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REVIEW

Molecular mechanisms and clinical implications of multiple forms of mitophagy in the heart

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Abstract

Mitochondria, the primary ATP-producing organelles, are highly abundant in cardiomyocytes. Mitochondrial function readily deteriorates in the presence of stress and, thus, maintenance of mitochondrial quality is essential for sustaining pump function in the heart. Cardiomyocytes under stress attempt to maintain mitochondrial quality primarily through dynamic changes in their morphology, namely fission and fusion, degradation, and biogenesis. Mitophagy, a mitochondria-specific form of autophagy, is a major mechanism of degradation. The level of mitophagy is altered in stress conditions, which, in turn, significantly affects mitochondrial function, cardiomyocyte survival, and death and cardiac function. Thus, mitophagy has been emerging as a promising target for treatment of cardiac conditions. To develop specific interventions, modulating the activity of mitophagy in the heart, understanding how mitochondria are degraded in a given condition is important. Increasing lines of evidence suggest that there are multiple mechanisms by which mitochondria are degraded through mitophagy in the heart. For example, in addition to the well-established mechanism commonly utilized by general autophagy, involving Atg7 and LC3, recent evidence suggests that an alternative mechanism, independent of Atg7 and LC3, also mediates mitophagy in the heart. Here, we describe molecular mechanisms through which mitochondria are degraded in the heart and discuss their functional significance. We also discuss molecular interventions to modulate the activity of mitophagy and their potential applications for cardiac conditions.

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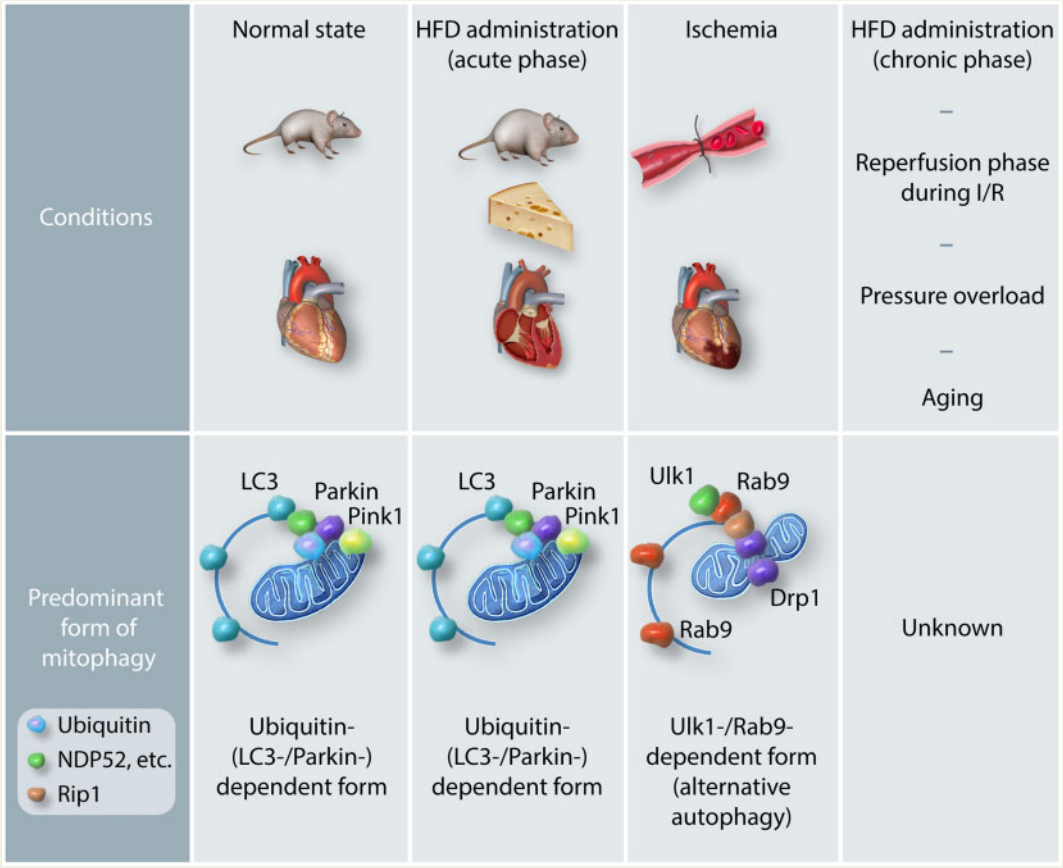
Graphical Abstract



Keywords Mitophagy • Alternative mitophagy • Parkin • Rab9 • Drp1

1. Introduction

The heart continuously demands a vast amount of energy for contractions and relaxations. While cardiac mitochondria supply the majority of ATP in the heart at baseline, the damaged mitochondria therein become a source of reactive oxidative species (ROS) and cell-death signalling under pathological conditions.[1] The failing heart manifests mitochondrial dysfunction regardless of its aetiology, and mitochondrial dysregulation broadly contributes to the pathophysiology of heart failure. Accordingly, mitochondrial quality control is highly critical in the heart and has been investigated extensively in the last decade.

The mitochondria quality control mechanism is intimately intertwined with mitochondrial dynamics, e.g. fusion and fission, degradation primarily mediated through mitochondria-specific autophagy (mitophagy), and biogenesis.[2] The fusion of two mitochondria is mainly mediated by GTPases, mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy protein 1 (Opa1). Mfn1 and Mfn2 regulate fusion of the outer mitochondrial membranes (OMMs), whereas Opa1 regulates fusion of the inner mitochondrial membranes (IMMs) and cristae remodelling.[3] Fission is mainly controlled by dynamin-related protein 1 (Drp1) and its receptors, mitochondrial fission 1 protein (Fis1), mitochondrial division protein 1 (Mdv1), and mitochondrial fission factor (Mff).[3] Fission generates uneven daughter mitochondria and those with reduced membrane potential have a reduced probability of fusing again, so that damaged mitochondria are eventually eliminated through mitophagy and replaced through expansion of pre-existing mitochondria (biogenesis).[4] When this mechanism is disturbed, the depolarized portion of mitochondria accumulates, thereby leading to global impairment of mitochondrial function.[5] Mitophagy is thus considered an essential mechanism for maintaining the quality of mitochondria.

Autophagy is a major mechanism of lysosome-dependent cellular degradation. Although macroautophagy, the most well studied form of autophagy, characterized by the presence of double-membrane vesicles termed autophagosomes, degrades cytosolic materials, and organelles in a non-selective manner, it can also degrade specific targets in a selective manner. The latter is termed selective autophagy and includes mitophagy. Although non-selective autophagy and mitophagy share mechanisms mediating autophagosome formation and degradation, it appears that additional mechanisms are involved in the induction of mitophagy and recognition of mitochondria to be degraded. We have shown recently that autophagy and mitophagy are activated with distinct time courses in the stressed heart, suggesting that mitophagy may be regulated by mechanisms that are distinct from those that regulate non-selective autophagy in the heart. Increasing lines of evidence suggest that mitophagy is

involved in modulation of heart disease conditions. Thus, it is important to understand how mitophagy is regulated in response to stress in the heart. In this review, we summarize the molecular mechanisms and the functional significance of mitophagy in the heart at baseline and in response to stress and discuss their clinical implications.

2. Molecular mechanisms of mitophagy

2.1 Ubiquitin-dependent mitophagy in mammalian cells

In mammalian cells, mitophagy is mediated through multiple mechanisms.[6,7] Depending upon how damaged mitochondria are labelled for sequestration by autophagosomes, mitophagy in mammalian cells can be broadly classified as either ubiquitin-dependent mitophagy or receptor-dependent mitophagy.[7] In the ubiquitin-dependent category, thus far, Pink1- and Parkin-mediated mitophagy has been characterized most intensively.[7] Pink1 is a serine/threonine kinase, whereas Parkin is an E3 ubiquitin kinase. Pink1 is anchored at the IMM and degraded by matrix processing peptidase and presenilin-associated rhomboid-like in intact mitochondria.[1] In response to depolarization of mitochondrial membrane potential or the unfolded protein response in mitochondria, Pink1 is accumulated in the OMM with its kinase domain facing the cytosol. Stabilization of Pink1 is also regulated by adenine-nucleotide translocator (ANT)-TIM44 mediated inhibition of the presequence translocase TIM23. The effect of ANT upon TIM23 and mitophagy is mediated independently of its nucleotide translocase catalytic activity. How ANT is involved in mitophagy activation under stress remains to be elucidated.[8] Evidence suggests that Parkin and Pink1 operate together in a common pathway for mitochondrial quality control.[9] Pink1 recruits Parkin to damaged mitochondria through phosphorylation of either ubiquitin at serine 65 or Mfn2 at threonine 111 and serine 442.[10] Pink1 also directly phosphorylates and activates Parkin.[11,12] Since Parkin can be recruited to peroxisomes or lysosomes when Pink1 is artificially overexpressed and targets these organelles,[9] phosphorylation of ubiquitin or the direct interaction between Parkin and Pink1 may be important. In contrast, transgenic expression of an Mfn2 mutant that is not phosphorylated by Pink1 (Tg-Mfn2 AA) in the heart results in lethality with dilated cardiomyopathy,[13] suggesting the importance of the Pink1-Mfn2 pathway in the heart at baseline. Parkin ubiquitinates various mitochondrial outer membrane proteins on depolarized mitochondria, including Tom20, VDAC, hexokinase I, Mitofusin, Miro, and MitoNEET/CISD1, whereas LC3-anchor proteins are involved in the recognition of ubiquitinated mitochondrial proteins, including NDP52, Optineurin, NBR1, and p62, by autophagosomes in cultured cells.[10] Further investigation is required to establish the significance of most of these mechanisms in mediating mitophagy in the heart *in vivo*. Compared to the molecular mechanism as to how damaged mitochondria are recognized, how autophagosomes are formed on site remains poorly understood. NDP52 and TBK1 in concert recruit and activate the Ulk1/FIP200 complex independently of AMPK and even in the presence of nutrient rich conditions, which in turn drives targeted autophagosome biogenesis.[14]

Currently, forced-expression of Parkin and/or CCCP treatment, an intervention that depolarizes mitochondrial membrane potential, is commonly used to investigate Pink1/Parkin-mediated mitophagy in mammalian cells. However, these experimental conditions may not necessarily faithfully reproduce conditions where mitophagy is induced in mammals *in vivo*.[9] Furthermore, Pink 1- and Parkin-dependent mechanisms have never been identified through unbiased screening of mitophagy mediators in mammalian cells.[15,16] Thus, more investigation is needed to establish Pink1- and Parkin-dependent mitophagy as the predominant form of mitophagy under various conditions *in vivo*.

2.2 Receptor-dependent mitophagy in mammalian cells

Several pathways have been reported to regulate mitophagy in a Parkin- or ubiquitin-independent manner. In these mechanisms, damaged mitochondria are directly recognized by autophagosomes via expression of proteins with the LC3-interacting region (LIR) on the OMM; thus, these mechanisms are called receptor-dependent mitophagy. One such example is Fun 14 domain-containing protein 1 (Fundc1), an OMM protein with an LIR, which mediates mitophagy in HeLa cells and MEFs.[17] In the resting state, CK2 and Src phosphorylate Fundc1 at serine 13 and tyrosine 18, respectively, and suppress the interaction between LC3 and Fundc1, thereby inhibiting mitophagy. However, under stress conditions, phosphorylation of Fundc1 by Ulk1 at serine 17 and dephosphorylation by PGAM5 at serine 13 promote interaction between LC3 and Fundc1 and induce mitophagy. In other examples of receptor mediated mitophagy, Bnip3- and Nix-mediated mechanisms play a significant role in mediating reticulocyte differentiation.[18,19]

Besides the aforementioned ubiquitin-dependent mitophagy and receptor-dependent mitophagy, recent evidence suggests that Ulk1-/Rab9-dependent alternative autophagy also plays a major role in mediating starvation- and hypoxia-induced mitophagy in HeLa cells.[20] Details concerning alternative autophagy are discussed in Sections 4 and 5.

3. Mitophagy in the heart

3.1 Mitophagy in cardiac homoeostasis and development

Mitochondria occupy a large percentage of the heart volume and continuously produce ATP and ROS, by-products of respiration, even at baseline. Mitophagy, autophagy targeting mitochondria, is essential for maintaining mitochondrial function and both the energetic and redox homoeostasis in cardiomyocytes. We summarized the cardiac phenotype of animal models of cardiac conditions with genetic manipulations related to the mitophagy pathways (*Table 1*).

In *Drosophila melanogaster*, knockout of Parkin induces heart failure with mitochondrial abnormality, suggesting that Parkin is essential for maintaining mitophagy and mitochondrial homoeostasis in the heart at baseline.[5] Heart-specific deletion of Parkin in mice during the perinatal period with *myh6*-driven MER-Cre-MER induces lethal cardiomyopathy without postnatal maturation of mitochondria,[13] suggesting that Parkin plays a vital role in the maintenance of the mammalian postnatal heart. In contrast, in mice with knockout of Parkin in the germ-line, normal function of the heart and mitochondria is maintained until 12 months of age.[21] In this case, germ-line knockout may activate compensatory mechanisms and mask the effect of Parkin-knockout. Similarly, however, tamoxifen-mediated ablation of Parkin at the adult stage in Parkin$^{flox/flox}$ *myh6*-driven MER-Cre-MER mice[22] also did not induce dysfunction in the mouse heart and mitochondria. This suggests that Parkin-dependent mitophagy may not play an essential role in maintaining mitochondrial function in the adult mouse heart. By inference, the abnormal cardiac phenotype observed in Tg-Mfn2 AA[13] may be mediated through

Table 1 Phenotypes of cardiac disease models with genetic interventions related to the mitophagy pathways

Target gene	Gene manipulation	Conditions	Mitophagy	Mitochondrial phenotype	Cardiac phenotype	Refs.
Ubiquitin-dependent mitophagy						
Parkin	Germ-line KO	Baseline	–	Normal	Normal	21
	Germ-line KO	MI	–	Dysfunction	Increased injury	21
	Germ-line KO	Baseline	Preserved	Mild dysfunction	Normal	79
	Germ-line KO	Sepsis	Preserved	Dysfunction	Normal	79
	Germ-line KO	DOX	Reduced	Dysfunction	Increased injury	80
	Germ-line KO	IPC	–	–	Increased injury	28
	Cardiac-specific inducible KO	Baseline (perinatal)	Reduced	Dysfunction	Lethal cardiomyopathy	13
	Cardiac-specific inducible KO	Baseline (adult)	–	Normal	Normal	22
	Cardiac-specific TG	Baseline	Increased	Normal	Normal	22
Pink1	Germ-line KO	Baseline	Reduced	Dysfunction	Dysfunction	81
	Germ-line KO	TAC	Reduced	Dysfunction	Increased injury	81
	Germ-line KO	Exercise	Reduced	–	–	82
	Cardiac-specific TG	Baseline	–	–	Normal	83
	Cardiac-specific TG	I/R	–	–	Reduced injury	83
Receptor-dependent mitophagy						
Bnip3	Germ-line KO	Baseline	–	–	Normal	84
	Germ-line KO	I/R	–	–	Reduced injury	84
	Cardiac-specific inducible TG	Baseline	–	–	Dysfunction	84
	Cardiac-specific inducible TG	MI	–	–	Increased injury	84
Nix	Germ-line KO	Baseline	–	–	Dysfunction	85
	Cardiac-specific KO	Baseline	–	–	Normal	86
	Cardiac-specific KO	TAC	–	–	Preserved function	86
	Cardiac-specific TG	Baseline	–	–	Dysfunction	87
Fundc1	Cardiac-specific KO	Baseline	–	Dysfunction	Dysfunction	88
	Cardiac-specific KO	MI	–	Dysfunction	Increased injury	88
Fusion/fission-related genes						
Mfn1/2	Cardiac-specific KO	Baseline	–	–	Lethal cardiomyopathy	89
	Cardiac-specific KO	Baseline	–	–	Lethal cardiomyopathy	90
	Cardiac-specific inducible KO	Baseline	–	Dysfunction	Dysfunction	90
	Cardiac-specific inducible KO	Baseline	–	Dysfunction	Dysfunction	91
	Cardiac-specific KO	Baseline	–	–	Lethal cardiomyopathy	92
	Cardiac-specific inducible KO	Baseline	–	Dysfunction	Dysfunction	92
	Cardiac-specific inducible KO	I/R	–	–	Reduced injury	93
Drp1	Muscle-specific KO	Baseline	–	Dysfunction	Lethal cardiomyopathy	94
	Cardiac-specific KO (homo)	Baseline	–	Dysfunction	Lethal cardiomyopathy	24
	Cardiac-specific KO (hetero)	Baseline	–	Dysfunction	Dysfunction	24
	Cardiac-specific inducible KO	Baseline	Increased	Dysfunction	Dysfunction	91
	Cardiac-specific inducible KO	Baseline	Reduced	Dysfunction	Dysfunction	25
	Cardiac-specific KO (hetero)	Baseline	Reduced	Dysfunction	Dysfunction	25
	Cardiac-specific KO (hetero)	I/R	Reduced	Dysfunction	Increased injury	25
	Cardiac-specific KO (hetero)	TAC	Reduced	Dysfunction	Increased injury	30
Opa1	Germ-line KO (hetero)	Baseline	–	Dysfunction	Normal	95
	Germ-line KO (hetero)	TAC	–	–	Increased injury	95

DOX, doxorubicin; I/R, ischaemia/reperfusion; IPC, ischaemic preconditioning; KO, knock-out; MI, myocardial infarction; TAC, transverse aortic constriction; TG, transgenic.

additional mechanisms besides the suppression of Parkin-mediated mitophagy.

The role of Drp1 in the heart has also been intensively investigated[23] homozygous deletion of cardiac Drp1 results in premature death due to heart failure in mice.[24,25] Analyses using heterozygous deletion have shown that down-regulation of endogenous Drp1 induces dilated cardiomyopathy with an accumulation of ubiquitinated and dysfunctional mitochondria,[24,25] suggesting a pivotal role of Drp1 in mediating mitophagy and mitochondrial homoeostasis in the heart. There have been conflicting reports, however, regarding whether mitophagy can be induced in the heart in the complete absence of Drp1. Although one report showed that mitophagy is mostly eliminated when Drp1 is down-

regulated in the heart,[25] other reports indicated that mitophagy can be activated without Drp1.[22,24] The remaining mitophagy appears to be activated through Parkin-dependent mechanisms and plays an either adaptive or maladaptive role in Drp1 KO hearts. One report suggested that Parkin-mediated mitophagy is critical in maintaining cardiac function in the absence of Drp1 in the heart,[24] whereas the other report suggested that simultaneous deletion of Parkin and Drp1 mitigated mitophagy and improved cardiac function, compared to ablation of Drp1 alone.[22] In this context, Parkin-mediated mitophagy may exacerbate cardiac dysfunction when mitochondrial division is reduced. Taken together, the induction and functional significance of Parkin-mediated mitophagy relative to Drp1-dependent mitophagy remain to be clarified in the heart. In addition, it will be essential to determine whether the mitophagy observed in Parkin KO mice is Drp1-dependent. If so, it is likely that Parkin and Drp1 mediate distinct forms of mitophagy in the heart. In other cell types, Drp1 has classically been observed to be essential in mediating the separation and mitophagic degradation of depolarized mitochondria.[4] However, since mitochondria are relatively fragmented in adult cardiomyocytes under baseline conditions and Drp1 also possesses fission-independent functions, whether or not Drp1 is directly involved in mitophagy in adult cardiomyocytes remains controversial.

A novel function of cardiac-resident macrophages was reported recently.[26] It was observed that cardiomyocytes eject LC3-positive membranous particles containing dysfunctional mitochondria and that cardiac macrophages take them up and digest them, thus contributing to mitochondrial homoeostasis in the heart. Each cardiomyocyte is surrounded by five cardiac macrophages, and each macrophage interacts with up to five cardiomyocytes. LC3-positve particles are generated through the autophagic process and preferentially contain dysfunctional mitochondria in cardiomyocytes. The phagocytic receptor, Mertk, plays a significant role in the uptake of these particles by cardiac macrophages. This mechanism plays an important role in maintaining cardiac function both at baseline and during stress, such as ischaemia or isoproterenol-overload. It is unknown, however, whether this mechanism plays a more important role in mediating mitochondrial quality control than mitophagic degradation by lysosomes. The availability of multiple options for the heart to remove damaged mitochondria may allow cardiomyocytes to survive even when one mechanism fails. Subsarcolemmal mitochondria are located close to the cellular surface and, thus, they may be subjected to ejection from cardiomyocytes more frequently than perinuclear or intermyofibrillar mitochondria, although this hypothesis remains to be tested.

3.2 Mitophagy in the heart during ischaemia and reperfusion

Growing evidence suggests that mitophagy is protective for the heart under ischaemic conditions and during post-myocardial infarction cardiac remodelling.[3,27] Since cardiac mitochondria are quite vulnerable to oxidative stress during reperfusion, a protective mechanism is essential as the first line of defense. Parkin is up-regulated in the ischaemic border zone from 8 to 48 h after coronary artery ligation.[21] Parkin-mediated mitophagy induces ischaemic preconditioning, thereby protecting the heart against recurrent ischaemia.[28] Parkin may not prevent acute ischaemic injury without preconditioning, however, since it is not activated at very early time points, such as 4 h, following acute ischaemia.[21] The heart chronically undergoes hypertrophy and dilation and its function decreases after myocardial infarction in a process termed cardiac remodelling. Again, mitochondrial dysfunction is commonly observed and precipitates the progression of heart failure after MI.[10] Parkin KO mice exhibit more severe cardiac remodelling and higher mortality, but less mitophagy after MI than wild-type mice.[21] Thus, Parkin-mediated mitophagy protects the heart during cardiac remodelling after MI. Contributions of Parkin to the overall level of mitophagy and the timing of Parkin-mediated mitophagy during cardiac remodelling remain to be established. Since the eventual outcome is the development of mitochondrial dysfunction and heart failure in many post-MI patients, activation of Parkin-mediated mitophagy alone appears insufficient to prevent the progression of cardiac remodelling.

Although rapid revascularization is essential for minimizing the size of MI, it induces reperfusion injury. In response to 20 min of ischaemia followed by 24 h of myocardial reperfusion, the myocardial injury was comparable between wild-type and Parkin KO mice.[28] These results suggest that Parkin may not be involved in mitochondrial quality control mechanisms during myocardial reperfusion. We have shown previously that cardiac-specific heterozygous Drp1 KO mice exhibit more extensive infarction with impairment of mitophagy than wild-type mice in response to 30 min of ischaemia followed by 24 h of reperfusion. Thus, endogenous Drp1 protects the heart against ischaemia/reperfusion (I/R) and this effect may be mediated through activation of mitophagy.[25] Further investigation is required to clarify the underlying molecular mechanism of mitophagy and the specific role of mitophagy during myocardial reperfusion.

3.3 Mitophagy in the heart during pressure-overload

Hypertension is a significant comorbidity in patients with heart failure. Among the potential underlying mechanisms of hypertension, ageing, and metabolic stress are commonly associated with an increase in vascular resistance.[29] Increasing evidence suggests a significant role of autophagy and mitophagy in cardiomyocytes in mediating the adaptive or maladaptive response of the heart against pressure afterload. A detailed time-course study showed that canonical autophagy is activated by pressure overload within a few hours of transverse aortic constriction when the heart becomes energetically starved due to increased cardiac afterload, and then quickly inactivated within 24 h.[30] Interestingly, mitophagy is activated more slowly, 3–5 days after the onset of pressure overload when canonical autophagy is inactivated, suggesting that mitophagy and canonical autophagy are mediated through distinct mechanisms. Dissociation between canonical autophagy and mitophagy was also observed in mouse embryonic fibroblasts in response to hypoxia.[20] Currently, the underlying molecular mechanisms by which mitophagy is induced in a manner independent of canonical autophagy during pressure overload remain elusive. The time course of mitophagy corresponds to that of mitochondrial division, but not Parkin-translocation to mitochondria. Down-regulation of Drp1 abolishes mitophagy and boosts cardiac dysfunction. These results imply that Drp1 plays a vital role in mitophagy and cardiac homoeostasis during pressure overload.

Activation of mitophagy in response to pressure overload is also transient, and it is no longer activated after 5 days of pressure overload. The mouse heart manifests mitochondrial dysfunction and heart failure, thereafter. Administration of TAT-Beclin 1, an autophagy-activating peptide that induces dissociation of Beclin 1 from GAPR-1, significantly induces mitophagy and ameliorates heart failure.[30] Thus, the inactivation of mitophagy during the chronic phase of pressure overload contributes to the development of heart failure. Reactivation of mitophagy by TAT-Beclin1 does not take place in Drp1 heterozygous knockout mice, again

suggesting the importance of Drp1 in mediating mitophagy during pressure overload.

3.4 Mitophagy in the heart during metabolic stress

More than half of diabetes patients develop cardiac abnormality, including hypertrophy and diastolic dysfunction, termed diabetic cardiomyopathy.[31] Diabetic cardiomyopathy is accompanied by mitochondrial dysfunction and myocardial accumulation of toxic lipid, including ceramide and diacylglycerol, termed lipotoxicity.[31] In the mouse model of type II diabetes, consumption of a high-fat diet (HFD), consisting of 60% saturated fatty acid, up-regulates autophagy in the heart, peaking at 6 weeks. Cardiac-specific knockout of Atg7 (Atg7cKO) or Parkin KO reduces mitophagy by half compared to in wild type, and induces lipid accumulation and cardiac dysfunction after 2 months of HFD consumption, suggesting that mitophagy mediated through autophagy or Parkin-dependent mechanisms protects the heart during the acute phase of HFD consumption. It should be noted that Parkin also plays a significant role in mediating lipid metabolism during HFD consumption. Parkin induces fatty acid uptake by hepatocytes and adipocytes through the ubiquitin-mediated stabilization of the lipid transporter CD36.[32] This demonstrates the diverse functions of Parkin besides mitophagy in mediating cellular homoeostasis. Thus, caution should be exercised when interpreting experimental results obtained using Parkin KO mice.

Obesity and metabolic syndrome are often long-term conditions. Our recent study showed that activation of autophagy in response to HFD consumption is transient and starts to decline rapidly after it reaches a peak around 6 weeks. On the other hand, mitophagy is progressively activated even after canonical autophagy is inactivated.[33] Thus, canonical autophagy and mitophagy appear to be activated with distinct time courses. Another study showed, however, that the protein level of cardiac Parkin is dramatically decreased after 12 weeks of HFD consumption.[34] Thus, whether mitophagy continues to be activated in a Parkin-independent manner during the chronic phase of HFD consumption and, if so, what underlying molecular mechanisms are involved and its functional significance remain to be elucidated.

4. Alternative autophagy

As described above, under some conditions, mitophagy is activated in the heart despite inactivation of non-selective autophagy.[30,33] Although the precise molecular mechanism remains elusive, our group recently demonstrated the existence of this unique form of mitophagy, which is mediated by mechanisms similar to the *alternative autophagy* originally reported by Nishida *et al.*,[35] in the heart and named it *alternative mitophagy*.[35] In the next two sections, we will describe the features of alternative autophagy and mitophagy.

4.1 Origin of alternative autophagosomes

Molecular mechanisms of autophagy, including how autophagosomes are generated, have been extensively investigated.[10] Primary mechanisms include autophagosomes formation through conjugation of ubiquitin-like proteins, including LC3 (Atg8), by autophagy-related (ATG) proteins. These mechanisms are also utilized in mitophagy when autophagosomes encapsulating mitochondria are formed. Besides the conventional mechanism of autophagy, another form of autophagy has been reported, called alternative autophagy or Golgi membrane-associated degradation (GOMED).[35,36] In alternative autophagy, an autophagosome-like structure with double membranes is induced even in the absence of some ATG proteins and it undergoes lysosomal degradation with its cargo. Although it is hard to distinguish the autophagosome-like structure in alternative autophagy morphologically from autophagosomes in canonical autophagy, it has been proposed that the former originates from an intracellular membrane source distinct from the latter. To date, multiple intracellular membranes have been identified as the origins of the autophagosome membrane in canonical autophagy, including the endoplasmic reticulum (ER), mitochondria, ER-mitochondria contact site, and plasma membrane.[37] It has been proposed that the *trans*-Golgi network is the origin of alternative autophagosomes, based on the following observations: (i) almost all autophagic vacuoles were localized near the Golgi apparatus in cells lacking ATG-conjugation, (ii) the Golgi ministack formation precedes autophagosome formation in these cells, (iii) some isolation membranes extended from the Golgi membrane, (iv) *trans*-Golgi proteins were detected on alternative autophagosomes and autolysosomes, and (v) the depletion of Golgi proteins inhibited alternative autophagy but not canonical autophagy.[37] In addition, Brefeldin A, a specific inhibitor of protein transport from the ER to the Golgi apparatus, significantly suppresses the formation of alternative autophagosomes, but not canonical ones.[35] These observations suggest that the *trans*-Golgi network is a significant membrane source of alternative autophagosomes.

4.2 Molecular mechanisms underlying alternative autophagy

Canonical and alternative forms of autophagy share up-stream molecular machinery, such as the Ulk1-Fip200 complex and phosphatidylinositol 3 kinase (PtdIns3K) complex, as evidenced by the fact that the autophagosome-like structure in cells lacking Atg5, namely alternative autophagy, is abolished by simultaneous silencing of Ulk1, Fip200, Beclin 1, and Vps34 (but not Atg7, Atg12, Atg16, or Atg9).[35] Accordingly, alternative autophagy or GOMED is often described as Ulk1-dependent autophagy. Following nutrient starvation or mTOR inhibition, the activated Ulk1 phosphorylates Beclin 1 at serine 14, thereby activating Atg14L-Vps34 complexes.[38] The Vps34-Beclin 1 complex serves as a binding partner for several proteins capable of either promoting (Atg14L, UVLAG, Bif1, and AMBRA-1) or inhibiting (Rubicon, Bcl-2, and Bcl-xl) autophagy. Vps34 produces phosphatidylinositol 3-phosphate (PtdIns(3)P), which promotes both canonical autophagy and retrograde trafficking from endosomes to the Golgi apparatus.[39] It is thus conceivable that these core complexes located upstream of canonical autophagy regulate Golgi membrane-associated degradation in alternative autophagy as well. Recently, the mechanism by which Ulk1 translocates to the Golgi has been reported using mouse embryonic fibroblasts (MEFs) (*Figure 1*).[40] During genotoxic stress-induced alternative autophagy, Rip3 interacts with Ulk1 in the cytosol and phosphorylates it at serine 746, corresponding to serine 747 of human Ulk1. This phosphorylation plays an essential role in mediating the dissociation of Ulk1 from the Fip200-Atg13 complex and its translocation to the Golgi (*Figure 1*).[40] Interestingly, the serine 746 residue is conserved in Ulk1, but not Ulk2, in higher vertebrates. This process is independent of canonical autophagy or Rip3-mediated necrosis.

In mammalian canonical autophagy, ATG-conjugation is vital for autophagosome closure, fusion with lysosomes, and degradation of the inner autophagosomes membrane.[41] In alternative autophagy, the membranes

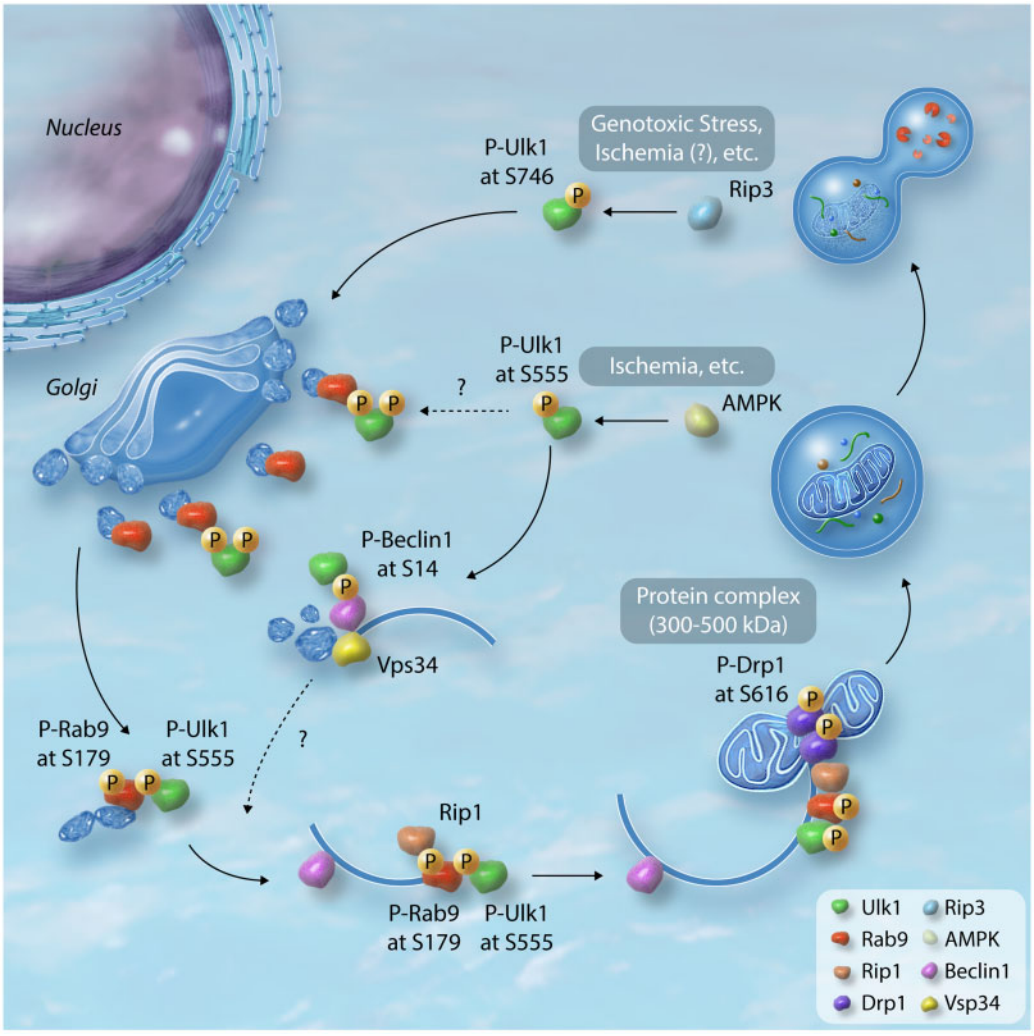


Figure 1 Proposed model of mitophagy mediated by Ulk1-dependent alternative autophagy. Ulk1 forms protein complex with Fip200 and Atg13 at the basal state. Upon stimuli, phosphorylation of Ulk1 at serine 746 by Rip3 allows for its dissociation from the complex and translocation to Golgi.[40] This mechanism was found in the MEFs under the genotoxic stress, however, its significance in the heart has yet to be clarified. The *trans*-Golgi network is proposed to be the origin of alternative autophagosomes. Upon energetic stress, such as ischaemia, multiple sites in Ulk1 are phosphorylated by AMPK. Of these, phosphorylation of serine 555 is important for its translocation to mitochondria in MEFs and skeletal muscle in mice.[59,60] Ulk1 phosphorylates Beclin 1 at serine 14, thereby inducing the Beclin 1-Vps34 complex. Vps34 is then activated at phagophore or endosomal membranes, which is pivotal for membrane remodelling, endosomal transport and autophagy.[39] Serine 555 phosphorylated Ulk1 acts as a scaffold to assemble a complex comprising Rab9, associated with trans-Golgi membranes. Ulk1 directly phosphorylates Rab9 at serine 179, that facilitates interaction between Rab9 and Rip1 and the consequent phosphorylation of Drp1 at serine 616 in cardiomyocytes.[57] Mitochondria labelled with phosphorylated Drp1 are subsequently sequestrated by phagophores assembled via Rab9 in cardiomyocytes.

that originate from the *trans*-Golgi network subsequently expand and close through fusion with other membranes from *trans*-Golgi or endosomal vesicles.[35] The GTPase activity of Rab9, an essential molecule for membrane and protein trafficking from the late endosomes to the *trans*-Golgi network, is required for this process.[35] Deletion of Rab9 reduces the number of autophagic vacuoles but not isolation membranes. Rab9 is thus pivotal for the maturation of alternative autophagosomes as a substitute for both ATG-conjugation and LC3. In response to genotoxic stress, Dram1 also participates in the closure of alternative autophagosomes in a p53-dependent manner.[42] Whether and how these molecules undergo post-translational modification during the maturation of alternative autophagosomes is currently unknown.

4.3 Biological significance of alternative autophagy

The relevance of alternative autophagy has been reported in various organs, including the heart. Before the discovery of alternative autophagy, autophagic degradation of mitochondria during erythroid maturation was documented in Atg5-depleted cells.[43] Further studies have

demonstrated the essential role of Ulk1-dependent alternative autophagy in this process.[44,45] Interestingly, the dominance of the Ulk1-dependent alternative mechanism in mitochondrial clearance has been reported in foetal definitive erythrocytosis, but not in adult erythrocytosis,[45] suggesting that mitophagy is executed through different forms of autophagy in a stimulus- or environment-dependent manner.

Alternative autophagy mediated mitophagy was also found to play a significant role in the development of induced pluripotent stem cells (iPSCs).[46] The reprogramming process requires a metabolic switch from oxidative phosphorylation to glycolysis and mitochondrial clearance is involved in this event. An earlier study reported the role of canonical autophagy in this process,[47] however, further analysis revealed that this highly efficient reprogramming in MEFs requires Ulk1-/Rab9-dependent alternative autophagy, not Atg5-dependent autophagy, for mitochondrial clearance followed by metabolic reprogramming and iPSCs development.[46] These studies agree that the deletion of Ulk1 or Rab9 impairs mitophagy more prominently than that of Atg7 or Atg5 under specific conditions. Ulk1-/Rab9-dependent alternative autophagy is thus neither a leaky phenotype of canonical autophagy nor merely residual autophagic activity detected in ATG-conjugation deficient cells. Rather, alternative autophagy is an important mechanism involved in mitochondrial clearance and cellular homoeostasis.

Substrates other than mitochondria, such as (pro)insulin granules and bacteria, have also been observed in alternative autophagosomes.[36,48–50] In pancreatic β cells where unique mechanisms of cellular degradation are utilized,[51] (pro)insulin granules can be engulfed by alternative autophagosomes labelled with Syntaxin 6, a marker of the Golgi membrane, when ATG-conjugation is deleted.[36] Since canonical autophagy is impaired in diabetic β cells, alternative autophagy may play a critical role in degrading (pro)insulin granules there. Under conditions of infectious disease, the role of autophagy in mediating the recognition and the elimination of intracellular pathogens is well-recognized. Interestingly, some bacteria have been shown to be sequestrated by autophagosomes in cells lacking Atg5.[49,50] In the intestinal epithelium, which interfaces with a variety of bacteria, the mitochondrial protein TRIM31 plays an essential role in forming autophagosomes independently of Atg5/Atg7.[48] However, the involvement of Rab9 or Syntaxin 6 in these mechanisms has not yet been elucidated.

5. Regulation of mitophagy by alternative autophagy in the heart

Various mechanisms have been identified as mitophagy pathways using cultured cells or animal models with gene manipulation. It is essential to determine which pathway predominantly regulates mitophagy in the heart under disease conditions, e.g. pressure overload, metabolic stress, and I/R, since cardiomyocytes and the mitochondria therein are subject to a variety of stresses. Mice expressing a mitophagy probe allow for the spatial and temporal quantitation of mitophagy in the heart, providing clues about the molecular mechanism mediating mitophagy under specific stress conditions.[52]

For example, a study with transgenic mice with mCherry-GFP-mtFIS1(101–152) fusion protein (mito-QC mice) showed that the deletion of Pink1 had no effect on mitophagy in the heart at baseline,[53] suggesting that mitophagy in the heart is mediated through Pink1-independent mechanisms. However, since mito-QC is expressed on the cytoplasmic side of OMM with a mitochondrial targeting sequence of Fis1 (101–152), it can be degraded more quickly through Parkin- and ubiquitin proteasome-dependent mechanisms compared to mitophagy.[54,55] Thus, caution should be exercised when interpreting the result obtained with mito-QC signal.[54] Mito-SRAI, a newer mitophagy probe circumvents the potential problem of mito-QC, and, thus, it may allow us to analyse the signalling mechanisms of mitophagy in a more accurate manner.[54]

Mice expressing another mitophagy probe targeted to mitochondrial matrix, mt-Keima, have also been generated by multiple groups.[56,57] Mice generated by crossing heart-specific transgenic mt-Keima mice (Mito-Keima-Tg) with Atg7cKO displayed nearly complete down-regulation of mitophagy in the heart at baseline, but mitophagy was preserved under conditions of starvation or ischaemia compared to in Mito-Keima-Tg without Atg7cKO.[57] While Mito-Keima-Tg crossed with Parkin KO exhibited well-preserved mitophagy under energy stress conditions, those with Ulk1cKO showed a significant decrease in mitophagy under conditions of starvation or ischaemia. These results suggest that mitophagy in the heart is executed through canonical autophagy at baseline, but is mediated mainly through Parkin-independent and/or Ulk1-dependent mechanisms under conditions of starvation or ischaemia. Since the mt-Keima signal during starvation and ischaemia was decreased in the presence of Brefeldin A and colocalized with Rab9 puncta, Ulk1-/Rab9-dependent alternative autophagy is thought to be the predominant form of mitophagy under conditions of starvation or ischaemia in the heart.[57] Ulk1 directly phosphorylates Rab9 at serine 179 in response to starvation or ischaemia and recruits a fission complex consisting of Ulk1, Rab9, Rip1, and Drp1 to damaged mitochondria (*Figure 1*). Drp1 is phosphorylated at serine 616 by Rip1, resulting in its activation and mitochondrial fragmentation. Rab9-mediated autophagosomes subsequently engulf the fragmented mitochondria (*Figure 1*). In this model, it is likely that mitochondrial division occurs simultaneously with autophagosome closure, which is consistent with the recent idea proposed by the Kanki group.[58] Interestingly, knock-in mice expressing a phosphorylation-resistant mutant of Rab9, Rab9(S179A), showed severely impaired mitophagy during ischaemia despite the preservation of canonical autophagy flux. This suggests that phosphorylation of Rab9 by Ulk1 is a specific mechanism involved in mitophagy that protects the heart against ischaemia, even in the absence of canonical autophagy. When Ulk1-/Rab9-dependent mitophagy takes place, a large protein complex 300–500 kDa in size, consisting of Ulk1, Rab9, Rip1, Drp1, and possibly other proteins, is formed (*Figure 1*). The large protein complex may consist of the machinery that forms Rab9-containing autophagosomes, as well as the fission complex, in close proximity to damaged mitochondria. The identity of proteins in the large protein complex and how Rab9-positive autophagosomes are formed and mitophagy is executed remain to be clarified.

6. Pharmacological tools that manipulate mitophagy

Autophagy and mitophagy have been considered attractive targets for pharmacological intervention and many drugs have been identified as autophagy inducers. For example, rapamycin, metformin, and polyphenols, including resveratrol, have been shown to induce autophagy and act protectively in the heart.[61,62] These compounds affect multiple cellular functions, and thus, caution must be exercised in determining whether activation of autophagy or mitophagy plays an essential role in mediating their salutary effects in a given condition. Recently, however, we and others have shown more selective and effective interventions,

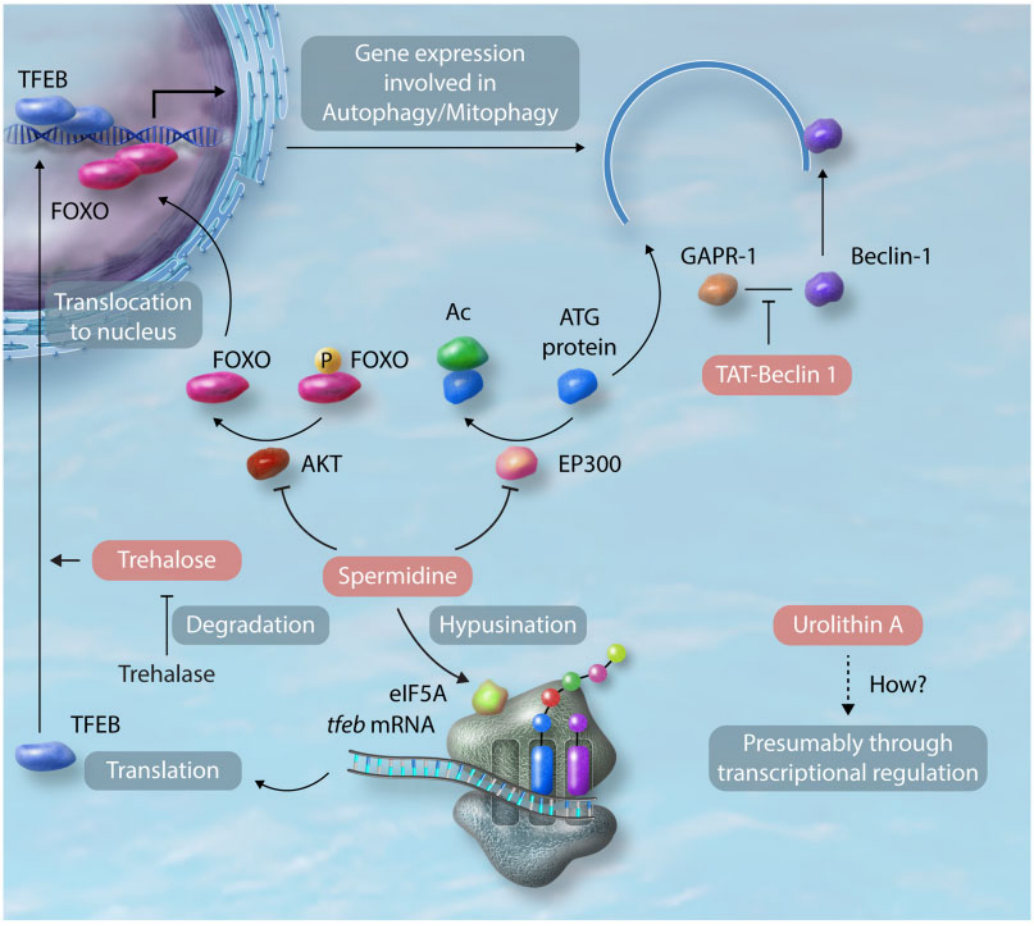


Figure 2 Molecular mechanisms of the effect of pharmacological interventions on autophagy/mitophagy. TAT-Beclin 1 competitively inhibits the interaction between endogenous Beclin 1 and its negative regulator, GAPR-1, thereby mobilizing Beclin 1 to stimulate autophagy.[67] Spermidine stimulates autophagy by inhibiting cytosolic EP300 acetyltransferase.[68] Furthermore, spermidine facilitates translocation of FOXO from the cytosol to nucleus and provokes epigenetic reprogramming through inhibition of histone acetyltransferases in the nucleus, thereby activating transcription of autophagy-related genes.[69] Spermidine also promotes hypusination of translational factor eIF5A, which uniquely contains the unusual amino acid hypusine, in memory B cells.[70] This post-translational modification of eIF5A makes the formation of the first peptide bonds more effective in the translation of TFEB mRNA. Trehalose promotes translocation of TFEB from the cytosol to nucleus, thereby inducing autophagy-related genes in macrophages and cardiomyocytes.[64,71] Trehalase expressed in the intestine in mice and humans can degrade Trehalose.[64] The detailed mechanism by which Urolithin A induces autophagy/mitophagy has yet to be clarified. Induction of autophagy/mitophagy-related genes and phosphorylation of AMPK were observed after treatment with Urolithin A in C2C12 myoblasts and skeletal muscles in mice and humans.[65,66]

including TAT-Beclin 1, spermidine, and trehalose, inducing autophagy and mitophagy in the heart.[30,63,64] Urolithin A also induces mitophagy in *Caenorhabditis elegans* and C2C12 myoblasts.[65] Treatment with spermidine or trehalose is being tested in patients with hypertension or ACS, respectively. The result of a human clinical trial of Urolithin A has been recently published.[66] These drugs appear to target proximal mechanisms regulating autophagy and mitophagy, and activation of autophagy and mitophagy plays a key role in mediating their effects (*Figure* 2).[61]

TAT-Beclin 1 competitively inhibits association of Beclin 1 with its negative regulator, GAPR-1, at the intracellular anchorage site, thereby releasing endogenous Beclin 1 and activating autophagy (*Figure* 2).[67] TAT-Beclin 1 activates autophagy and mitophagy in the heart and attenuates progression of heart failure in the presence of high blood pressure. Spermidine rapidly induces autophagy through inhibition of the acetyltransferase EP300 that primarily deacetylates autophagy-related proteins in cytosol in U2OS cells (*Figure* 2).[68] Spermidine provokes epigenetic reprogramming that activates transcription of autophagy-related genes (*Figure* 2).[69] Spermidine also promotes translation of TFEB mRNA, a master-transcription factor for genes involved in autophagy and lysosomal biogenesis, through hypusination of eIF5A in memory B cells (*Figure* 2).[70] Spermidine improves cardiac function in old hearts and its protective effect is accompanied by stimulation of mitophagy. Trehalose, a natural disaccharide, induces translocation of TFEB from cytosol to nucleus, thereby inducing autophagy and lysosomal biogenesis in macrophages and cultured cardiomyocytes (*Figure* 2).[64,71] Urolithin A induces expression of genes involved in autophagy and mitophagy in skeletal muscles, presumably through transcriptional regulation.[63,66] Importantly, the well-established salutary effects of caloric restriction and exercise upon organ functions and ageing are mediated through their ability to stimulate autophagy and mitophagy. Exploring signalling mechanisms and

developing small molecules mimicking the effect of caloric restriction or exercise have proven a productive avenue of research and remain promising for further discovery of effective interventions for heart disease.

7. A Clinical perspective on mitophagy in the heart

Recent advancements in cardiovascular medicine have successfully decreased the early mortality in patients with acute coronary syndrome (ACS). However, many survivors of MI develop heart failure due to increased haemodynamic overload and maladaptive remodelling. As a result, the occurrence of chronic heart failure has increased and cardiovascular disease remains the leading cause of death in western countries. Mitochondrial dysfunction is commonly observed in the failing heart and often precedes the transition from the compensated to the decompensated form of heart failure. Mitochondrial dysfunction is also seen in patients suffering heart failure with preserved ejection fraction. Although mitophagy is activated in the stressed heart, the level of mitophagy appears insufficient and mitochondrial dysfunction eventually develops in the heart. As we discussed, mitophagy is activated only transiently after the initiation of pressure overload; it is inactivated thereafter and mitochondrial dysfunction and heart failure follow. Restoring the level of mitophagy could be a rational intervention to delay cardiac decompensation. Currently, the reason mitophagy activation is only transient in the stressed heart remains unclear. We have shown that mammalian sterile 20 like kinase 1 (Mst1), a potent inhibitor of autophagy, is activated 1 week after pressure overload. Mst1 phosphorylates Beclin 1 at threonine 108, thereby inducing Beclin1-Bcl-2 interaction and inhibiting both autophagy and mitophagy.[72] It is also possible that proteins involved in autophagy and mitophagy may become depleted after prolonged stress. Thus, it is essential to evaluate whether interventions to reactivate mitophagy would be effective in such a negative environment. Furthermore, whether the aforementioned mitophagy inducers activate mitophagy mediated by the canonical mechanism or the alternative mechanism remains to be clarified.

Modulation of autophagy is used to treat or prevent various diseases. For instance, cancer cells acquire resistance against chemotherapy by activating autophagy. The inhibition of autophagy is thus a therapeutic strategy against this type of cancer, and several drugs are being tested in clinical trials.[73] Likewise, autophagy is being investigated as a therapeutic target in the cardiovascular field as well. Earlier studies suggested that the activation of autophagy protects the heart against ischaemia alone but that further activation of autophagy during reperfusion is detrimental.[74,75] Although there was a long-standing controversy regarding whether autophagy is a cause of cell death or not, a new form of autophagic cell death, called autosis and morphologically characterized by a concave nucleus and swollen perinuclear space, was identified in the rat brain subjected to hypoxia-ischaemia.[76] Interestingly, recent evidence has shown that excessive activation of canonical autophagy during I/R induces autosis and exacerbates cell death in the heart.[77] Down-regulation of Rubicon or inhibition of Na^+, K^+-ATPase, a regulator of autosis, significantly ameliorates cardiac injury during I/R. These results suggest that it is important to maintain the activity of canonical autophagy within an appropriate range during I/R in the heart, where mitochondrial clearance is still required. Accordingly, selective induction of mitophagy, without activating canonical autophagy, may be a novel therapeutic strategy against I/R. Stimulating Ulk1-dependent mitophagy through the phosphorylation of Rab9 may be an attractive approach to applying this strategy. It should be noted that excessive activation of mitophagy induces cell death in doxorubicin-induced cardiotoxicity.[78] Thus, it is important to evaluate the levels of autophagy and mitophagy in the heart in a given condition. Currently, it is challenging to evaluate the levels of autophagy and mitophagy in humans in a non-invasive manner. Thus, the development of a convenient, accurate, and non-invasive method for measuring autophagy and mitophagy in the heart *in vivo* is urgently needed.

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