162]. And while MuRF1 −/− and MuRF2 −/− hearts have similar metabolomics profiles [138], they have independent roles in pressure overload-induced cardiac hypertrophy [10].

Recent studies have demonstrated that MuRF2's expression parallels the expression of multiple proteins that regulate autophagy, including the proteins nbr1 (an autophagy receptor containing LC3- and ubiquitin-binding domains), p62 (a functionally similar autophagy receptor to nbr1), and LC3 (a marker of autophagy) [163]. Additionally, Y2H interaction assays have shown that MuRF2 binds the autophagy cargo recognizing adaptor proteins nbr1 and p62 [164], while other studies have shown that the MuRF2A isoform can interact with p62 while MuRF2B contains a domain for interacting with LC3 [165]. Despite these detailed interactions of MuRF2 with the autophagy machinery, its role in regulating autophagic flux is not clear. However, recent studies have demonstrated that increasing cardiac MuRF1 enhances autophagy flux, with MuRF1 deletion significantly inhibiting autophagy [166,167]. The specific molecular targets of MuRF1 have not been validated, but together these suggest that MuRF1 and MuRF2 may support autophagy through specific regulation of key autophagy steps at the molecular level, which may be utilized in a disease-specific manner to modulate autophagic flux and possibly support cardioprotection. However, the therapeutic implications of these findings need to be tested directly before any conclusions can be made.

With increasing appreciation of the link between cardiomyocyte energy metabolism and protein quality control by the UPS and lysosomal autophagy system, we presented recent studies identifying cardioprotection through both enhanced or inhibited autophagy, depending upon the disease-context and duration (Fig. 2). We then discussed emerging evidence that specific ubiquitin ligases regulate autophagy at two key points, linking the UPS and autophagy pathways to each other through multiple mechanisms (Fig. 3). In the future, targeting these specific mechanisms may allow a more precise and controlled regulation of autophagy to promote cardioprotection in a less haphazard way. Therapeutic targeting of muscle-specific E3's, such as Atrogin-1, may be one way to target cardiac autophagy specifically, allowing the regulation of autophagy in metabolically favorable ways without disrupting broader systemic balances in autophagy in non-cardiac tissues.

Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Transparency document

The Transparency document associated with this article can be found, in online version.

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