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REVIEW

Comprehensive autophagy evaluation in cardiac disease models

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Abstract Autophagy is a highly conserved recycling mechanism essential for maintaining cellular homeostasis. The pathophysiological role of autophagy has been explored since its discovery 50 years ago, but interest in autophagy has grown exponentially over the last years. Many researchers around the globe have found that autophagy is a critical pathway involved in the pathogenesis of cardiac diseases. Several groups have created novel and powerful tools for gaining deeper insights into the role of autophagy in the aetiology and development of pathologies affecting the heart. Here, we discuss how established and emerging methods to study autophagy can be used to unravel the precise function of this central recycling mechanism in the cardiac system.

Keyword Autophagy • Heart • Chaperone-mediated autophagy • Autosis • Mitophagy • Cardiac diseases

1. Introduction

Autophagy is an evolutionarily conserved mechanism driven by autophagy-related (ATG) proteins devoted to the intracellular recycling of almost every cytoplasmic element, such as senescent organelles and unfolded proteins.[1–5] Three major types of autophagy exist: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy refers to the formation of double-membrane vesicles called autophagosomes that engulf damaged, dysfunctional and redundant proteins, and organelles. Sequestered cargo is then delivered to lysosomes for its degradation. Depending on the cargo that is degraded, macroautophagy can be classified into several subtypes, including mitophagy for mitochondria, lipophagy for lipid droplets (LDs), or ERphagy for endoplasmic reticulum (ER).[2–6] Microautophagy involves the direct engulfment of cytoplasmic cargo by lysosomes or vacuoles (in yeast and plants).[7,8] Although generally considered to be a non-selective

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process, microautophagy is also cargo-specific. Several types of selective microautophagy have been identified: micropexophagy (for peroxisomes),[9] micromitophagy (for mitochondria),[10] microlipophagy (for LDs),[11] and piecemeal microautophagy (for nucleus portions).[12] The third pathway is CMA, which entails the recognition of soluble proteins bearing a KFERQ-like amino acid sequence by the chaperone heat shock cognate 70 (HSC70). The targeted protein is translocated across the lysosomal membrane for degradation with the assistance of the CMA receptor, lysosome-associated membrane protein type 2A (LAMP-2A).[13] Autophagy contributes to the maintenance of cellular functions and survival in both lower organisms, plants, and mammals under basal conditions. Furthermore, this recycling mechanism ensures quality control and regulates the synthesis of new cellular components by providing amino acids, fatty acids, and sugars obtained from the turnover of superfluous cellular constituents. Recent evidence indicates that autophagy may also regulate cellular secretion and intracellular trafficking of specific cellular molecules.[2–6] In addition, all autophagic pathways can be modulated under stress conditions. It has been demonstrated that a plethora of different stress stimuli (e.g. nutritional, metabolic, chemical, physical, etc.) can affect macroautophagy, microautophagy, and CMA regulation.[8,14–16]

Amongst the different subtypes of macroautophagy, mitophagy is the best characterized form of selective macroautophagy, and is responsible for the degradation of redundant or damaged mitochondria via their sequestration into autophagosomes.[17–19] By maintaining the mitochondrial quality control, mitophagy helps to preserve cellular energy production and overall cell function and viability. Mitophagy is typically activated following hallmarks of mitochondrial damage: membrane potential ($\Delta\Psi_m$) collapse, reactive oxygen species (ROS) production, and low ATP levels. Changes in mitochondrial dynamics represent the first step in response to mitochondrial damage, and it is thought that mitochondrial fragmentation is a prerequisite for mitophagy to occur.[17–19] Mitochondrial fusion and fission processes are mediated by different protein subsets. For instance, dynamin-related protein 1 (DRP1) and its adaptor proteins human fission protein (hFIS), mitochondrial dynamics proteins (MID49 and MID51), and mitochondrial fission factor (MFF) regulate mitochondrial division, while mitofusins 1 and 2 (MFN1, MFN2), and optic atrophy protein-1 (OPA1) control mitochondrial fusion.[20–27] Thus, proteins that regulate mitochondrial dynamics also play essential roles in autophagy, and both *in vitro* and *in vivo* models have confirmed that mitochondrial dynamics and mitophagy are indeed highly integrated processes.[18,24,28,29] Regarding the molecular effectors driving mitophagy, defective or senescent mitochondria are selectively targeted for degradation either through pathways that can be dependent or independent on PTEN-induced putative kinase1 (PINK1)-Parkin. PINK1/Parkin-independent mitophagy may constitute an adaptation to chronic Parkin loss and is mediated, in part, by mitochondrial BCL2 Interacting Protein 3 Like L (NIX/BNIP3L) that can directly bind to microtubule-associated protein 1A/1B-light chain 3 (MAP1LC3; best known as LC3) to form the mitophagosome.[2,6,30] Studies suggest that the PINK1/Parkin-dependent pathway primarily functions as an inducible stress-response mechanism in the adult heart.[31] Indeed, loss of $\Delta\Psi_m$ results in the stabilization and accumulation of PINK1 at the mitochondria. PINK1 then phosphorylates the outer mitochondrial membrane (OMM) fusion protein MFN2 and promotes its ubiquitination by recruiting the E3 ubiquitin ligase Parkin onto the OMM, thereby initiating mitophagic clearance.[24,32]

Lipid autophagy (lipophagy), another subtype of macroautophagy, is involved in the catabolism of LDs, including neutral lipids (i.e. triglycerides, cholesterol).[33] Lipophagy is characterized by the engulfment of small LDs or portions of large LDs by autophagosomes, which in turn deliver the lipid cargo to lysosomes, where it is degraded. Degradation products, such as non-esterified free fatty acids (FFAs), are then released into the cytoplasm and reused for other cellular purposes. Lipophagy plays a pivotal role in the regulation of lipid accumulation as well as in lipid homeostasis control.[34] Although lipophagy was first described in the liver, it is now clear that it occurs in many other types of tissues and cells including neurons. This process is induced during fasting and is deregulated in numerous human pathologies, such as liver and neurodegenerative diseases and cancer.[35–37] For example, previous work showed that the absence of Toll-interacting protein (Tollip), an innate immune molecule involved in the formation of autolysosomes, deregulates lipophagy and intensifies liver steatosis and atherosclerosis in mice.[38] Several studies also revealed that lipophagy is involved in lipid synthesis, LD biogenesis, and adipogenesis. Uchiyama and colleagues demonstrated that the Atg conjugation system is critical for lipid synthesis and LD development in liver cells and cardiac myocytes. Moreover, in mice with liver ATG7 deficiency, a defective LC3 conjugation inhibited LD formation.[39,40] Besides, mice with (global) ATG5- and adipose-specific ATG7-deficiency presented a reduction of adipose mass; depletion of *Atg5* or *Atg7* significantly inhibited adipocyte differentiation in 3T3-L1 cells and mouse embryo fibroblasts.[41–43] These results suggest that lipophagy regulates lipid accumulation in adipose tissue and controls adipocyte differentiation. Altogether, these data indicate that lipophagy prevents excess lipid accumulation and modulates lipid metabolism; for these reasons, we could assume that lipophagy should be a promising target for the treatment of cardiometabolic diseases such as atherosclerosis.[36]

Given these findings, one might expect that compromised autophagy and specific forms of macroautophagy are associated with the development of human diseases. In fact, defects in autophagy contribute to the development of cancer, neurodegenerative disorders, excessive lipid accumulation and metabolic defects, or inflammatory disorders. Moreover, autophagy is progressively altered in old age, obesity, and metabolic syndrome, which likely contributes to the abnormalities typically associated with these conditions.[2–6]

Along similar lines, alterations in the CMA pathway have extensively been tied to human diseases. In this regard, it is well known that CMA is required for the maintenance of cellular energetic balance via the finely tuned regulated degradation of enzymes involved in major metabolic routes and through the timely removal of structural proteins from the surface of LDs.[44] Thus, it is not surprising that selective blockage of CMA alters cellular ATP levels and results in aberrant lipid accumulation and metabolic dysregulation.[45] As CMA declines with age, this may contribute to increased susceptibility to stress in aging individuals and worsen age-related disorders.[46,47] Furthermore, it has been shown that deficient CMA activity due to aging negatively affects the adaptive immune system.[48] Reduced CMA activity has also been reported after high-fat/high-cholesterol diets[49] and in several metabolic disorders, such as diabetes mellitus.[50] The rising expansion of the western diets and the fact that our societies tend to have an increasing number of elderly leads to a growing interest in understanding the role of CMA in cardiac disease (CD).

The purpose of this review is to discuss the evidence on the role of different forms of autophagy in the regulation of heart function and to describe the state-of-the-art approaches to study autophagy in cardiac pathophysiology. In particular, we will comprehensively discuss new tools and genetically modified animal models available for the investigation of the activity and function of autophagy/mitophagy and CMA during cardiac stress.

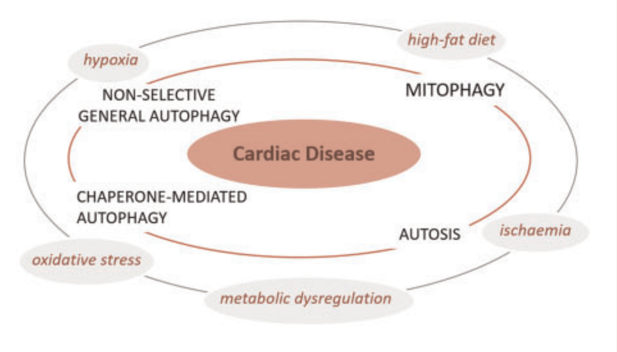

Figure 1 Autophagy and CD. Different stressors such as ischaemia, hypoxia, oxidative stress, and metabolic dysregulation are known to affect non-selective general autophagy, mitophagy, chaperone-mediated autophagy and autosis, which in turn will adversely impact cardiac function to cause CD. Model depicts a converging paradigm wherein loss or exacerbation of distinct forms of autophagy results in the development of CD.

2. The role of autophagy in the heart

Autophagy plays unique, tissue-specific roles in maintaining homeostasis. In the recent years, a number of studies have demonstrated that autophagy is a major factor in the regulation of cardiac homeostasis under basal conditions and during cardiac adaptation to stress (*Figure 1*).[20] An overview of the role of autophagy in different cardiac conditions is outlined below.

2.1 Autophagy: baseline vs. aging

Growing lines of evidence suggest that autophagy plays a pivotal role in preserving cardiac structure and function. Mice with inducible cardiomyocyte-specific *Atg5* gene deletion display contractile dysfunction and left ventricular dilation, along with early mortality. At the cellular level, *Atg5*-deleted cardiomyocytes exhibit protein aggregation and mitochondrial dysfunction[51] that likely contribute to heart failure. Autophagy is also required for delaying cardiac aging. Indeed, age-associated progressive loss in autophagic activity contributes to the development of cardiac senescence, hypertrophy, and diastolic dysfunction.[22] Conversely, systemic overexpression of *Atg5* and expression of a gain-of-function mutation of *Beclin 1* increase levels of autophagy and extend lifespan in mice while reducing age-related cardiac fibrosis.[23,52] In accordance with these data, it was also recently demonstrated that administration of spermidine, a natural polyamine, significantly reduces cardiac aging and extends lifespan through autophagy activation. In fact, when administered to cardiac-specific *Atg7* knockout mice, spermidine failed to confer cardioprotection, indicating that this benefit is mediated via autophagy.[24] Spermidine induces autophagy by inhibiting the activity of the acetyltransferase adenovirus early region 1A (E1A)-binding protein p300 (EP300),[53,54] a major repressor of autophagy.[55] Autophagic clearance of mitochondria also appears to be reduced during aging. A reduction of p53-induced Parkin translocation to mitochondria represents one of the mechanisms underlying this mitophagy defect.[24] Consistent with this idea, overexpression of Parkin has been shown to reduce age-associated cardiac abnormalities.[25]

2.2 Autophagy in myocardial ischaemia and reperfusion

Cardiac tissue is exquisitely sensitive to ischaemia resulting from atherosclerotic blockage and/or thrombotic occlusion in one or more coronary arteries, a major cause of mortality worldwide. Autophagy and mitophagy each play pivotal roles in cellular homeostasis and adaptation to injury in the heart.[20] Studies of CDs in animal models have shown that activation of autophagy in myocardium limits cardiac senescence, ischaemic injury, chronic cardiac remodelling, genetic cardiomyopathy, and heart failure.[52,56–58] Similar beneficial effects are elicited when mitophagy is stimulated.[59] During an ischaemic episode, activation of autophagy promotes quality control by degrading dysfunctional mitochondria and lowering ROS levels. Induction of autophagy also provides substrates for ATP production and thereby sustains cellular energy levels.[2–6] Evidence for this notion comes from studies showing that cardiac-specific overexpression of a dominant-negative form of 5’ AMP-activated protein kinase (AMPK) or forced activation of mechanistic target of rapamycin complex 1 (mTORC1), through the overexpression of Ras homologue enriched in brain (RHEB), each inhibited autophagy and significantly exacerbated myocardial injury following prolonged ischaemia without reperfusion.[26,27] Similarly, cardiac-specific deletion of *Nox4*, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoform, showed impaired autophagy activation during ischaemia and increased ischaemic injury.[28]

While much is known about autophagy during ischaemia, the role of autophagy activation during reperfusion is largely unclear. When compared to ischaemic injury, reperfusion injury is mediated by distinct pathophysiological mechanisms. Generally, during reperfusion, a marked accumulation of ROS is observed, contributing to the progression of myocardial injury. It has been shown that autophagy is stimulated during reperfusion through Beclin 1-dependent mechanisms.[26] Accordingly, mice with systemic heterozygous *Beclin 1* gene deletion displayed a significant reduction in ischaemic injury suggesting that *Beclin 1*-dependent upregulation of autophagy is deleterious during reperfusion.[26] Consistent with this hypothesis, partial glycogen synthase kinase-3β (GSK3β) inhibition reduced ischaemia/reperfusion injury via mTOR-dependent suppression of autophagy.[29] In contrast, pharmacological inhibition of histone deacetylases decreased ischaemia/reperfusion injury through autophagy stimulation.[30] Nevertheless, Ma *et al.*[31] reported that autophagosomes accumulate in the heart in response to reperfusion injury as a result of the concomitant impairment in autophagic flux and increased autophagosome formation due to Beclin 1 induction. The authors showed that, beyond its primordial function in the initiation of the autophagic cascade, Beclin 1 expression can also be associated with a decrease in total LAMP-2 levels resulting in reduced autophagosome-lysosome fusion. Surprisingly, heterozygous *Beclin 1* gene deletion normalized autophagosome levels, likely by supporting the efficient clearance of autophagosomes, and reduced reperfusion injury. All in all, the precise role of autophagy in ischaemia/reperfusion injury remains under debate.

As previously discussed, the physiological level of autophagy activation in basal conditions or following an acute pathologic insult is generally considered cardioprotective. However, excessive or uncontrolled levels of autophagy activation possibly trigger cardiac cell death in some circumstances, thus contributing to myocardial injury.[20] The so-called ‘autophagy-dependent cell death’ is a type of regulated cell death that relies on the autophagic machinery (or components thereof) as defined in the latest nomenclature for cell death guidelines.[60] For instance, cardiac cell death is attenuated by autophagy inhibition in some stress conditions including ischaemia/reperfusion,[26] pressure overload,[21] and doxorubicin-induced cardiomyopathy,[61] implicating a detrimental role of autophagy in the heart. Doxorubicin, a member of anthracyclines molecule class, has been used for a long time as an anti-neoplastic drug because of its ability to intercalate with DNA and inhibit topoisomerase II. Unfortunately, the therapeutic efficacy of doxorubicin is limited by its severe side effects, particularly on the myocardium, resulting in arrhythmias, ventricular dysfunction, and heart failure. Doxorubicin-induced cardiotoxicity is mostly caused by oxidative and ER stress, lipid peroxidation, calcium deregulation, and inhibition of the autophagic flux that leads to accumulation of autophagosomes/autolysosomes and undegraded material.[62–64] However, whether autophagy really contributes to cardiomyocyte death is still controversial. Equally elusive are the morphological or biochemical features of cell death induced by autophagy. Recently, Dr Levine's group provided evidence that excessive autophagy induces a unique type of autophagic cell death with characteristic morphological and biochemical features. This form of cell death, specifically relying on the Na^+/K^+-ATPase, was termed autosis.[65] Consistent with the physiological relevance of this process, administration of Na^+/K^+-ATPase inhibitors, such as cardiac glycosides, confers neuroprotection in a rat model of neonatal hypoxia–ischaemia.[65]

There is consensus that mitochondrial clearance limits ischaemia/reperfusion injury in the heart. Mice with cardiac-specific heterozygous deletion of *DRP1*, a mitochondrial fission protein, showed an increase in ischaemia/reperfusion injury that correlated with mitochondrial dysfunction and inhibition of mitophagy.[32] Similarly, mice knocked-out for the mitochondrial protein phosphoglycerate mutase family member 5 (PGAM5), accelerated necroptosis in the heart following ischaemia/reperfusion injury. This effect occurred through the inhibition of mitophagy that caused mitochondrial abnormalities and oxidative stress.[66]

In accordance with its role as a stress-response mechanism, CMA has been shown to be activated during hypoxia/ischaemia and oxidative stress thus contributing to cell survival during these conditions.[67,68] Consequently, it is plausible that changes in CMA (e.g. its decline associated with aging) may impact cardiac function in response to ischaemic and/or oxidative insults.

2.3 Autophagy during CD

Numerous studies have demonstrated that autophagy is necessary for the ability of the heart to respond to mechanical stress. Mice with cardiac-specific *Atg5* gene deletion displayed cardiac hypertrophy, left ventricular dilation, and contractile dysfunction when subjected to transverse aortic constriction.[51] This functional decline was paralleled by increased protein ubiquitination, disorganized sarcomere, and accumulation of dysfunctional mitochondria in the heart.[51] Indeed, partial activation of autophagy during severe mechanical stress is beneficial. In fact, Zhu *et al.*[21] demonstrated that cardiac overexpression of *Beclin 1* accentuates pathologic remodelling whereas heterozygous deletion of *Beclin 1* gene blunts this process in mice subjected to severe pressure overload. It is now largely accepted that, during chronic ischaemic remodelling, autophagy activation exerts protective effects. Consistent with this notion, activation of autophagy by deleting mammalian STE20-like kinase 1 (*Mst1*) gene reduced infarct size, cardiac dysfunction, and dilation in response to chronic myocardial infarction.[69] Trehalose, a natural disaccharide synthesized by simpler organisms and previously described as an autophagy inducer, is able to reduce chronic cardiac remodelling induced by myocardial infarction. These effects are autophagy-dependent since the beneficial effects of trehalose were in part attenuated in *Beclin 1*$^{+/-}$ mice.[58] Aspirin is also an important drug for the secondary prevention of CD, reducing both the risk of CD and mortality.[70,71] Interestingly, it was recently reported that aspirin and its active metabolite salicylate trigger autophagy via the inhibition of the EP300 activity, suggesting a novel molecular mechanism underlying the pro-health effects of these compounds.[72]

As one might expect, the benefits of autophagy in preventing chronic cardiac stress are mediated in part through mitophagy. In agreement with this idea, mice with cardiac-specific *Drp1* gene knockout display impaired mitophagy in response to pressure overload and develop mitochondrial dysfunction, cardiac hypertrophy, and heart failure.[73] Mitophagy is also involved in cardiac hypertrophy after myocardial infarction. Indeed, Kubli *et al.*[74] demonstrated the presence of cardiac hypertrophy and larger infarct sizes in *Parkin*-deficient mice after myocardial infarction. Accordingly, wild-type animals were characterized by increased Parkin expression and stimulation of mitophagy in areas bordering the myocardial infarct. Interestingly, autophagy-mediated mitochondrial turnover/quality control is important to reduce the amount of mitochondrial DNA released from the injured tissue, which is thought to drive myocardial inflammation and heart failure during mechanical stress.[75]

2.4 Autophagy and metabolic cardiomyopathy

Several studies have investigated the impact of diabetes on autophagy in the heart. The majority of these studies have demonstrated that metabolic alterations impact cardiac autophagy at multiple steps.[76] Under pathological conditions, cardiac autophagy and mitophagy are both inhibited in models of diabetes and metabolic syndrome, likely with deleterious consequences.[27,77] Decreased myocardial autophagy was reported at basal levels and in response to ischaemia in mice fed with high-fat diet (HFD). A reduction of cardiac autophagy was also detected in the heart of hypercholesterolaemic pigs.[78,79] Reactivation of autophagy via rapamycin administration partially rescued the deleterious effect of HFD on ischaemic injury.[27] Levine and Mentzer labs each observed a significant reduction in autophagosome formation in mice with HFD-induced obesity.[80–82] Other studies corroborated these findings by demonstrating that HFD inhibits autophagosome formation in the heart, which can be reversed by a number of interventions including macrophage migration inhibitory factor (*Mif*) gene disruption,[83] overexpression of catalase,[84] and ablation of the toll-like receptor 4 (*Tlr4*) gene.[85] Consistent with this notion, cardiac autophagic flux is impaired in hearts of mice with type II diabetes. Indeed, autophagosome clearance is significantly suppressed in hearts of mice fed with HFD.[86] Similarly, autophagosome maturation was found to be impaired in obese mice due to AKT2-dependent mTOR activation,[87] a well-established inhibitor of autophagy. Both type I and type II diabetes are associated with reduced cardiac autophagy. However, the functional consequence of such impairment remains under-explored.

Since AMPK activity leads to induction of autophagy, numerous studies have explored the effect of AMPK on cardiac function and found that AMPK activation improves cardiac function by reactivating autophagy in the heart.[77,88] The mechanisms through which this occurs remain unclear, but it may involve improved fuel utilization and fuel-type switch in the face of acute myocardial injury. There are contrasting studies suggesting that decreasing levels of a subset of autophagy proteins protects against diabetic cardiomyopathy. For example, overexpression of *Beclin 1* exacerbates diabetic cardiomyopathy, while inhibiting *Atg16* gene attenuates cardiac derangements in mice with type I diabetes. Surprisingly, inhibition of *Atg16* gene expression restores mitophagy (likely in an ATG16-independent manner) in diabetic mice through the upregulation of PINK1 and Parkin, suggesting that stimulation of mitophagy could be a potential therapeutic approach against diabetic cardiomyopathy.[89]

CMA selectively degrades not only enzymes involved in carbohydrate and lipid metabolism but also proteins important for mitochondrial homeostasis.[90] Indeed, CMA activity is compromised in mice with HFD-induced obesity.[49] The decline in CMA activity in these lipogenic stress conditions is due to changes in the lipids at the lysosomal membrane, resulting in decreased stability of the CMA receptor LAMP-2A. Interestingly, these HFD-triggered changes of the lysosomal membrane composition resemble those observed in aging.[49] Given the metabolic relevance of this autophagic pathway, the role of CMA in cardiomyocytes is currently the focus of investigation.

2.5 Mutations in human ATG genes associated with CD

Several mutations in genes linked to or required for autophagy and lysosomal function are associated with the development of CDs. Among them, the mutation in the *LAMP-2* gene that causes Danon's cardiomyopathy is perhaps the best characterized one.[91,92] In this regard, the pathological hallmark of the disease is the accumulation of intracytoplasmic vacuoles containing autophagic material and glycogen in skeletal and cardiac muscle cells. Recessive mutations in Ectopic P-Granules autophagy protein 5 homologue (*EPG5*), a key factor implicated in the autophagolysosomal fusion and maturation of autolysosomes, play a causative role in Vici syndrome.[93,94] This is a recessively inherited multisystem disorder characterized by callosal agenesis, cataracts, hypopigmentation, cardiomyopathy, psychomotor retardation, and immunodeficiency with cleft lip and palate. Mutations in *GBA1*, gene encoding the lysosomal enzyme glucocerebrosidase, result in defects in autophagic-lysosomal function. GBA1 loss of function is responsible for the Gaucher's disease, in which glucocerebroside accumulates in cells and organs. Gaucher's disease type 3C is also defined as the cardiovascular form; cardiovascular abnormalities consist of calcification of the ascending aorta and of the aortic and mitral valves, leading to reduced lifespan.[95] From a cardiometabolic point of view, a recent study identified single-nucleotide polymorphisms in several autophagy genes, such as *ATG7, MAP1LC3A, ATG3, ULK1*, or different *ATG4*s, associated with alterations in systolic and diastolic blood pressure and coronary artery disease.[96] Moreover, exclusive DNA sequence variants of *ATG7* gene promoter have been recently found in acute myocardial infarction patients.[97]

3. Methods for the assay of different forms of autophagy

In the following sections, several techniques useful for the adequate and precise study of the different forms of autophagy are described (*Table 1*).

3.1 Non-selective general autophagy

In the 1950s, autophagy and lysosomal biology were largely studied at the morphological/ultrastructural level by electron microscopy (EM) or at the biochemical level by determining the degradation of long-lived proteins.[98] In the 1990s, ATG molecules were identified using simpler systems (yeast cells) and genetic approaches, and since then, the analysis of autophagy has undergone significant advances. In principle, given that autophagic degradation is a highly dynamic process, and because of differences in the rates of cargo degradation across different cell types and in response to various stressors, the assessment of autophagy requires the measurement of autophagic flux involving the use of lysosomal inhibitors. Nevertheless, given the dynamic nature of the autophagic process, multiple complementary approaches should be considered for its evaluation. A common strategy is to estimate the number of autophagosomes by observing and quantifying their number. However, the net/steady-state level of autophagosomes in a given moment is the balance between the rate of their generation and degradation. This underscores the need for measuring the complete flux for accurate assessment of autophagy activity. Monitoring the number of autophagosomes is typically carried out by EM, fluorescence detection of LC3, and biochemical assays as discussed below.

3.1.1 Electron microscopy

Despite new methods have been developed to study autophagy, EM remains the gold standard to analyse the ultrastructural details of autophagic structures to date (*Figure 2*).

An autophagosome is a double-membrane structure generated from an isolation membrane at distinct sites in the cell, though it is still unknown which cellular structures contribute to the formation of autophagosomes. These vesicles, as discussed earlier, sequester cargo and deliver it to lysosomes. EM images of an autophagosome typically reveal a double-membrane vacuole with a clear gap between the two membranes (*Figure 2*, arrow). EM is also used to determine the nature of the contents sequestered, allowing to distinguish between autophagosomes and those that have already fused with lysosomes (*Figure 2*, asterisk). Ultrastructural analyses reveal that sequestered organelles are largely intact in autophagosomes and allow for visualization of individual mitochondria engulfed during mitophagy. Mitochondria sequestered within autophagosomes can be readily detected through identification of cristae enclosed within the double-membrane autophagosomes. However, autophagosome fusion with lysosomes (forming the autolysosomes) leads to the rapid digestion of the cargo, which is apparent by the presence of material at various stages of degradation. Since the inner autophagosomal membrane is digested upon fusion with lysosomes (*Figure 2*, arrowhead), an autolysosome is typically observed as a single-membrane vacuole with an amorphous structure and high electron density.[99] Indeed, late stage mitophagy is reflected by the single-membrane autolysosomes with residual mitochondria that might be difficult to identify.[100] Immunogold EM for specific mitochondrial markers could also help confirm the nature of mitochondria in these late stages of mitophagy. Although EM is a powerful tool to evaluate the ultrastructural details of autophagic structures, it does not allow to determine whether autophagy is active or suppressed. In addition, this time-consuming approach is subject to possible artefacts in case fixation of samples and tissue sectioning have been inappropriately performed. Moreover, it may be difficult to preserve the integrity of the membranes. The ferrocyanide-reduced osmium method has proven useful for the preservation and contrast of membranous structures,[101] and thus, is a method of choice for the detection of autophagosomes and autolysosomes. However, caution must be exerted when visualizing representative areas in a reasonably large number of replicates as this method is largely non-quantitative due to the limited numbers of sections and cells, and in differentiating autophagic structures from organelles such as rough ER,

Table 1 Methods for the assay of different forms of autophagy

Form of autophagy assayed	Method	Sensor/marker	Features observed	Limitations
Non-selective general autophagy	Electron microscopy	–	Double-membrane structures (autophagosomes); Single-membrane, electron dense vacuoles (autolysosomes)	Subject to artefacts, large number of replicates required
	Fluorescence microscopy	GFP-LC3	GFP-LC3 puncta indicative of autophagosome levels	Aggregates formation, GFP fluorescence quenched at low pH (no information on autophagy flux)
		mRFP-GFP-LC3	Green and red fluorescent puncta indicate autophagosomes, red-only puncta indicate autolysosomes	–
		GFP-LC3-RFP-LC3ΔG	GFP/RFP ratio indicates autophagic flux	Poor time resolution
		DAPGreen/DALGreen	Enhanced fluorescence under lipophilic (DAPGreen) or acidic (DALGreen) conditions	
	Western Blot	LC3-II	LC3-II levels and turnover directly correlate with the autophagic flux/activity	Assess in the presence and absence of lysosomal inhibitors
		p62	p62 levels inversely correlate with the autophagic flux/activity	p62 levels are also transcriptionally regulated; p62 can be degraded by the proteasome
Mitophagy	Electron microscopy	–	Individual mitochondria engulfed by autophagosomes or autolysosomes	Non-quantitative due to limited number of sections, large sample variability
	Fluorescence microscopy	Mito-Keima	Ratio between green (mitochondria) and red fluorescence (lysosomes)	Fixation disrupts the lysosomal pH, excitation spectra partially overlap
		Mito-Timer	Shift from green to red fluorescence over time	–
		Mito-QC	Predominant red fluorescence upon mitophagy induction	–
		Co-labeling for GFP-LC3 or lysosomes and mitochondrial proteins	Colocalization of mitochondria with autophagosomes and/or lysosomes	Mitochondrial degradation confirmed upon addition of lysosomal inhibitors, GFP-LC3 aggregation
	Assessment of mitochondrial mass by western blot and real time PCR	Levels of mitochondrial proteins, mitochondrial-to-nuclear DNA ratio	Altered levels of mitochondrial proteins and mtDNA	Some mitochondrial proteins are degraded through proteasome or CMA; mtDNA levels may change due to alterations in mitochondrial biogenesis
Chaperone-mediated autophagy	Fluorescence microscopy	KFERQ-Dendra2	Fluorescent puncta indicative of CMA activity	Indicates only the binding step in the CMA pathway
		Co-staining for HSC70 and LAMP-2A	Indicates the number of CMA active lysosomes	
	Western blot	LAMP-2A	Lysosomal LAMP-2A levels are proportional to CMA activity	CMA activation may occur without *de novo* synthesis of LAMP-2A
		Lysosomal uptake of CMA substrates	The amount of translocated substrate is proportional to CMA activity	
	Metabolic labeling	Radio-labelled proteins	Accumulation of radio-labelled proteins with inhibitors of lysosomal proteases or macroautophagy	CMA and microautophagy cannot be differentiated
Autosis	Electron microscopy	–	Intense vacuolization, swelling of the perinuclear space, nuclear concavity	Nuclear concavity may also appear in other cell types (i.e. endothelial cells)
	Fluorescence microscopy	Nuclear staining	Nuclear concavity	Nuclear concavity may also appear in other cell types (i.e. endothelial cells)
	Fluorescence microscopy	ER proteins immunostaining	ER fragmentation	Not a definitive marker of autosis

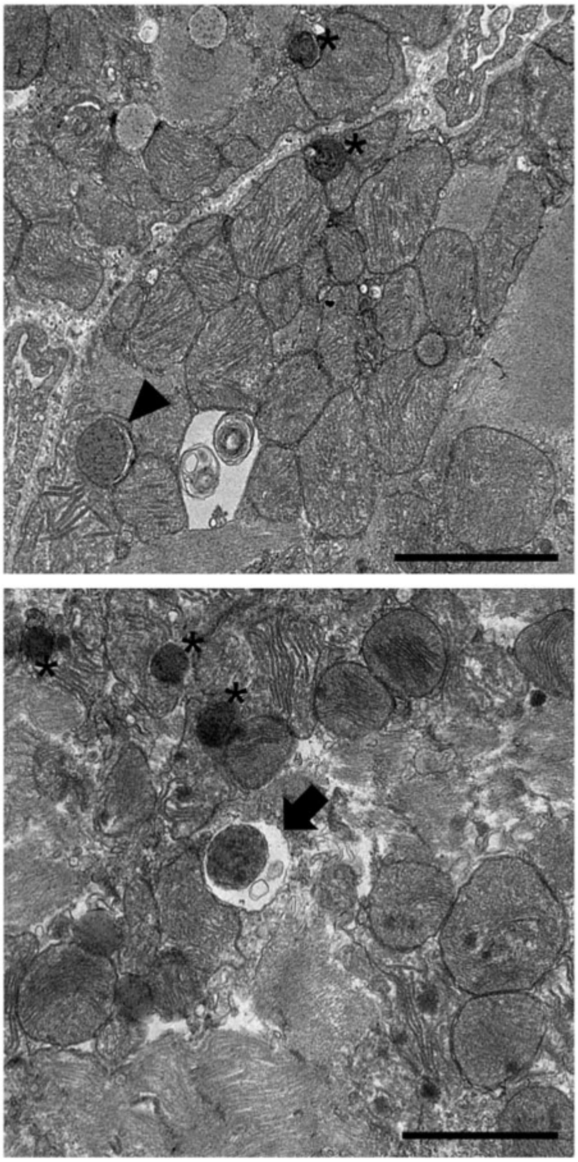

Figure 2 Electron microscopy analysis of autophagic structures. Three-month old male C57BL/6 mice were subjected to 30 min ischaemia and 2 h reperfusion, and hearts with infarction border area were analysed by electron microscopy. Representative images indicate an autophagosome (arrowhead), an autolysosome (arrow), and a lysosome (asterisk). Scale bar: 2 µm.

swollen mitochondria, and vacuoles of uncertain origin. Thus, EM must be used in combination with other assays for the accurate assessment of autophagic activity.

3.1.2 Fluorescence microscopy

The identification of LC3, a mammalian homologue of yeast Atg8 and the molecular marker of autophagosomes, undoubtedly facilitated autophagy monitoring. Pro-LC3, the unprocessed form of LC3, undergoes proteolytic cleavage by ATG4, which converts pro-LC3 to LC3-I. LC3-I is the soluble cytosolic form of LC3. Upon autophagic stimulus, LC3-I is conjugated to phosphatidylethanolamine (PE) through a ubiquitin-like reaction, resulting in the formation of LC3-II, the membrane-bound form of LC3. LC3-II is the only protein that specifically localizes to autophagosomes throughout the process, from the formation of the isolation membrane to its lysosomal degradation. Therefore, assessing LC3 lipidation is the most accurate way to follow the process of autophagy.[102,103]

Consequently, the development of modern tools to study autophagy evolved around LC3. Green fluorescent protein (GFP) fused to the *N*-terminus of LC3 (GFP-LC3) is broadly used as an autophagosome reporter. GFP-LC3 is thought to behave in the same way as the endogenous LC3.[104] When expressed in cells, GFP-LC3 distributes diffusely throughout the cytoplasm and nucleus reflecting the soluble LC3-I form. Upon autophagy induction, punctate LC3 signals can be observed in the cytoplasm, representing the autophagosome-bound LC3. Therefore, increases in autophagosome content are reflected by the number of LC3 puncta per cell or the number of cells with an augmented number of LC3 puncta, as compared to cells maintained in the basal/non-stimulated state. Since ATG5 is required for the conversion of LC3-I to LC3-II, LC3-positive punctate structures do not form in *Atg5*-deficient cells[105] or in cells expressing LC3 mutated at the amino acid residue required for PE conjugation,[106] indicating the specificity of GFP-LC3 in measuring autophagosome levels. This molecule is also useful for tracking the formation, localization, and turnover of autophagosomes in live cells. The identification of LC3 as an autophagosome marker and the development of tools based on the specificity provided by LC3 is a great advantage over the use of EM.

There are, however, several pitfalls in monitoring autophagy activity using the GFP-LC3 reporter. First, since GFP itself can be aggregate-prone, LC3-positive aggregates may be misinterpreted as true LC3 puncta. Also, LC3 may bind to other membranes in the cell. Thus, complementary analysis should be performed to further confirm its presence in autophagosomes. This is particularly true when GFP-LC3 is transiently overexpressed or co-expressed with other aggregation-prone proteins. To circumvent this issue, the use of transformants that stably express GFP-LC3 is highly recommended. Protein aggregates in cells could also cause LC3 puncta formation through the incorporation of p62/sequestosome1 (SQSTM1), a well-established cargo adapter for autophagy.[107] More importantly, as mentioned in the EM section, the number of autophagosomes calculated from GFP-LC3 puncta do not reflect autophagic activity. In other words, GFP-LC3 only reveals the steady-state levels of LC3 but not autophagy activity or flux. This problem can be solved by assessing autophagy flux.

In context of fluorescence-based approaches, a construct that combines a red fluorescent protein with GFP-LC3 (mRFP-GFP-LC3) wherein mRFP and GFP are tandemly fused to the *N*-terminus of LC3 is a useful tool for measurement of autophagy flux (*Figure 3*).[108] Upon delivery of the reporter to the lysosome, the GFP signal is quenched by the low pH/acidic environment. In contrast, the RFP is acid-stable and continues to fluoresce in the acidic environment. Using this construct, yellow (merged RFP and GFP signal) puncta indicate autophagosomes while red (RFP-positive and GFP-negative) puncta indicate autolysosomes. When both yellow and red puncta are increased, autophagic flux is considered to be high; however, robust autophagy induction is also reflected by a significant increase in red puncta, while the yellow puncta are unaffected in number. On the other hand, increases or no changes in yellow puncta without an accompanying increase in the red punctate signal indicate that autophagy flux/activity is blocked (*Figure 3*).[102]

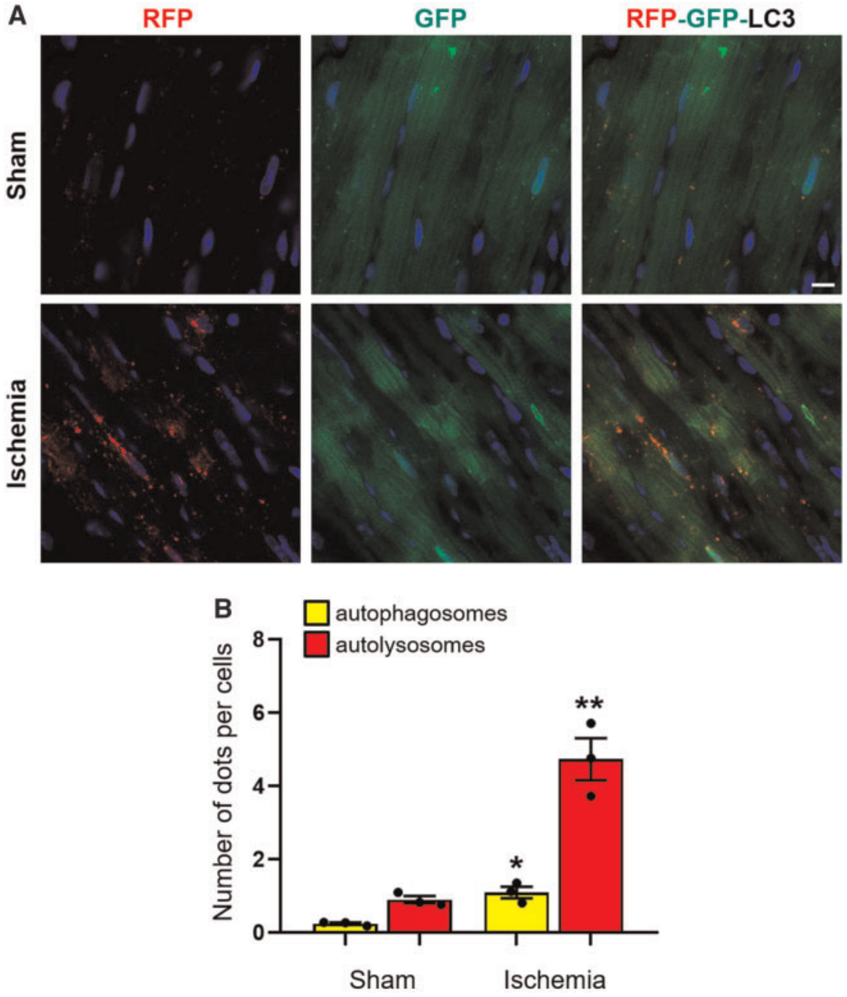

Figure 3 Cardiac-specific-tandem-fluorescence mRFP-GFP-LC3 (tf-LC3): (*A*) three-month-old male C57BL/6 transgenic mice expressing cardiac-specific-tandem-fluorescence mRFP-GFP-LC3 (tf-LC3) were subjected to 30 min ischaemia and hearts with infarction border area were analysed by confocal microscopy to assess autophagic flux (scale bar: 10 µm). (*B*) The quantification of autophagosomes (yellow puncta) and autolysosomes (red puncta) is shown (mean values ± SE, $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. sham).

In addition to the genetic approaches mentioned above, fluorescent small molecules can also be used to visualize autophagosomes and monitor autophagy. Though these probes are easy to use without the need for genetic engineering, there are limitations such as low specificity, cytotoxicity, the relative lack of information on materials and methodology for their optimal use across different systems, as well as the requirement of specialized instruments/training in generating these molecules. Recently, novel small molecule probes DALGreen and DAPGreen that stain autophagosomes were developed.[109] It appears that the terminal amino group of DALGreen and DAPGreen is necessary for the incorporation of these dyes into the autophagosomal membrane by mimicking intramembrane phospholipids such as phosphatidylethanolamine. DAPGreen detects both autophagosomes and autolysosomes given that it is incorporated into the autophagosome during double-membrane formation and emits fluorescence under hydrophobic conditions without being affected by the pH. On the other hand, the fluorescence of DALGreen is enhanced in the acidic pH range and is suitable for monitoring autophagy activity/flux. It is likely that in the future, these probes could find wide use in the evaluation of autophagy across diverse model systems.

3.1.3 Flow cytometry

In the recent past, conventional or multispectral imaging flow cytometry have been proposed as alternative approaches for the high-throughput assessment of autophagic flux in live or fixed cells.[102,110] These methodologies, which have been optimized for cells growing in suspension or circulating leukocytes, account for the study of autophagy dynamics in a large number of cells. The imaging flow cytometry combines the statistical power of flow cytometry with the information content of fluorescent microscopy. Associating the flow cytometry analysis of autophagic flux with that of other parameters such as cellular morphology, complexity, and (as in the case of concomitant detection of surface markers) phenotype, permits to accurately capture cell type-specific heterogeneity in autophagy levels.

Flow cytometry monitoring of autophagic flux relies on the expression of GFP-LC3 probe by target cells. Since GFP-LC3 is targeted for lysosomal degradation, total fluorescence intensity of GFP-LC3 can be used as a proxy to infer autophagy levels in cells; in conditions of autophagy induction (i.e. starvation) total GFP fluorescence intensity decreases. Alternatively, transient extraction with saponin (which depletes from cells soluble GFP-LC3I but not autophagosome-bound GFP-LC3II) can be performed prior to flow cytometry analysis; in this second scenario, an increase in GFP residual fluorescence will indicate an upregulation of the autophagic activity. In both settings, autophagy-inducing treatments should be compared in presence or absence of lysosomal inhibitors (see below) for an accurate measurement of autophagic flux.[102] In line with these observations, a recent work has demonstrated that flow cytometry assessment of GFP-LC3 fluorescence could be successfully adopted to monitor autophagy in HL-1 cardiomyocytes.[111]

Despite the potential application of flow cytometry to monitor autophagic flux, these strategies are currently under used. In most of the cases, these methods only allow to measure global changes in GFP-LC3 levels, regardless of its transcriptional or translational fluctuations.

In addition, preparation of single cell suspensions of adherent cells or saponin extraction procedures impose elevated levels of stress to target cells, which in turn may lead to autophagy induction secondary to treatment. To bypass this issue, it is highly recommended to stain cells with viability dyes and to carefully titrate saponin concentration in order to minimize cellular toxicity and GFP-LC3 self-aggregation.

3.1.4 Biochemical assays

LC3 is commonly used to assess autophagosome number and autophagy activity also via biochemical approaches. The conversion of LC3-I to LC3-II, reflecting LC3 lipidation, is easily detectable by immunoblotting. The hydrophobicity of lipidated LC3-II increases its electrophoretic mobility in the SDS-PAGE, causing its separation from LC3-I. However, pro-LC3 (which is recognized by commercially available antibodies) also shows higher electrophoretic mobility than LC3-I[112] and may be mistaken with LC3-II. Thus, it is always important to get a good separation of the bands using specific gels (e.g. Tris-glycine or gradient gels), as well as trying to visualize both LC3-I and LC3-II bands, as it will confirm the correct lipidation of LC3. Whatever the case may be, the amount of LC3-II correlates well with the number of autophagosomes in the cell.[113] Although some investigators recommend the use of polyvinylidene difluoride membrane with 0.20 µm pore size for the immunoblotting of LC3,[114–116] nitrocellulose membrane provides sufficient sensitivity to distinguish between differences in LC3-II in different samples. Most importantly, it is necessary to perform complete autophagic flux assays (*Figure 4A–C*), which will evaluate whether the increased number of autophagosomes is due to autophagy induction (*Figure 4C*, scenario 1) or is rather a consequence of an impairment in autophagosome-lysosome fusion or blocked degradation in lysosomes (*Figure 4C*, scenario 2). The differences in the intensity (densitometric values) of LC3-II between samples in the presence or absence of lysosomal inhibitors represent autophagic flux. For this purpose, inhibitors of lysosomal proteases such as bafilomycin A (that blocks autophagosome-lysosome fusion), or a combination of ammonium chloride (a base that dissipates lysosomal pH) and leupeptin (inhibitor of lysosomal proteases), as well as E64 and pepstatin A, are commonly used. *Ex vivo* flux of the tissue of interest can also be used to assess the autophagic activity (*Figure 4B*). Chloroquine and NH_4Cl/Leupeptin have been successfully used to block lysosomal degradation in cardiac cells. In primary cultures of adult mouse cardiomyocytes or neonatal rat ventricular myocytes, it is sufficient to treat the cells 2–4 h prior to cell lysis either with 50 µM chloroquine, 50 nM bafilomycin A, or 20 mM/100 µM NH_4Cl/leupeptin ([117] and data not published). It is important that chloroquine and ammonium chloride solutions are freshly prepared each time. To inhibit the autophagic flux *in vivo*, different groups have used chloroquine concentrations ranging from 10 to 50 mg/kg injected i.p. in mice 4–6 h before sample collection.[52,117–119] Another study reported the use of bafilomycin A for *in vivo* experiments; mice were injected with 6 µmol/kg i.p. and sacrificed after 30 min.[89]. In these *in vivo* experiments it is essential to synchronize autophagy induction/suppression in the animals by starvation/re-feeding regimen (24 h of starvation plus 3 h of feeding) thereby reducing the variability due to differences in food intake. The evaluation of autophagic substrates protein levels may also be used to study autophagy flux in combination with additional supporting methods. However, caution should be exercised regarding the inclusion of controls to confirm that changes in levels of these additional proteins in response to a given treatment are not due to alterations in gene expression. One such protein is p62/SQSTM1, a well-studied autophagy substrate which directly binds to LC3 and is degraded by autophagy. One concern is that p62/SQSTM1 can also be degraded by the proteasome;[120,121] consequently, the assessment of autophagy flux/activity must be done in the presence of lysosomal inhibitors. In principle, the protein level of p62/SQSTM1 is considered to inversely correlate with autophagic activity.[122] However, levels of p62/SQSTM1 are also transcriptionally regulated during autophagy and/or in response to stressors,[120,121] which may be misinterpreted as an increase in autophagic activity. Alternatively, information about autophagic flux status can accurately be inferred by monitoring the degradation of radioactively[123] or non-radioactively[124] labelled long-lived proteins.

3.2 Mitophagy

Several approaches and molecular tools have been established to study mitophagy both in neonatal and adult cardiomyocytes, or other cell types in the heart (for instance, cardiac fibroblasts). The most commonly used methods to study mitophagy in cells assess changes in mitochondrial mass, ultrastructural changes in mitochondria via transmission EM (described in Section 3.1), and fluorescence microscopy for colocalization of mitochondria with autophagosomes or lysosomes.[100,102] More recently, several novel assays have been developed, including MitoTimer, Mito-Keima, and Mito-QC, as discussed in Section 4.2. Importantly, these assays can be used to monitor and quantify mitophagy *in vivo* in diverse tissues.

3.2.1 Assessment of mitochondrial mass

Analysis of mitochondrial mass is the most reliable readout of mitophagy. This can be done by immunoblotting, measuring the relative amount of mitochondrial proteins in relation to levels of classical cellular protein loading markers. It is recommended to cover all mitochondrial compartment proteins, including the outer [e.g. translocase of outer membrane-20 (TOM20), voltage-dependent anion-selective channel protein (VDAC)] and inner mitochondrial membrane proteins [such as cytochrome C oxidase (COX) or ADP/ATP translocase 1 (ANT)], intermembrane space (cytochrome C), and matrix proteins [manganese superoxide dismutase (MnSOD), heat shock protein-60 (HSP60)]. This precaution is justified by the fact that some OMM proteins are degraded by the proteasome and are therefore not reliable markers of mitophagy,[100] while others have been identified as substrates for CMA.[90] In this regard, measurement of citrate synthase activity, a citric acid cycle enzyme generally not affected by the depletion of mtDNA or respiratory

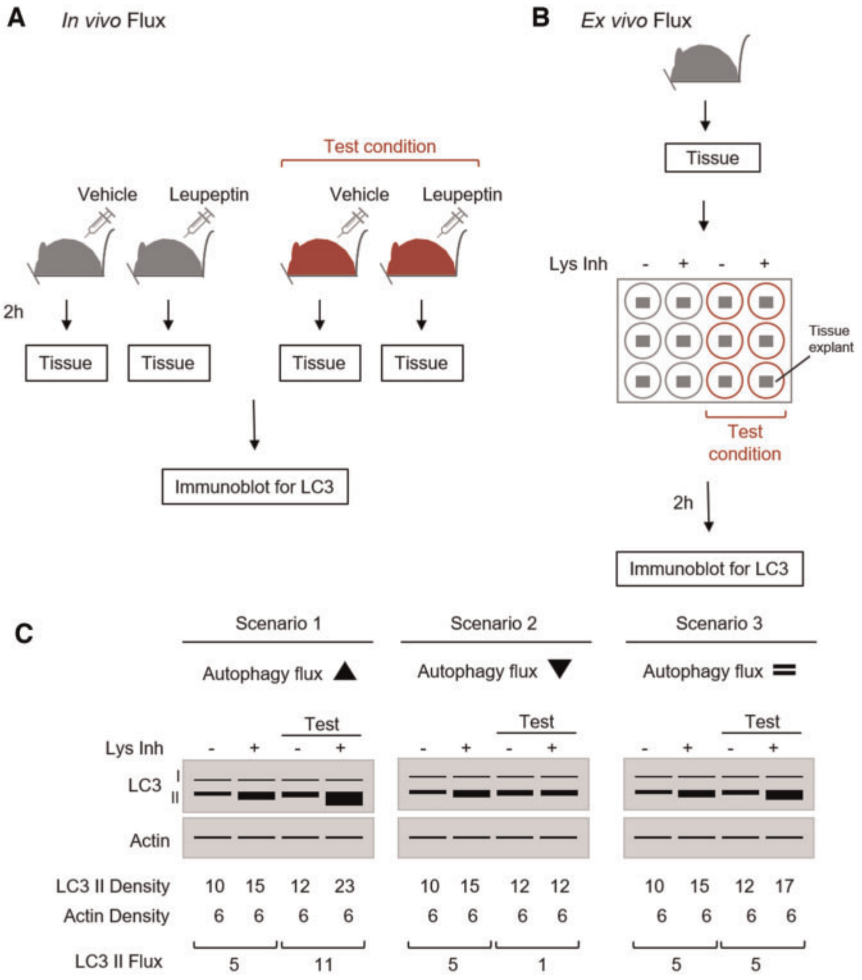

Figure 4 Autophagic flux *in vivo* and *ex vivo* via LC3 immunoblot. (*A*) *In vivo* flux. Mice (control or relevant test condition) can be injected with vehicle or leupeptin (30 mg/kg i.p.) 2 h before harvesting. Collected tissue(s) can be processed for SDS-PAGE and immunoblot for LC3. (*B*). *Ex vivo* flux. Tissue(s) harvested from a mouse can be cut into 0.5 cm^3 explants and cultured in media under control or test conditions with vehicle or lysosomal inhibitors (Lys Inh) at 37°C in a CO_2 incubator for 2 h with occasional swirling. Then, they can be processed for SDS-PAGE and immunoblot for LC3. (*C*). Schematic representation of LC3 blots depicting increased (left), decreased (middle), or unchanged (right) autophagic flux. Autophagic flux or LC3-II flux is calculated by subtracting the density values (after actin normalization) of + Lys Inh and control for each condition.

chain inhibition, is frequently used to measure mitochondrial mass in cells and tissues. Nevertheless, it must be considered that citrate synthase is one of the citric acid cycle enzymes identified as a CMA substrate.[90] In addition, quantitative PCR can be used to quantify mitochondrial-to-nuclear DNA ratio, using 16S rRNA as an index of mtDNA, and hexokinase 2 for nuclear DNA.[100] However, in order to interpret these results correctly, it is essential to assess mitochondrial biogenesis as well, to ensure that it is not impaired. It must be also noted that high levels of mitophagy may be required to detect net loss of mitochondrial proteins.

3.2.2 Fluorescence-based approaches

Fluorescence microscopy for colocalization of mitochondria with autophagosomes and lysosomes helps with the assessment of the degree to which the autophagic machinery sequesters and degrades mitochondria in the presence of a stressor. Co-labelling with GFP-LC3 and MitoTracker Red (for live cells) or immunostaining for mitochondrial proteins such as TOM20, VDAC, or COX-IV with antibodies (for fixed cells) offers information regarding colocalization between mitochondria and GFP-LC3-positive autophagosomes. Quantitative colocalization provides information on the degree of sequestration and serves as a marker for mitophagy. In addition, co-labelling for mitochondria and lysosomes using either LysoTracker or antibodies directed against lysosomal protein (LAMPs for instance) can be used as another assay for the assessment of mitophagy through colocalization of mitochondria with lysosomal markers. Although these assays may indicate the association of mitochondria with LC3-positive autophagosomes or lysosomes, mitochondrial degradation can only be confirmed following addition of lysosomal inhibitors. As discussed above, the propensity of GFP-LC3 to form aggregates requires caution since not all puncta will necessarily represent autophagosomes. The combination of electron and fluorescence microscopy tends to facilitate the interpretation of results related to the localization of LC3-positive double membranes around mitochondria. Given its complexity, one assay is unlikely to provide a clear

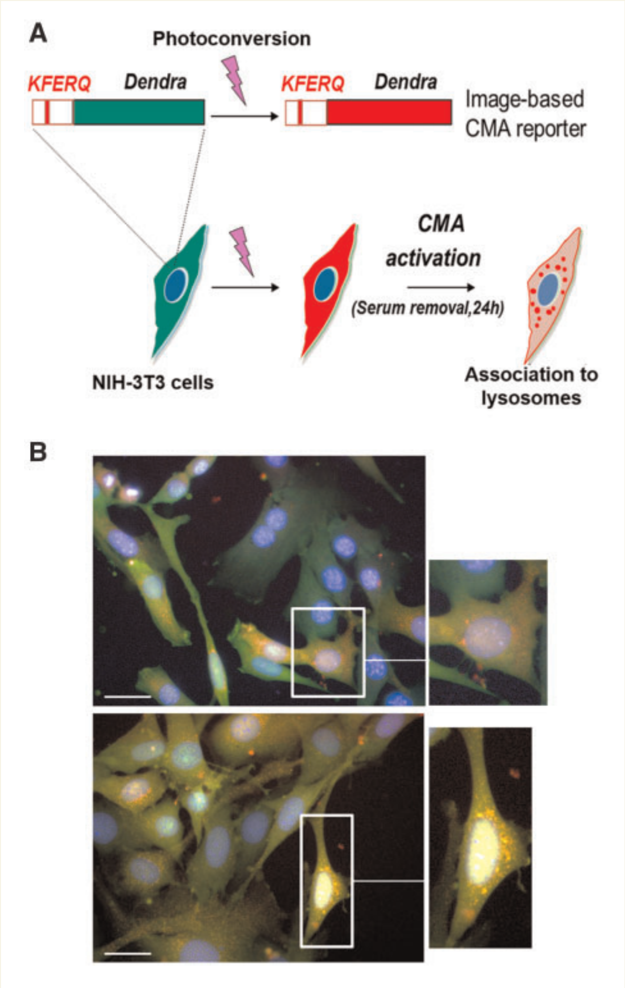

Figure 5 KFERQ-Dendra reporter to measure CMA activity. (*A*) Scheme of the experimental design to monitor CMA activity in cultured cells using the KFERQ–Dendra2 reporter. (*B*) NIH-3T3 cells expressing the Dendra2-KFERQ photoswitchable CMA reporter cultured in the presence (top panel and magnification) or absence (lower panel and magnification) of serum. Scale bar: 20 µm.

picture of the biology so it is recommended to use complementary approaches for its assessment.

3.3 Chaperone-mediated autophagy

3.3.1 CMA components

Determining the abundance and distribution of key CMA components and CMA-active lysosomes can be used as an indirect way to quantify CMA activity, but these methods yield only snapshots and should be complemented with functional assays. Lysosomal LAMP-2A is rate-limiting for CMA and changes in LAMP-2A levels usually correlate with CMA activity.[80] Nonetheless, not all LAMP-2A-positive lysosomes are equipped for CMA, as lysosomal HSC-70 (lys-HSC70) needs to be present as well, which is the case for 30% of LAMP-2A-positive lysosomes under basal conditions in organs such as liver. Additionally, lysosomal LAMP-2A levels are proportional to CMA activity and thus blotting lysosome-enriched fractions or membranous cell fractions is superior to blotting whole cell lysates. Given the sole involvement of LAMP-2A in CMA, antibodies specific for LAMP-2A should be utilized, rather than total LAMP-2 (that recognize the three isoforms of the *lamp-2* gene—LAMP-2A, LAMP-2B, and LAMP-2C). CMA activation often occurs without *de novo* synthesis of LAMP-2A, but in some conditions such as oxidative stress,[48,68] hypoxia,[125] or genotoxic stress it is transcriptionally upregulated.[68] Thus, *LAMP-2A* mRNA could be proportional to CMA activity; however, absence of changes in *LAMP-2A* mRNA does not necessarily mean that CMA is not altered. Regarding HSC70, total cellular levels measured via immunoblot or mRNA quantification are not informative of the CMA status. Assessment of lys-HSC70 in lysosomal fractions is more helpful than analyses of whole cell lysates. It is important to note that, when analysing lys-HSC70 in lysosome-enriched fractions, one should use antibodies specifically recognizing HSC70 and not HSP70.

3.3.2 Functional assays to track CMA activity

Comprehensive assessment of CMA activity in cells, tissues, and organelles requires tracking this pathway over time.[126] Below we detail a number of assays to determine CMA activity.

3.3.2.1 CMA reporter

The use of photoconvertible CMA reporters is a good approach to assess CMA activity since it can be performed in intact cells, even with limited sample size (*Figure 5*).[127] The principle behind this assay is the use of a photo-switchable protein that contains the KFERQ motif. Upon exposure to 405 nm light, it becomes possible to distinguish the photo-converted protein from the newly synthesized protein. Upon CMA activation in response to a given treatment, the fluorescent protein localizes to lysosomal membranes giving rise to visible fluorescent puncta. The number of puncta per cell is indicative of CMA activity (*Figure 5*). The CMA reporter ceases to fluoresce upon reaching the lysosomal lumen because of the unfolding required for the translocation across the lysosomal membrane and its rapid degradation inside the organelle. Consequently, this assay is only indicative of the binding step in the CMA pathway. One key advantage is that the pulse-chase nature of this assay allows testing CMA activity in response to different conditions and therapeutics.

3.3.2.2 Lysosomal uptake

The most reliable method to assess CMA activity is reconstituting the process with isolated lysosomes that contribute exclusively to CMA. This *in vitro* assay uses intact lysosomes, pretreated or not with lysosomal protease inhibitors (PI), and incubated with a known CMA substrate such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or ribonuclease A (RNase A). Samples are centrifuged after the incubation and the subsequent immunoblot for the CMA substrate used is a measure of the amount of substrate either bound to lysosomal membrane (those untreated with PI) or associated with lysosomes (those pretreated with PI). The amount of substrate translocated into the lysosomal lumen can be calculated by subtracting the amount of bound substrate from the total amount of substrate associated with lysosomes.

3.3.2.3 Metabolic labelling

Pulse and chase experiments can be used to track the degradation of potential CMA substrates by using radio-labelled amino acids ^{3}H-leucine or ^{3}H-valine in conjunction with inhibitors of either lysosomal proteases or macroautophagy to distinguish between macroautophagy and CMA-sensitive proteolysis. For example, contrary to macroautophagy, CMA is insensitive to phosphatidylinositol 3-kinase (PtdIns3K) inhibitors.[128] When using inhibitors of macroautophagy, it must be noted that

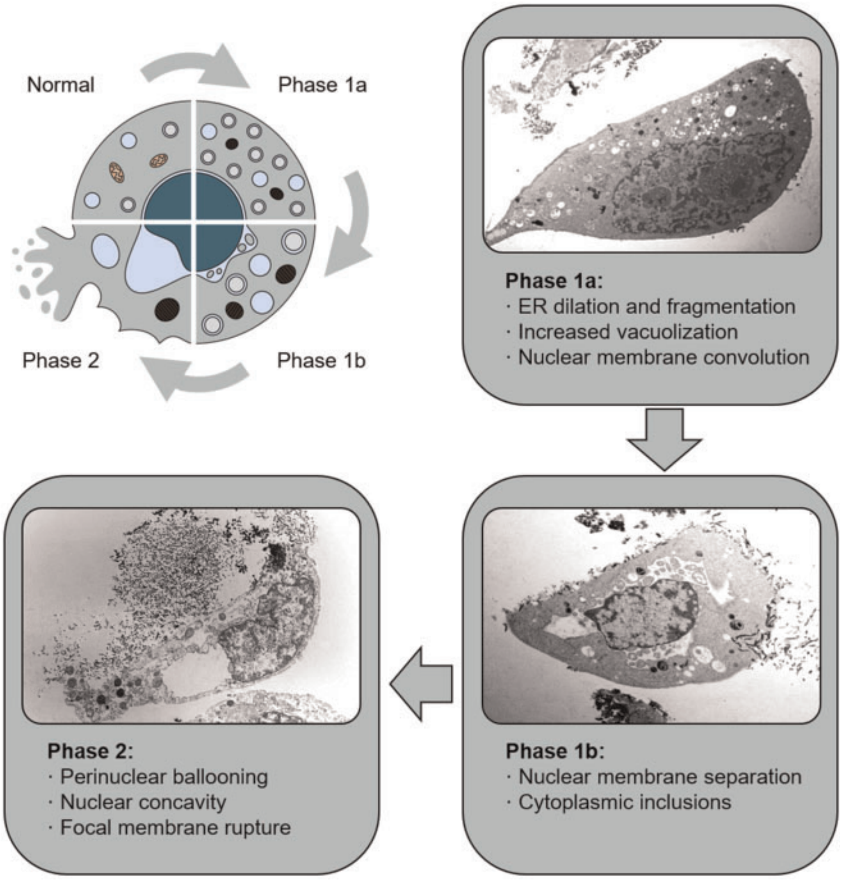

Figure 6 Autosis. Schematic diagram and representative images of the different phases of autosis in HeLa cells, showing the most characteristic features of this type of cell death. Images from Liu and Levine licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

prolonged blockage of macroautophagy leads to an upregulation in CMA. Thus, analyses of activity of these pathways should be assessed at early time-points (<24 h). Otherwise, the fraction of cellular proteins degraded through CMA could be overestimated.

3.3.2.4 Immunofluorescence

Changes in the number of CMA-active lysosomes is an indirect measurement of CMA activity. Co-staining via immunofluorescence or use of dual immunogold staining for HSC70 and LAMP-2A usually offers good correlation with CMA activity. As previously stated, increases in LAMP-2A or lys-HSC70 levels positively correlate with CMA activity.[25,26]

3.4 Autosis

Although autophagy is often triggered as a pro-survival pathway, it may lead to cell death in specific conditions.[60] In fact, several studies demonstrated how autophagy repression may ameliorate some pathological alterations in CD. For this reason, it is important to further explore the possible role of autosis in cardiac pathologies.

Unique morphological features characterize this type of cell death, including accumulation of autophagosomes and autolysosomes, disappearance of intracellular organelles, nuclear convolution, and characteristic expansion of the perinuclear space (*Figure 6*). Therefore, EM analysis remains the best method to detect autotic cells. In this regard, both the early phase (intense vacuolization) and the late phase (swollen perinuclear space and nuclear concavity) have been described in some pathological conditions *in vivo* (rat brain or human liver) using this technique.[65,129] It is important to distinguish autosis features from the ones present in endothelial cells, such as the concavity of the nucleus. Immunofluorescence analysis could help in the identification of autotic cells, for instance detecting ER fragmentation and nuclear concavity.[46,47] Nevertheless, double membranes are not discernible with this technique, as it would be impossible to distinguish the swelling of the perinuclear space from a random alteration in the shape of the whole nucleus.

Little is known about the complete molecular pathway underlying autosis, along with possible biochemical markers that could help in the identification and quantitation of this type of cell death. However, a unique feature of autosis is its dependency on autophagy proteins and the Na^+, K^+-ATPase.[65] In this regard, another way to confirm autotic cell death relies on the blockage of this process by depleting ATGs or inhibiting Na^+, K^+-ATPase.

4. Animal models for the study of autophagy and CD

In the following sections, we discuss the animal models useful for studying autophagy in the context of CD (*Table 2*).

Table 2 Genetic mouse models employed for the study of different macroautophagy forms in the cardiac system

Mouse line	Effect on autophagy/mitophagy	Cardiac disease model	Effect on cardiac function	References
Atg5$^{-/-}$	Inhibited	Pressure overload	Exacerbated hypertrophy, LV dilation and dysfunction	51
Atg5 Tg overexpression	Increased	Aging	Reduced age-related cardiac fibrosis, lifespan extension	23
Beclin 1$^{+/-}$	Attenuated	I/R injury, pressure overload, diabetic cardiomyopathy, sepsis	Cardioprotective during reperfusion, blunted LV remodelling	21,26,87,130,131
Beclin 1 Tg overexpression	Increased	Pressure overload; Diabetic cardiomyopathy	Exacerbated LV remodelling	21,87
	Increased	Sepsis	Cardioprotective	130
Beclin 1 F121A knock-in	Increased	Aging	Reduced age-associated cardiac alterations, increased lifespan	52
ATG16L1-HM	Macroautophagy inhibited, mitophagy restored	Diabetic cardiomyopathy	Attenuated cardiac derangements	87
Mst1$^{-/-}$	Increased	Myocardial infarction	Reduced infarct size, cardioprotective	69
RHEB overexpression	Inhibited	Ischaemia	Exacerbated injury	27
AMPK dominant-negative Tg overexpression	Inhibited	Ischaemia	Exacerbated injury	26
Parkin Tg overexpression	Increased	Aging	Reduction in age-associated cardiac abnormalities	25
Parkin$^{-/-}$	Reduced	Myocardial infarction	Increased infarct size, hypertrophy	74
DRP1$^{-/-}$	Inhibited	I/R injury, pressure overload	Exacerbated injury, heart failure	32,73
PGAM5$^{-/-}$	Inhibited	I/R injury	Exacerbated injury	66

4.1 Non-selective general autophagy

Mouse models for the measurement of autophagy *in vivo* have been established to precisely monitor the autophagic process. Transgenic mice expressing GFP fused to the autophagosome marker LC3 were generated in 2004 by Dr Noboru Mizushima in Dr Yoshinori Ohsumi's group.[104] Expression of GFP-LC3 did not affect autophagy *per se*, and these mice do not exhibit an abnormal phenotype. Since then, the GFP-LC3 transgenic mice have been widely used to evaluate and measure autophagy by fluorescence microscopy in multiple tissues, including the heart. Autophagosomes are visualized as puncta or vesicles labelled by GFP. However, treatment of these mice with lysosomal inhibitors to inhibit the degradation of autophagic vesicles is required to accurately assess the autophagy flux.

Other mouse models have been developed to better visualize the autophagy process and to overcome the limitation of GFP fluorescence typically quenched by the low lysosomal pH. The mRFP-GFP-LC3 tandem fluorescent-tagged LC3 reporter mouse allows for the detection of autophagosomes labelled with both mRFP and pEGFP (*Figure 3*).[108,132] This tool has been useful to monitor autophagic activity during cardiac injury through cardiomyocyte-specific expression of mRFP-GFP-LC3 reporter[40] (*Figure 3*). More recently, GFP-LC3-RFP-LC3ΔG fluorescent probe was designed to monitor autophagic flux *in vitro* and *in vivo*. Upon cleavage by endogenous ATG4, equimolar amounts of GFP-LC3 and RFP-LC3ΔG are generated. GFP-LC3 is degraded through autophagy, whereas RFP-LC3ΔG is not and remains in the cytosol, acting as an internal control. The GFP/RFP ratio reflects autophagic flux.[133]

Besides monitoring autophagy with reporter mice, the function of autophagy in tissues has been analysed using genetically engineered mice deficient in key autophagy genes. Knockout mice for non-redundant *Atgs* are lethal in the neonatal or embryonic stage. Consequently, conditional tissue-specific *Atg* gene knockout mice have been developed to assess the contribution of autophagy to diverse pathophysiological conditions. For example, cardiac-specific *Atg5* knockout mice revealed the importance of autophagy in preserving cardiac function.[51] Autophagy has also been investigated in adult mice at the whole body level. Mice heterozygous for the loss of the autophagy gene *Beclin 1* have significantly reduced autophagy activity.[134] These mice have been used to study autophagy in models of ischaemia/reperfusion and sepsis in the heart, demonstrating important cardioprotective roles of autophagy.[26,31,130] Similar to the *Beclin 1* deficient animals, ATG16L1 hypomorphic mice, or animals lacking autophagins (the mammal orthologues of yeast Atg4s), exhibit decreased levels of autophagy.[135,136] Other approaches include the tamoxifen-inducible *Cre*-mediated deletion of floxed *Atg5* or *Atg7* alleles, leading to selective and temporal deletion of autophagy genes in adulthood.[137–139] Using this strategy, autophagy activity is inhibited within 1–2 weeks of treatment with tamoxifen, following which these mice begin to show tissue-specific abnormalities over a period of few weeks. Another approach is to study the pathophysiological consequences of increased basal autophagy activity in mice. For instance, transgenic mice overexpressing *Atg5* have been developed; however, it is unclear whether and how autophagy is indeed increased in these

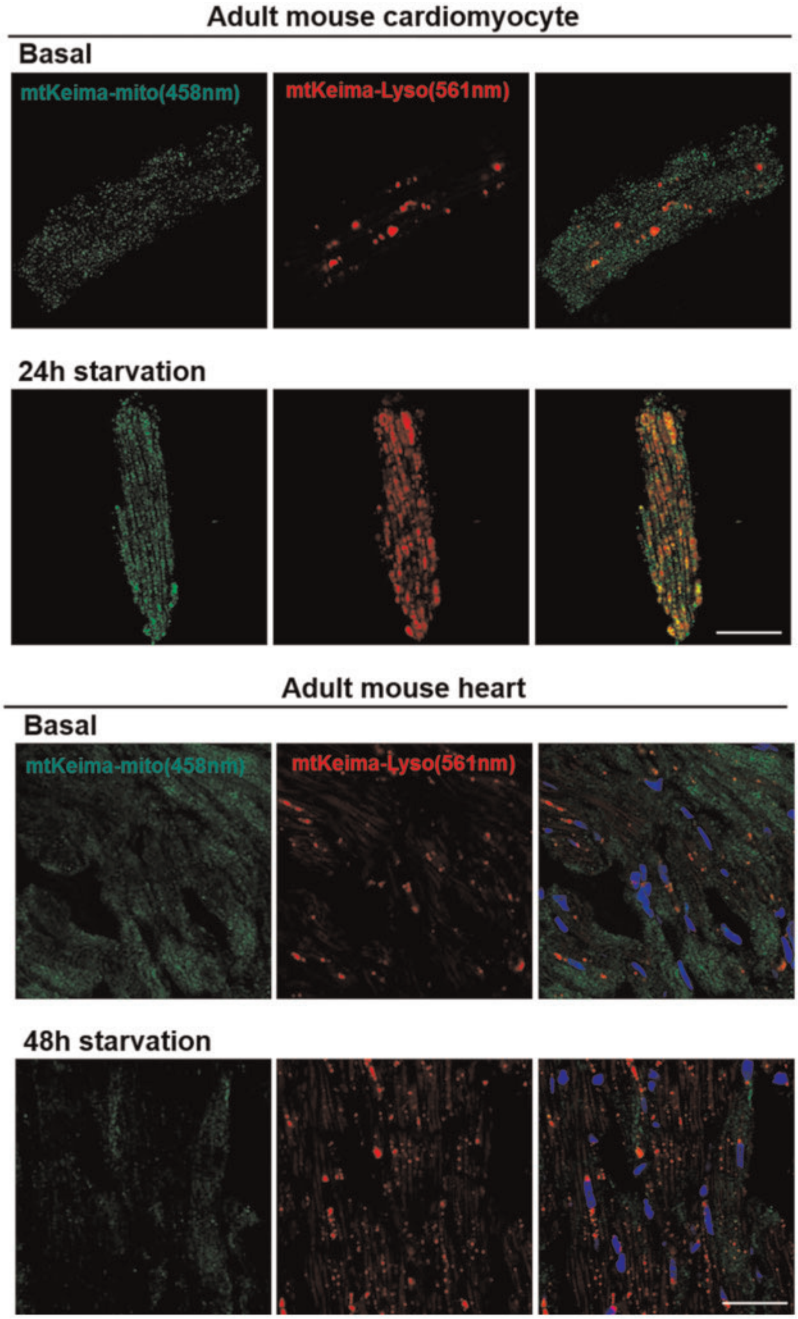

Figure 7 Mito-Keima as a mitophagy reporter to study cardiac mitophagy in mice. (Upper panel) Isolated adult mouse cardiomyocytes from 16-week-old male Mito-Keima mice with no treatment (basal) or 24 h starvation. (Lower panel) Mouse heart tissue from Mito-Keima mice with no treatment (basal) or after 48 h of starvation. Images were taken with a confocal microscope with sequential 458 nm and 561 nm excitations. Normal mitochondria are presented as green and mitophagy (mitochondria fused with lysosome) is presented as red. Scale bar: 25 µm.

mice since, although required for the autophagy to occur, ATG5 is not involved in the regulation of autophagy levels.[23]

More recent studies have described the Beclin 1-F121A knock-in mice, expressing a mutation in the BH3 domain of Beclin 1 that leads to constitutively increased basal autophagy in multiple tissues.[52,140,141] The Beclin 1-F121A mice with increased levels of autophagy throughout adulthood have an increased lifespan and improved healthspan including decreased age-associated cardiac alterations.[52] On a similar note, cardiac-specific transgenic mice overexpressing Beclin 1 have also been developed and show increase in stress-induced autophagy.[21,130]

4.2 Mitophagy

Currently, the large majority of mitophagy studies monitor the colocalization of mitochondrial probes with typical markers of autophagy.[102,142] Dr Toren Finkel's group generated a reporter mouse expressing the fluorescent reporter Mito-Keima, which results from the fusion of the fluorescent protein Keima to the mitochondrial matrix protein COX8 (*Figure 7*).[143] Keima is a pH-sensitive fluorescent protein whose emission spectra differ in mitochondria (458 nm, green fluorescence at pH 8.0) compared to the acidic environment of lysosomes (561 nm, red fluorescence at pH 4.5).[144] Transgenic mitochondria-localized Keima mice have been used to assess mitophagy since this model provides the most sensitive and specific analysis of mitochondrial flux to lysosomes (*Figure 7*). However, there are downsides to assessing mitophagy using the Mito-Keima probe. For instance, the Keima excitation wavelengths for green and red fluorescence partially overlap, rendering the quantitation and interpretation of the data difficult. It should also be noted that Mito-Keima fluorescence fades upon prolonged light exposure. In addition, fixation disrupts the lysosomal pH gradient required for this probe to work.[144–146] To optimize these conditions, it is important to increase the cross-link reaction while suppressing tissue damage. Augmenting the concentration of formaldehyde is not an option since its amount is usually in excess. Higher temperatures promote autolysis of the tissue. A longer reaction time may result in an inappropriate diffusion of denatured protein. Thus, pH may be the sole parameter that can be adjusted to achieve this purpose. Since the affinity of formaldehyde for an unprotonated amino group is much higher than for a protonated group, the formation of the aminomethylol group is highly pH sensitive. As the pH increases, more groups lose their proton and react with formaldehyde. In fact, the advantage of alkaline fixation for effective cross-linking has been reported in the context of immunohistochemistry and *in situ* hybridization.[147,148] Moreover, alkaline fixation counter-neutralizes the intracellular pH, thus preventing inappropriate acidic Mito-Keima signals due to cell death-dependent intracellular acidification. To observe mitophagy using Mito-Keima mice, heart is fixed with alkaline formaldehyde for 30 min.[117] Vibratome-mediated sectioning and microscopic analysis are conducted immediately after fixation. The high-ratio signal (561/458 nm), indicating localization of Mito-Keima protein in an acidic environment, is upregulated in response to starvation or ischaemia in the wild-type heart and associated with the induction of autophagy (*Figure 7*, unpublished data). Treatment with Tat-Beclin1, an autophagy-inducing peptide,[149] also increased this signal. On the other hand, the hearts from cardiac-specific *Atg7* knockout mice do not show any high-ratio signal at baseline.[143] This suggests that the high-ratio signal of Mito-Keima obtained with alkaline fixation clearly reflects mitophagy. One cautionary note with the use of Mito-Keima mice is that it is difficult to exclude the possibility that ischaemia may contribute to the formation of acidic puncta. It is recommended to complete the procedure within 2 h.

Since the rate of mitochondrial turnover is slow in the heart (approximately every 2 weeks),[25,74] measuring mitochondrial turnover and biosynthesis *in vivo* has proven difficult. Consequently, several novel methods have been developed to monitor mitophagy *in vivo*, including the use of fluorescent probes such as MitoTimer and Mito-QC (quality control).

MitoTimer is a useful tool for monitoring mitochondrial turnover *in vivo*. The fluorescent timer protein, also called DsRed-E5, is a mutant form of the red fluorescent protein DsRed that changes its colour from green to red over a period of time as the protein matures.[150] The timer protein was fused with mitochondria targeting sequence of COX8A to generate MitoTimer targeted to the mitochondrial matrix.[151,152] Subsequently, Stotland and Gottlieb generated transgenic mice with cardiac-specific expression of MitoTimer driven by the cardiac α-myosin heavy chain (α-MHC) promoter.[153] Despite the fact that α-MHC is equally expressed in atrial and ventricular myocardium, MitoTimer is largely expressed in the left and right ventricles and to the lesser extent in the atria. MitoTimer expression *in vivo* facilitates its use in experiments exploring roles of mitophagy in skeletal and cardiac muscle.

The Mito-QC probe is another new tool developed for assessing mitophagy and visualizing the mitochondrial network.[145] Like Mito-Keima, Mito-QC is a pH-sensitive mitochondrial fluorescent probe, consisting in the tandem mCherry-GFP tag fused to the OMM protein FIS1. Mito-QC shows green and red fluorescence in normal conditions but reveals a predominant mCherry (red) signal when mitophagy is induced and mitochondria are delivered to the lysosomes. McWilliams *et al.*[145] generated the Mito-QC transgenic mice, which can be used to monitor changes in mitophagy during stress. Unlike for the Mito-Keima reporter, there is no evidence of an overlap in the Mito-QC excitation spectra. Furthermore, it has been reported that samples from Mito-QC mouse can be fixed prior to microscopic analysis.[145] However, Mito-QC also requires the pH gradient across the lysosomal membrane for an accurate detection of mitophagy. Since acid-induced reduction of GFP fluorescence is reversible upon re-neutralization of pH,[154,155] the acidic signal of Mito-QC (red alone) should theoretically only be maintained in the presence of the pH gradient across the lysosomal membrane. We therefore speculate that optimized fixation aimed at preserving lysosomal membrane structure is essential not only for Mito-Keima but also for Mito-QC.

Despite technical limitations, these tools present significant advantages that allow the assessment of mitophagy *in vivo*. However, extensive research is necessary for the development of the state-of-the-art techniques to better understand the regulation of mitophagy in CD.

4.3 Chaperone-mediated autophagy

Assessing the CMA status in different tissues from various rodent models can be performed via the assays mentioned earlier. For example, CMA has been investigated using lysosomes isolated from liver, brain, kidney, and spleen.[13] The *lamp-2* gene gives rise to three splice variants, *lamp-2a*, *b,* and *c*. The resulting proteins, LAMP-2A, B, and C, have been associated with CMA,[156] macroautophagy,[91] and RNAphagy[157] respectively. A previous model with deletion of the whole *LAMP-2* gene that targets all three isoforms of LAMP2 proved unsuitable for investigating CMA due to interference with other autophagic pathways and defective lysosomal biogenesis and stability.[92] A Cre-loxP system was used to disrupt an exon specific to domains of LAMP-2A without affecting the other two LAMP-2 isoforms. This first *in vivo* approach demonstrated the importance of CMA in the regulation of metabolism, since mice lacking LAMP-2A in the liver displayed severe dysfunction in glucose and lipid metabolism.[90] Implication of CMA in the immune system was shown by Valdor *et al*. This study showed that CMA is required for proper T-cell activation and that restoration of LAMP-2A in aged cells results in an improved T-cell response.[48]

4.4 Autosis

As a unique biochemical feature, autosis is regulated by the Na^+, K^+-ATPase. Cardiac glycosides like digoxin, a chemical inhibitor of the Na^+, K^+-ATPase, block autosis and markedly reduce brain injury in response to cerebral hypoxia–ischaemia in neonatal rats.[65] Of note, unlike the human α-isoforms of the Na^+, K^+-ATPase, the mouse α1 isoform of the Na^+, K^+-ATPase is insensitive to cardiac glycosides. Since the expression level of α1 isoform is predominant over the other α-isoforms, the Na^+, K^+-ATPase cannot be inhibited by digoxin-like molecules in the rodent heart. This limitation can be avoided using cardiac-glycoside sensitive Na^+, K^+-ATPase α1 knock-in mice.[158] Replacement of specific amino acid residues in the mouse Na^+, K^+-ATPase α1 (Arg-111 and Asp-122) with residues naturally present in the human α1 isoform (to Glu-111 and Asn-122) confers sensitivity to cardiac glycosides. It should be noted that these compounds are used for the treatment of systolic heart failure and for heart rate control in the presence of atrial fibrillation. These effects are mediated by an increase of intracellular calcium concentration in cardiomyocytes. It is reasonable to speculate that some of the beneficial effects of digitalis on cardiac contractility may be partially mediated by the reduction of autosis.

5. Conclusions and perspectives

The literature reviewed here strongly indicates that modulation of autophagy represents a potential therapeutic approach to treat CD upon various stress conditions. Several therapeutic strategies have been proposed to stimulate autophagy. Inhibitors of mTORC1, such as rapalogues (rapamycin analogues), or activators of AMPK, such as metformin and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), confer cardioprotection in different stress conditions, including aging.[27,159,160] Natural compounds can also be considered valid options because of their lower toxicity. For example, resveratrol, a polyphenol present in red wine, stimulates cardiac autophagy through activation of NAD-dependent protein deacetylase sirtuin-1 (SIRT1) and AMPK.[161,162] Spermidine and trehalose are inducers of autophagy and may be considered novel cardioprotective agents as well.[24,58] Synthetic peptides that have the ability to selectively activate autophagy were recently developed and showed to benefit cardiac function. For instance, administration of a low dose of Tat-Beclin 1 counteracted heart failure induced by pressure overload via induction of mitophagy.[73] Recently, it has been shown that aspirin, a worldwide drug used to prevent heart attack in high risk patients, induces autophagy, suggesting a novel molecular mechanism underlying the pro-health effects of this drug.[72] However, most of the molecules that induce autophagy, often, also impact other molecular pathways and this problem limits their use. Certainly, the development of more specific autophagy inducers is a major issue for forthcoming researches. In particular, natural activators of autophagy may represent a valid option for activating autophagy in patients because of their low adverse effects.[163] Another point that needs to be considered is the study of the impact of standard pharmacological agents for the treatment of CD on autophagy. In the future, it will be interesting to evaluate the cardioprotective role of CMA and lipophagy stimulation for the treatment of cardiac and metabolic diseases, also in response to triggers relevant to CD, such as oxidative stress, hypoxia, and lipogenic challenges, or its age-associated decline.

Although in most conditions autophagy activation reduces myocardial injury, in certain pathological situations it appears to be maladaptive in the heart. Further investigations are needed to better understand the mechanisms underlying this paradoxical consequence. Although it is possible that exaggerated autophagy activation is deleterious, it may also be possible that the signalling mechanisms promoting autophagy may determine whether the outcome is beneficial or toxic. It is now known that alternative pathways regulating autophagy exist and recent work has demonstrated that specific molecules regulating the autophagic machinery could also trigger other forms of cell death. Clarifying these aspects is key to understanding when autophagy needs to be activated and when this activation should be avoided. In this regard, the use of cardiac-specific conditional knockout models for autophagy may prove beneficial.

Finally, methods to study autophagy require further improvements. Since autophagy is a dynamic process, misinterpretation of data leads to inaccurate evaluation of autophagy and autophagic flux. In addition, several results from previous studies were obtained in *in vitro* models, which are important for dissecting the signalling pathways regulating autophagy but may not be able to mimic the *in vivo* environment and may have limited physiological relevance. To date, several detection tools have been developed to precisely quantify autophagy in diverse model systems, and these methodologies were recently summarized and standardized in guidelines for the evaluation of mammalian autophagy.[102] The evaluation of autophagy in human subjects remains yet another important challenge. It would be critical to understand how levels of cardiac autophagy correlate with cardiac outcomes in subjects affected by CD. This is possible when myocardial biopsies are available. Unfortunately, this approach is not always feasible, particularly in larger cohorts of subjects. Therefore, it is important to develop new techniques to monitor autophagy *in vivo* or to identify novel circulating markers associated with cardiac autophagy and dysfunction, which may be used to monitor autophagy in human subjects.

Author contributions

M.C.M., A.F., and S.S.[12,13] conceived the manuscript idea and together with J.M.M. and N.K. coordinated and supervised the work. All the authors participated in manuscript writing, critically reviewed the content and agree to be accountable for the work. S.K. performed major scientific editing. A.F., M.C.M., F.C., J.N., J.M.M., N.K., S.K., and Y.C. prepared figures and tables.

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References

1. Levine B, Kroemer G. Biological functions of autophagy genes: a disease perspective. *Cell* 2019;**176**:11–42.
2. Choi AM, Ryter SW, Levine B. Autophagy in human health and disease. *N Engl J Med* 2013;**368**:1845–1846.
3. Kroemer G. Autophagy: a druggable process that is deregulated in aging and human disease. *J Clin Invest* 2015;**125**:1–4.
4. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell* 2011; **147**:728–741.
5. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature* 2008;**451**:1069–1075.
6. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 2008;**132**: 27–42.
7. Galluzzi L, Baehrecke EH, Ballabio A, Boya P, Bravo-San Pedro JM, Cecconi F, Choi AM, Chu CT, Codogno P, Colombo MI, Cuervo AM, Debnath J, Deretic V, Dikic I, Eskelinen EL, Fimia GM, Fulda S, Gewirtz DA, Green DR, Hansen M, Harper JW, Jaattela M, Johansen T, Juhasz G, Kimmelman AC, Kraft C, Ktistakis NT, Kumar S, Levine B, Lopez-Otin C, Madeo F, Martens S, Martinez J, Melendez A, Mizushima N, Munz C, Murphy LO, Penninger JM, Piacentini M, Reggiori F, Rubinsztein DC, Ryan KM, Santambrogio L, Scorrano L, Simon AK, Simon HU, Simonsen A, Tavernarakis N, Tooze SA, Yoshimori T, Yuan J, Yue Z, Zhong Q, Kroemer G. Molecular definitions of autophagy and related processes. *EMBO J* 2017;**36**:1811–1836.
8. Li WW, Li J, Bao JK. Microautophagy: lesser-known self-eating. *Cell Mol Life Sci* 2012;**69**:1125–1136.
9. Sakai Y, Koller A, Rangell LK, Keller GA, Subramani S. Peroxisome degradation by microautophagy in *Pichia pastoris*: identification of specific steps and morphological intermediates. *J Cell Biol* 1998;**141**:625–636.

10. Lemasters JJ. Variants of mitochondrial autophagy: types 1 and 2 mitophagy and micromitophagy (Type 3). *Redox Biol* 2014;**2**:749–754.
11. Seo AY, Lau PW, Feliciano D, Sengupta P, Gros MAL, Cinquin B, Larabell CA, Lippincott-Schwartz J. AMPK and vacuole-associated Atg14p orchestrate μ-lipophagy for energy production and long-term survival under glucose starvation. *Elife* 2017;**6**:e2169.
12. Roberts P, Moshitch-Moshkovitz S, Kvam E, O'Toole E, Winey M, Goldfarb DS. Piecemeal microautophagy of nucleus in *Saccharomyces cerevisiae*. *Mol Biol Cell* 2003; **14**:129–141.
13. Kaushik S, Cuervo AM. The coming of age of chaperone-mediated autophagy. *Nat Rev Mol Cell Biol* 2018;**19**:365–381.
14. Galluzzi L, Bravo-San Pedro JM, Levine B, Green DR, Kroemer G. Pharmacological modulation of autophagy: therapeutic potential and persisting obstacles. *Nat Rev Drug Discov* 2017;**16**:487–511.
15. Galluzzi L, Pietrocola F, Levine B, Kroemer G. Metabolic control of autophagy. *Cell* 2014;**159**:1263–1276.
16. Madrigal-Matute J, Cuervo AM. Regulation of liver metabolism by autophagy. *Gastroenterology* 2016;**150**:328–339.
17. Green DR, Galluzzi L, Kroemer G. Mitochondria and the autophagy-inflammation-cell death axis in organismal aging. *Science* 2011;**333**:1109–1112.
18. Sun N, Youle RJ, Finkel T. The mitochondrial basis of aging. *Mol Cell* 2016;**61**: 654–666.
19. Saito T, Sadoshima J. Molecular mechanisms of mitochondrial autophagy/mitophagy in the heart. *Circ Res* 2015;**116**:1477–1490.
20. Sciarretta S, Maejima Y, Zablocki D, Sadoshima J. The role of autophagy in the heart. *Annu Rev Physiol* 2018;**80**:1–26.
21. Zhu H, Tannous P, Johnstone JL, Kong Y, Shelton JM, Richardson JA, Le V, Levine B, Rothermel BA, Hill JA. Cardiac autophagy is a maladaptive response to hemodynamic stress. *J Clin Invest* 2007;**117**:1782–1793.
22. Shirakabe A, Ikeda Y, Sciarretta S, Zablocki DK, Sadoshima J. Aging and autophagy in the heart. *Circ Res* 2016;**118**:1563–1576.
23. Pyo JO, Yoo SM, Ahn HH, Nah J, Hong SH, Kam TI, Jung S, Jung YK. Overexpression of Atg5 in mice activates autophagy and extends lifespan. *Nat Commun* 2013;**4**:2300.
24. Eisenberg T, Abdellatif M, Schroeder S, Primessnig U, Stekovic S, Pendl T, Harger A, Schipke J, Zimmermann A, Schmidt A, Tong M, Ruckenstuhl C, Dammbrueck C, Gross AS, Herbst V, Magnes C, Trausinger G, Narath S, Meinitzer A, Hu Z, Kirsch A, Eller K, Carmona-Gutierrez D, Büttner S, Pietrocola F, Knittelfelder O, Schrepfer E, Rockenfeller P, Simonini C, Rahn A, Horsch M, Moreth K, Beckers J, Fuchs H, Gailus-Durner V, Neff F, Janik D, Rathkolb B, Rozman J, de Angelis MH, Moustafa T, Haemmerle G, Mayr M, Willeit P, von Frieling-Salewsky M, Pieske B, Scorrano L, Pieber T, Pechlaner R, Willeit J, Sigrist SJ, Linke WA, Mühlfeld C, Sadoshima J, Dengjel J, Kiechl S, Kroemer G, Sedej S, Madeo F. Cardioprotection and lifespan extension by the natural polyamine spermidine. *Nat Med* 2016;**22**: 1428–1438.
25. Hoshino A, Mita Y, Okawa Y, Ariyoshi M, Iwai-Kanai E, Ueyama T, Ikeda K, Ogata T, Matoba S. Cytosolic p53 inhibits Parkin-mediated mitophagy and promotes mitochondrial dysfunction in the mouse heart. *Nat Commun* 2013;**4**:2308.
26. Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B, Sadoshima J. Distinct roles of autophagy in the heart during ischemia and reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. *Circ Res* 2007; **100**:914–922.
27. Sciarretta S, Zhai P, Shao D, Maejima Y, Robbins J, Volpe M, Condorelli G, Sadoshima J. Rheb is a critical regulator of autophagy during myocardial ischemia: pathophysiological implications in obesity and metabolic syndrome. *Circulation* 2012; **125**:1134–1146.
28. Sciarretta S, Zhai P, Shao D, Zablocki D, Nagarajan N, Terada LS, Volpe M, Sadoshima J. Activation of NADPH oxidase 4 in the endoplasmic reticulum promotes cardiomyocyte autophagy and survival during energy stress through the protein kinase RNA-activated-like endoplasmic reticulum kinase/eukaryotic initiation factor 2alpha/activating transcription factor 4 pathway. *Circ Res* 2013;**113**: 1253–1264.
29. Zhai P, Sciarretta S, Galeotti J, Volpe M, Sadoshima J. Differential roles of GSK-3beta during myocardial ischemia and ischemia/reperfusion. *Circ Res* 2011;**109**: 502–511.
30. Xie M, Kong Y, Tan W, May H, Battiprolu PK, Pedrozo Z, Wang ZV, Morales C, Luo X, Cho G, Jiang N, Jessen ME, Warner JJ, Lavandero S, Gillette TG, Turer AT, Hill JA. Histone deacetylase inhibition blunts ischemia/reperfusion injury by inducing cardiomyocyte autophagy. *Circulation* 2014;**129**:1139–1151.
31. Ma X, Liu H, Foyil SR, Godar RJ, Weinheimer CJ, Hill JA, Diwan A. Impaired autophagosome clearance contributes to cardiomyocyte death in ischemia/reperfusion injury. *Circulation* 2012;**125**:3170–3181.
32. Ikeda Y, Shirakabe A, Maejima Y, Zhai P, Sciarretta S, Toli J, Nomura M, Mihara K, Egashira K, Ohishi M, Abdellatif M, Sadoshima J. Endogenous Drp1 mediates mitochondrial autophagy and protects the heart against energy stress. *Circ Res* 2015;**116**: 264–278.
33. Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, Tanaka K, Cuervo AM, Czaja MJ. Autophagy regulates lipid metabolism. *Nature* 2009;**458**:1131–1135.
34. Liu K, Czaja MJ. Regulation of lipid stores and metabolism by lipophagy. *Cell Death Differ* 2013;**20**:3–11.
35. Zhou K, Yao P, He J, Zhao H. Lipophagy in nonliver tissues and some related diseases: pathogenic and therapeutic implications. *J Cell Physiol* 2019;**234**:7938–7947.
36. Zechner R, Madeo F, Kratky D. Cytosolic lipolysis and lipophagy: two sides of the same coin. *Nat Rev Mol Cell Biol* 2017;**18**:671–684.
37. Yang M, Zhang Y, Ren J. Autophagic regulation of lipid homeostasis in cardiometabolic syndrome. *Front Cardiovasc Med* 2018;**5**:38.
38. Chen K, Yuan R, Zhang Y, Geng S, Li L. Tollip deficiency alters atherosclerosis and steatosis by disrupting lipophagy. *J Am Heart Assoc* 2017;**6**:e004078.
39. Shibata M, Yoshimura K, Furuya N, Koike M, Ueno T, Komatsu M, Arai H, Tanaka K, Kominami E, Uchiyama Y. The MAP1-LC3 conjugation system is involved in lipid droplet formation. *Biochem Biophys Res Commun* 2009;**382**:419–423.
40. Shibata M, Yoshimura K, Tamura H, Ueno T, Nishimura T, Inoue T, Sasaki M, Koike M, Arai H, Kominami E, Uchiyama Y. LC3, a microtubule-associated protein1A/B light chain3, is involved in cytoplasmic lipid droplet formation. *Biochem Biophys Res Commun* 2010;**393**:274–279.
41. Baerga R, Zhang Y, Chen PH, Goldman S, Jin S. Targeted deletion of autophagy-related 5 (ATG5) impairs adipogenesis in a cellular model and in mice. *Autophagy* 2009;**5**:1118–1130.
42. Singh R, Xiang Y, Wang Y, Baikati K, Cuervo AM, Luu YK, Tang Y, Pessin JE, Schwartz GJ, Czaja MJ. Autophagy regulates adipose mass and differentiation in mice. *J Clin Invest* 2009;**119**:3329–3339.
43. Zhang Y, Goldman S, Baerga R, Zhao Y, Komatsu M, Jin S. Adipose-specific deletion of autophagy-related gene 7 (atg7) in mice reveals a role in adipogenesis. *Proc Natl Acad Sci USA* 2009;**106**:19860–19865.
44. Kaushik S, Cuervo AM. Degradation of lipid droplet-associated proteins by chaperone-mediated autophagy facilitates lipolysis. *Nat Cell Biol* 2015;**17**:759–770.
45. Massey AC, Kaushik S, Sovak G, Kiffin R, Cuervo AM. Consequences of the selective blockage of chaperone-mediated autophagy. *Proc Natl Acad Sci USA* 2006;**103**: 5805–5810.
46. Cuervo AM, Dice JF. Age-related decline in chaperone-mediated autophagy. *J Biol Chem* 2000;**275**:31505–31513.
47. Kiffin R, Kaushik S, Zeng M, Bandyopadhyay U, Zhang C, Massey AC, Martinez-Vicente M, Cuervo AM. Altered dynamics of the lysosomal receptor for chaperone-mediated autophagy with age. *J Cell Sci* 2007;**120**:782–791.
48. Valdor R, Mocholi E, Botbol Y, Guerrero-Ros I, Chandra D, Koga H, Gravekamp C, Cuervo AM, Macian F. Chaperone-mediated autophagy regulates T cell responses through targeted degradation of negative regulators of T cell activation. *Nat Immunol* 2014;**15**:1046–1054.
49. Rodriguez-Navarro JA, Kaushik S, Koga H, Dall'Armi C, Shui G, Wenk MR, Di Paolo G, Cuervo AM. Inhibitory effect of dietary lipids on chaperone-mediated autophagy. *Proc Natl Acad Sci USA* 2012;**109**:E705–714.
50. Sooparb S, Price SR, Shaoguang J, Franch HA. Suppression of chaperone-mediated autophagy in the renal cortex during acute diabetes mellitus. *Kidney Int* 2004;**65**: 2135–2144.
51. Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsumura Y, Asahi M, Nishida K, Hori M, Mizushima N, Otsu K. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. *Nat Med* 2007;**13**:619–624.
52. Fernandez AF, Sebti S, Wei Y, Zou Z, Shi M, McMillan KL, He C, Ting T, Liu Y, Chiang WC, Marciano DK, Schiattarella GG, Bhagat G, Moe OW, Hu MC, Levine B. Disruption of the beclin 1-BCL2 autophagy regulatory complex promotes longevity in mice. *Nature* 2018;**558**:136–140.
53. Morselli E, Marino G, Bennetzen MV, Eisenberg T, Megalou E, Schroeder S, Cabrera S, Benit P, Rustin P, Criollo A, Kepp O, Galluzzi L, Shen S, Malik SA, Maiuri MC, Horio Y, Lopez-Otin C, Andersen JS, Tavernarakis N, Madeo F, Kroemer G. Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome. *J Cell Biol* 2011;**192**:615–629.
54. Pietrocola F, Lachkar S, Enot DP, Niso-Santano M, Bravo-San Pedro JM, Sica V, Izzo V, Maiuri MC, Madeo F, Mariño G, Kroemer G. Spermidine induces autophagy by inhibiting the acetyltransferase EP300. *Cell Death Differ* 2015;**22**:509–516.
55. Madeo F, Pietrocola F, Eisenberg T, Kroemer G. Caloric restriction mimetics: towards a molecular definition. *Nat Rev Drug Discov* 2014;**13**:727–740.
56. Valentim L, Laurence KM, Townsend PA, Carroll CJ, Soond S, Scarabelli TM, Knight RA, Latchman DS, Stephanou A. Urocortin inhibits Beclin1-mediated autophagic cell death in cardiac myocytes exposed to ischaemia/reperfusion injury. *J Mol Cell Cardiol* 2006;**40**:846–852.
57. Kanamori H, Takemura G, Goto K, Maruyama R, Ono K, Nagao K, Tsujimoto A, Ogino A, Takeyama T, Kawaguchi T, Watanabe T, Kawasaki M, Fujiwara T, Fujiwara H, Seishima M, Minatoguchi S. Autophagy limits acute myocardial infarction induced by permanent coronary artery occlusion. *Am J Physiol Heart Circ Physiol* 2011;**300**: H2261–2271.
58. Sciarretta S, Yee D, Nagarajan N, Bianchi F, Saito T, Valenti V, Tong M, Del Re DP, Vecchione C, Schirone L, Forte M, Rubattu S, Shirakabe A, Boppana VS, Volpe M, Frati G, Zhai P, Sadoshima J. Trehalose-induced activation of autophagy improves cardiac remodeling after myocardial infarction. *J Am Coll Cardiol* 2018;**71**: 1999–2010.

59. Wang B, Nie J, Wu L, Hu Y, Wen Z, Dong L, Zou MH, Chen C, Wang DW. AMPKalpha2 protects against the development of heart failure by enhancing mitophagy via PINK1 phosphorylation. *Circ Res* 2018;**122**:712–729.
60. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, Alnemri ES, Altucci L, Amelio I, Andrews DW, Annicchiarico-Petruzzelli M, Antonov AV, Arama E, Baehrecke EH, Barlev NA, Bazan NG, Bernassola F, Bertrand MJM, Bianchi K, Blagosklonny MV, Blomgren K, Borner C, Boya P, Brenner C, Campanella M, Candi E, Carmona-Gutierrez D, Cecconi F, Chan FK-M, Chandel NS, Cheng EH, Chipuk JE, Cidlowski JA, Ciechanover A, Cohen GM, Conrad M, Cubillos-Ruiz JR, Czabotar PE, D'Angiolella V, Dawson TM, Dawson VL, De Laurenzi V, De Maria R, Debatin K-M, DeBerardinis RJ, Deshmukh M, Di Daniele N, Di Virgilio F, Dixit VM, Dixon SJ, Duckett CS, Dynlacht BD, El-Deiry WS, Elrod JW, Fimia GM, Fulda S, García-Sáez AJ, Garg AD, Garrido C, Gavathiotis E, Golstein P, Gottlieb E, Green DR, Greene LA, Gronemeyer H, Gross A, Hajnoczky G, Hardwick JM, Harris IS, Hengartner MO, Hetz C, Ichijo H, Jäättelä M, Joseph B, Jost PJ, Juin PP, Kaiser WJ, Karin M, Kaufmann T, Kepp O, Kimchi A, Kitsis RN, Klionsky DJ, Knight RA, Kumar S, Lee SW, Lemasters JJ, Levine B, Linkermann A, Lipton SA, Lockshin RA, López-Otín C, Lowe SW, Luedde T, Lugli E, MacFarlane M, Madeo F, Malewicz M, Malorni W, Manic G, Marine J-C, Martin SJ, Martinou J-C, Medema JP, Mehlen P, Meier P, Melino S, Miao EA, Molkentin JD, Moll UM, Muñoz-Pinedo C, Nagata S, Nuñez G, Oberst A, Oren M, Overholtzer M, Pagano M, Panaretakis T, Pasparakis M, Penninger JM, Pereira DM, Pervaiz S, Peter ME, Piacentini M, Pinton P, Prehn JHM, Puthalakath H, Rabinovich GA, Rehm M, Rizzuto R, Rodrigues CMP, Rubinsztein DC, Rudel T, Ryan KM, Sayan E, Scorrano L, Shao F, Shi Y, Silke J, Simon H-U, Sistigu A, Stockwell BR, Strasser A, Szabadkai G, Tait SWG, Tang D, Tavernarakis N, Thorburn A, Tsujimoto Y, Turk B, Vanden Berghe T, Vandenabeele P, Vander Heiden MG, Villunger A, Virgin HW, Vousden KH, Vucic D, Wagner EF, Walczak H, Wallach D, Wang Y, Wells JA, Wood W, Yuan J, Zakeri Z, Zhivotovsky B, Zitvogel L, Melino G, Kroemer G. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ* 2018;**25**:486–541.
61. Kobayashi S, Volden P, Timm D, Mao K, Xu X, Liang Q. Transcription factor GATA4 inhibits doxorubicin-induced autophagy and cardiomyocyte death. *J Biol Chem* 2010;**285**:793–804.
62. Renu K, V.G A, P.B TP, Arunachalam S. Molecular mechanism of doxorubicin-induced cardiomyopathy—an update. *Eur J Pharmacol* 2018;**818**:241–253.
63. Koleini N, Kardami E. Autophagy and mitophagy in the context of doxorubicin-induced cardiotoxicity. *Oncotarget* 2017;**8**:46663–46680.
64. Li DL, Wang ZV, Ding G, Tan W, Luo X, Criollo A, Xie M, Jiang N, May H, Kyrychenko V, Schneider JW, Gillette TG, Hill JA. Doxorubicin blocks cardiomyocyte autophagic flux by inhibiting lysosome acidification. *Circulation* 2016;**133**: 1668–1687.
65. Liu Y, Shoji-Kawata S, Sumpter RM Jr, Wei Y, Ginet V, Zhang L, Posner B, Tran KA, Green DR, Xavier RJ, Shaw SY, Clarke PG, Puyal J, Levine B. Autosis is a Na^+, K^+-ATPase-regulated form of cell death triggered by autophagy-inducing peptides, starvation, and hypoxia-ischemia. *Proc Natl Acad Sci USA* 2013;**110**: 20364–20371.
66. Lu W, Sun J, Yoon JS, Zhang Y, Zheng L, Murphy E, Mattson MP, Lenardo MJ. Mitochondrial protein PGAM5 regulates mitophagic protection against cell necroptosis. *PLoS One* 2016;**11**:e0147792.
67. Dohi E, Tanaka S, Seki T, Miyagi T, Hide I, Takahashi T, Matsumoto M, Sakai N. Hypoxic stress activates chaperone-mediated autophagy and modulates neuronal cell survival. *Neurochem Int* 2012;**60**:431–442.
68. Kiffin R, Christian C, Knecht E, Cuervo AM. Activation of chaperone-mediated autophagy during oxidative stress. *Mol Biol Cell* 2004;**15**:4829–4840.
69. Maejima Y, Kyoi S, Zhai P, Liu T, Li H, Ivessa A, Sciarretta S, Del Re DP, Zablocki DK, Hsu CP, Lim DS, Isobe M, Sadoshima J. Mst1 inhibits autophagy by promoting the interaction between Beclin1 and Bcl-2. *Nat Med* 2013;**19**:1478–1488.
70. Madeo F, Carmona-Gutierrez D, Hofer SJ, Kroemer G. Caloric restriction mimetics against age-associated disease: targets, mechanisms, and therapeutic potential. *Cell Metab* 2019;**29**:592–610.
71. Cuzick J, Thorat MA, Bosetti C, Brown PH, Burn J, Cook NR, Ford LG, Jacobs EJ, Jankowski JA, La Vecchia C, Law M, Meyskens F, Rothwell PM, Senn HJ, Umar A. Estimates of benefits and harms of prophylactic use of aspirin in the general population. *Ann Oncol* 2015;**26**:47–57.
72. Pietrocola F, Castoldi F, Markaki M, Lachkar S, Chen G, Enot DP, Durand S, Bossut N, Tong M, Malik SA, Loos F, Dupont N, Marino G, Abdelkader N, Madeo F, Maiuri MC, Kroemer R, Codogno P, Sadoshima J, Tavernarakis N, Kroemer G. Aspirin recapitulates features of caloric restriction. *Cell Rep* 2018;**22**:2395–2407.
73. Shirakabe A, Zhai P, Ikeda Y, Saito T, Maejima Y, Hsu CP, Nomura M, Egashira K, Levine B, Sadoshima J. Drp1-dependent mitochondrial autophagy plays a protective role against pressure overload-induced mitochondrial dysfunction and heart failure. *Circulation* 2016;**133**:1249–1263.
74. Kubli DA, Zhang X, Lee Y, Hanna RA, Quinsay MN, Nguyen CK, Jimenez R, Petrosyan S, Murphy AN, Gustafsson AB. Parkin protein deficiency exacerbates cardiac injury and reduces survival following myocardial infarction. *J Biol Chem* 2013; **288**:915–926.
75. Oka T, Hikoso S, Yamaguchi O, Taneike M, Takeda T, Tamai T, Oyabu J, Murakawa T, Nakayama H, Nishida K, Akira S, Yamamoto A, Komuro I, Otsu K. Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. *Nature* 2012;**485**:251–255.
76. Sciarretta S, Boppana VS, Umapathi M, Frati G, Sadoshima J. Boosting autophagy in the diabetic heart: a translational perspective. *Cardiovasc Diagn Ther* 2015;**5**:394–402.
77. Xie Z, He C, Zou MH. AMP-activated protein kinase modulates cardiac autophagy in diabetic cardiomyopathy. *Autophagy* 2011;**7**:1254–1255.
78. Li ZL, Woollard JR, Ebrahimi B, Crane JA, Jordan KL, Lerman A, Wang SM, Lerman LO. Transition from obesity to metabolic syndrome is associated with altered myocardial autophagy and apoptosis. *Arterioscler Thromb Vasc Biol* 2012;**32**:1132–1141.
79. Osipov RM, Bianchi C, Feng J, Clements RT, Liu Y, Robich MP, Glazer HP, Sodha NR, Sellke FW. Effect of hypercholesterolemia on myocardial necrosis and apoptosis in the setting of ischemia-reperfusion. *Circulation* 2009;**120**:S22–30.
80. Andres AM, Kooren JA, Parker SJ, Tucker KC, Ravindran N, Ito BR, Huang C, Venkatraman V, Van Eyk JE, Gottlieb RA, Mentzer RM Jr. Discordant signaling and autophagy response to fasting in hearts of obese mice: implications for ischemia tolerance. *Am J Physiol Heart Circ Physiol* 2016;**311**:H219–228.
81. Gottlieb RA, Andres AM, Sin J, Taylor DP. Untangling autophagy measurements: all fluxed up. *Circ Res* 2015;**116**:504–514.
82. He C, Bassik MC, Moresi V, Sun K, Wei Y, Zou Z, An Z, Loh J, Fisher J, Sun Q, Korsmeyer S, Packer M, May HI, Hill JA, Virgin HW, Gilpin C, Xiao G, Bassel-Duby R, Scherer PE, Levine B. Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. *Nature* 2012;**481**:511–515.
83. Xu X, Ren J. Macrophage migration inhibitory factor (MIF) knockout preserves cardiac homeostasis through alleviating Akt-mediated myocardial autophagy suppression in high-fat diet-induced obesity. *Int J Obes* 2015;**39**:387–396.
84. Liang L, Shou XL, Zhao HK, Ren GQ, Wang JB, Wang XH, Ai WT, Maris JR, Hueckstaedt LK, Ma AQ, Zhang Y. Antioxidant catalase rescues against high fat diet-induced cardiac dysfunction via an IKKbeta-AMPK-dependent regulation of autophagy. *Biochim Biophys Acta* 2015;**1852**:343–352.
85. Hu N, Zhang Y. TLR4 knockout attenuated high fat diet-induced cardiac dysfunction via NF-kappaB/JNK-dependent activation of autophagy. *Biochim Biophys Acta Mol Basis Dis* 2017;**1863**:2001–2011.
86. Jaishy B, Zhang Q, Chung HS, Riehle C, Soto J, Jenkins S, Abel P, Cowart LA, Van Eyk JE, Abel ED. Lipid-induced NOX2 activation inhibits autophagic flux by impairing lysosomal enzyme activity. *J Lipid Res* 2015;**56**:546–561.
87. Xu X, Hua Y, Nair S, Zhang Y, Ren J. Akt2 knockout preserves cardiac function in high-fat diet-induced obesity by rescuing cardiac autophagosome maturation. *J Mol Cell Biol* 2013;**5**:61–63.
88. Xie Z, Lau K, Eby B, Lozano P, He C, Pennington B, Li H, Rathi S, Dong Y, Tian R, Kem D, Zou MH. Improvement of cardiac functions by chronic metformin treatment is associated with enhanced cardiac autophagy in diabetic OVE26 mice. *Diabetes* 2011;**60**:1770–1778.
89. Xu X, Kobayashi S, Chen K, Timm D, Volden P, Huang Y, Gulick J, Yue Z, Robbins J, Epstein PN, Liang Q. Diminished autophagy limits cardiac injury in mouse models of type 1 diabetes. *J Biol Chem* 2013;**288**:18077–18092.
90. Schneider JL, Suh Y, Cuervo AM. Deficient chaperone-mediated autophagy in liver leads to metabolic dysregulation. *Cell Metab* 2014;**20**:417–432.
91. Nishino I, Fu J, Tanji K, Yamada T, Shimojo S, Koori T, Mora M, Riggs JE, Oh SJ, Koga Y, Sue CM, Yamamoto A, Murakami N, Shanske S, Byrne E, Bonilla E, Nonaka I, DiMauro S, Hirano M. Primary LAMP-2 deficiency causes X-linked vacuolar cardiomyopathy and myopathy (Danon disease). *Nature* 2000;**406**: 906–910.
92. Tanaka Y, Guhde G, Suter A, Eskelinen EL, Hartmann D, Lullmann-Rauch R, Janssen PM, Blanz J, von Figura K, Saftig P. Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice. *Nature* 2000;**406**:902–906.
93. Cullup T, Kho AL, Dionisi-Vici C, Brandmeier B, Smith F, Urry Z, Simpson MA, Yau S, Bertini E, McClelland V, Al-Owain M, Koelker S, Koerner C, Hoffmann GF, Wijburg FA, ten Hoedt AE, Rogers RC, Manchester D, Miyata R, Hayashi M, Said E, Soler D, Kroisel PM, Windpassinger C, Filloux FM, Al-Kaabi S, Hertecant J, Del Campo M, Buk S, Bodi I, Goebel HH, Sewry CA, Abbs S, Mohammed S, Josifova D, Gautel M, Jungbluth H. Recessive mutations in EPG5 cause Vici syndrome, a multisystem disorder with defective autophagy. *Nat Genet* 2013;**45**:83–87.
94. Hori I, Otomo T, Nakashima M, Miya F, Negishi Y, Shiraishi H, Nonoda Y, Magara S, Tohyama J, Okamoto N, Kumagai T, Shimoda K, Yukitake Y, Kajikawa D, Morio T, Hattori A, Nakagawa M, Ando N, Nishino I, Kato M, Tsunoda T, Saitsu H, Kanemura Y, Yamasaki M, Kosaki K, Matsumoto N, Yoshimori T, Saitoh S. Defects in autophagosome-lysosome fusion underlie Vici syndrome, a neurodevelopmental disorder with multisystem involvement. *Sci Rep* 2017;**7**:3552.
95. Chabas A, Cormand B, Grinberg D, Burguera JM, Balcells S, Merino JL, Mate I, Sobrino JA, Gonzalez-Duarte R, Vilageliu L. Unusual expression of Gaucher's disease: cardiovascular calcifications in three sibs homozygous for the D409H mutation. *J Med Genet* 1995;**32**:740–742.
96. Portilla-Fernandez E, Ghanbari M, van Meurs JBJ, Danser AHJ, Franco OH, Muka T, Roks A, Dehghan A. Dissecting the association of autophagy-related genes with cardiovascular diseases and intermediate vascular traits: a population-based approach. *PLoS One* 2019;**14**:e0214137.
97. Zhang P, Zhang J, Zhang Y, Wang S, Pang S, Yan B. Functional variants of the ATG7 gene promoter in acute myocardial infarction. *Mol Genet Genomic Med* 2018;**6**: 1209–1219.

98. Novikoff AB, Beaufay H, De Duve C. Electron microscopy of lysosomerich fractions from rat liver. *J Biophys Biochem Cytol* 1956;**2**:179–184.

99. Eskelinen EL. Fine structure of the autophagosome. *Methods Mol Biol* 2008;**445**: 11–28.

100. Dorn GW. Parkin-dependent mitophagy in the heart. *J Mol Cell Cardiol* 2016;**95**: 42–49. 2nd.

101. Willingham MC, Rutherford AV. The use of osmium-thiocarbohydrazide-osmium (OTO) and ferrocyanide-reduced osmium methods to enhance membrane contrast and preservation in cultured cells. *J Histochem Cytochem* 1984;**32**:455–460.

102. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K, Adhihetty PJ, Adler SG, Agam G, Agarwal R, Aghi MK, Agnello M, Agostinis P, Aguilar PV, Aguirre-Ghiso J, Airoldi EM, Ait-Si-Ali S, Akematsu T, Akporiaye ET, Al-Rubeai M, Albaiceta GM, Albanese C, Albani D, Albert ML, Aldudo J, Algul H, Alirezaei M, Alloza I, Almasan A, Almonte-Beceril M, Alnemri ES, Alonso C, Altan-Bonnet N, Altieri DC, Alvarez S, Alvarez-Erviti L, Alves S, Amadoro G, Amano A, Amantini C, Ambrosio S, Amelio I, Amer AO, Amessou M, Amon A, An Z, Anania FA, Andersen SU, Andley UP, Andreadi CK, Andrieu-Abadie N, Anel A, Ann DK, Anoopkumar-Dukie S, Antonioli M, Aoki H, Apostolova N, Aquila S, Aquilano K, Araki K, Arama E, Aranda A, Araya J, Arcaro A, Arias E, Arimoto H, Ariosa AR, Armstrong JL, Arnould T, Arsov I, Asanuma K, Askanas V, Asselin E, Atarashi R, Atherton SS, Atkin JD, Attardi LD, Auberger P, Auburger G, Aurelian L, Autelli R, Avagliano L, Avantaggiati ML, Avrahami L, Awale S, Azad N, Bachetti T, Backer JM, Bae DH, Bae JS, Bae ON, Bae SH, Baehrecke EH, Baek SH, Baghdiguian S, Bagniewska-Zadworna A, Bai H, Bai J, Bai XY, Bailly Y, Balaji KN, Balduini W, Ballabio A, Balzan R, Banerjee R, Banhegyi G, Bao H, Barbeau B, Barrachina MD, Barreiro E, Bartel B, Bartolome A, Bassham DC, Bassi MT, Bast RC Jr, Basu A, Batista MT, Batoko H, Battino M, Bauckman K, Baumgarner BL, Bayer KU, Beale R, Beaulieu JF, Beck GR Jr, Becker C, Beckham JD, Bedard PA, Bednarski PJ, Begley TJ, Behl C, Behrends C, Behrens GM, Behrns KE, Bejarano E, Belaid A, Belleudi F, Benard G, Berchem G, Bergamaschi D, Bergami M, Berkhout B, Berliocchi L, Bernard A, Bernard M, Bernassola F, Bertolotti A, Bess AS, Besteiro S, Bettuzzi S, Bhalla S, Bhattacharyya S, Bhutia SK, Biagosch C, Bianchi MW, Biard-Piechaczyk M, Billes V, Bincoletto C, Bingol B, Bird SW, Bitoun M, Bjedov I, Blackstone C, Blanc L, Blanco GA, Blomhoff HK, Boada-Romero E, Bockler S, Boes M, Boesze-Battaglia K, Boise LH, Bolino A, Boman A, Bonaldo P, Bordi M, Bosch J, Botana LM, Botti J, Bou G, Bouche M, Bouchecareilh M, Boucher MJ, Boulton ME, Bouret SG, Boya P, Boyer-Guittaut M, Bozhkov PV, Brady N, Braga VM, Brancolini C, Braus GH, Bravo-San Pedro JM, Brennan LA, Bresnick EH, Brest P, Bridges D, Bringer MA, Brini M, Brito GC, Brodin B, Brookes PS, Brown EJ, Brown K, Broxmeyer HE, Bruhat A, Brum PC, Brumell JH, Brunetti-Pierri N, Bryson-Richardson RJ, Buch S, Buchan AM, Budak H, Bulavin DV, Bultman SJ, Bultynck G, Bumbasirevic V, Burelle Y, Burke RE, Burmeister M, Butikofer P, Caberlotto L, Cadwell K, Cahova M, Cai D, Cai J, Cai Q, Calatayud S, Camougrand N, Campanella M, Campbell GR, Campbell M, Campello S, Candau R, Caniggia I, Cantoni L, Cao L, Caplan AB, Caraglia M, Cardinali C, Cardoso SM, Carew JS, Carleton LA, Carlin CR, Carloni S, Carlsson SR, Carmona-Gutierrez D, Carneiro LA, Carnevali O, Carra S, Carrier A, Carroll B, Casas C, Casas J, Cassinelli G, Castets P, Castro-Obregon S, Cavallini G, Ceccherini I, Cecconi F, Cederbaum AI, Cena V, Cenci S, Cerella C, Cervia D, Cetrullo S, Chaachouay H, Chae HJ, Chagin AS, Chai CY, Chakrabarti G, Chamilos G, Chan EY, Chan MT, Chandra D, Chandra P, Chang CP, Chang RC, Chang TY, Chatham JC, Chatterjee S, Chauhan S, Che Y, Cheetham ME, Cheluvappa R, Chen CJ, Chen G, Chen GC, Chen G, Chen H, Chen JW, Chen JK, Chen M, Chen M, Chen P, Chen Q, Chen Q, Chen SD, Chen S, Chen SS, Chen W, Chen WJ, Chen WQ, Chen W, Chen X, Chen YH, Chen YG, Chen Y, Chen Y, Chen Y, Chen YJ, Chen YQ, Chen Y, Chen Z, Chen Z, Cheng A, Cheng CH, Cheng H, Cheong H, Cherry S, Chesney J, Cheung CH, Chevet E, Chi HC, Chi SG, Chiacchiera F, Chiang HL, Chiarelli R, Chiariello M, Chieppa M, Chin LS, Chiong M, Chiu GN, Cho DH, Cho SG, Cho WC, Cho YY, Cho YS, Choi AM, Choi EJ, Choi EK, Choi J, Choi ME, Choi SI, Chou TF, Chouaib S, Choubey D, Choubey V, Chow KC, Chowdhury K, Chu CT, Chuang TH, Chun T, Chung H, Chung T, Chung YL, Chwae YJ, Cianfanelli V, Ciarcia R, Ciechomska IA, Ciriolo MR, Cirone M, Claerhout S, Clague MJ, Claria J, Clarke PG, Clarke R, Clementi E, Cleyrat C, Cnop M, Coccia EM, Cocco T, Codogno P, Coers J, Cohen EE, Colecchia D, Coletto L, Coll NS, Colucci-Guyon E, Comincini S, Condello M, Cook KL, Coombs GH, Cooper CD, Cooper JM, Coppens I, Corasaniti MT, Corazzari M, Corbalan R, Corcelle-Termeau E, Cordero MD, Corral-Ramos C, Corti O, Cossarizza A, Costelli P, Costes S, Cotman SL, Coto-Montes A, Cottet S, Couve E, Covey LR, Cowart LA, Cox JS, Coxon FP, Coyne CB, Cragg MS, Craven RJ, Crepaldi T, Crespo JL, Criollo A, Crippa V, Cruz MT, Cuervo AM, Cuezva JM, Cui T, Cutillas PR, Czaja MJ, Czyzyk-Krzeska MF, Dagda RK, Dahmen U, Dai C, Dai W, Dai Y, Dalby KN, Dalla Valle L, Dalmasso G, D'Amelio M, Damme M, Darfeuille-Michaud A, Dargemont C, Darley-Usmar VM, Dasarathy S, Dasgupta B, Dash S, Dass CR, Davey HM, Davids LM, Davila D, Davis RJ, Dawson TM, Dawson VL, Daza P, de Belleroche J, de Figueiredo P, de Figueiredo RC, de la Fuente J, De Martino L, De Matteis A, De Meyer GR, De Milito A, De Santi M, de Souza W, De Tata V, De Zio D, Debnath J, Dechant R, Decuypere JP, Deegan S, Dehay B, Del Bello B, Del Re DP, Delage-Mourroux R, Delbridge LM, Deldicque L, Delorme-Axford E, Deng Y, Dengjel J, Denizot M, Dent P, Der CJ, Deretic V, Derrien B, Deutsch E, Devarenne TP, Devenish RJ, Di Bartolomeo S, Di Daniele N, Di Domenico F, Di Nardo A, Di Paola S, Di Pietro A, Di Renzo L, Di Antonio A, Diaz-Araya G, Diaz-Laviada I, Diaz-Meco MT, Diaz-Nido J, Dickey CA, Dickson RC, Diederich M, Digard P, Dikic I, Dinesh-Kumar SP, Ding C, Ding WX, Ding Z, Dini L, Distler JH, Diwan A, Djavaheri-Mergny M, Dmytruk K, Dobson RC, Doetsch V, Dokladny K, Dokudovskaya S, Donadelli M, Dong XC, Dong X, Dong Z, Donohue TM Jr, Doran KS, D'Orazi G, Dorn GW 2nd, Dosenko V, Dridi S, Drucker L, Du J, Du LL, Du L, du Toit A, Dua P, Duan L, Duann P, Dubey VK, Duchen MR, Duchosal MA, Duez H, Dugail I, Dumit VI, Duncan MC, Dunlop EA, Dunn WA Jr, Dupont N, Dupuis L, Duran RV, Durcan TM, Duvezin-Caubet S, Duvvuri U, Eapen V, Ebrahimi-Fakhari D, Echard A, Eckhart L, Edelstein CL, Edinger AL, Eichinger L, Eisenberg T, Eisenberg-Lerner A, Eissa NT, El-Deiry WS, El-Khoury V, Elazar Z, Eldar-Finkelman H, Elliott CJ, Emanuele E, Emmenegger U, Engedal N, Engelbrecht AM, Engelender S, Enserink JM, Erdmann R, Erenpreisa J, Eri R, Eriksen JL, Erman A, Escalante R, Eskelinen EL, Espert L, Esteban-Martinez L, Evans TJ, Fabri M, Fabrias G, Fabrizi C, Facchiano A, Faergeman NJ, Faggioni A, Fairlie WD, Fan C, Fan D, Fan J, Fang S, Fanto M, Fanzani A, Farkas T, Faure M, Favier FB, Fearnhead H, Federici M, Fei E, Felizardo TC, Feng H, Feng Y, Feng Y, Ferguson TA, Fernandez AF, Fernandez-Barrena MG, Fernandez-Checa JC, Fernandez-Lopez A, Fernandez-Zapico ME, Feron O, Ferraro E, Ferreira-Halder CV, Fesus L, Feuer R, Fiesel FC, Filippi-Chiela EC, Filomeni G, Fimia GM, Fingert JH, Finkbeiner S, Finkel T, Fiorito F, Fisher PB, Flajolet M, Flamigni F, Florey O, Florio S, Floto RA, Folini M, Follo C, Fon EA, Fornai F, Fortunato F, Fraldi A, Franco R, Francois A, Francois A, Frankel LB, Fraser ID, Frey N, Freyssenet DG, Frezza C, Friedman SL, Frigo DE, Fu D, Fuentes JM, Fueyo J, Fujitani Y, Fujiwara Y, Fujiya M, Fukuda M, Fulda S, Fusco C, Gabryel B, Gaestel M, Gailly P, Gajewska M, Galadari S, Galili G, Galindo I, Galindo MF, Galliciotti G, Galluzzi L, Galluzzi L, Galy V, Gammoh N, Gandy S, Ganesan AK, Ganesan S, Ganley IG, Gannage M, Gao FB, Gao F, Gao JX, Garcia Nannig L, Garcia Vescovi E, Garcia-Macia M, Garcia-Ruiz C, Garg AD, Garg PK, Gargini R, Gassen NC, Gatica D, Gatti E, Gavard J, Gavathiotis E, Ge L, Ge P, Ge S, Gean PW, Gelmetti V, Genazzani AA, Geng J, Genschik P, Gerner L, Gestwicki JE, Gewirtz DA, Ghavami S, Ghigo E, Ghosh D, Giammarioli AM, Giampieri F, Giampietri C, Giatromanolaki A, Gibbings DJ, Gibellini L, Gibson SB, Ginet V, Giordano A, Giorgini F, Giovannetti E, Girardin SE, Gispert S, Giuliano S, Gladson CL, Glavic A, Gleave M, Godefroy N, Gogal RM Jr, Gokulan K, Goldman GH, Goletti D, Goligorsky MS, Gomes AV, Gomes LC, Gomez H, Gomez-Manzano C, Gomez-Sanchez R, Goncalves DA, Goncu E, Gong Q, Gongora C, Gonzalez CB, Gonzalez-Alegre P, Gonzalez-Cabo P, Gonzalez-Polo RA, Goping IS, Gorbea C, Gorbunov NV, Goring DR, Gorman AM, Gorski SM, Goruppi S, Goto-Yamada S, Gotor C, Gottlieb RA, Gozes I, Gozuacik D, Graba Y, Graef M, Granato GE, Grant GD, Grant S, Gravina GL, Green DR, Greenhough A, Greenwood MT, Grimaldi B, Gros F, Grose C, Groulx JF, Gruber F, Grumati P, Grune T, Guan JL, Guan KL, Guerra B, Guillen C, Gulshan K, Gunst J, Guo C, Guo L, Guo M, Guo W, Guo XG, Gust AA, Gustafsson AB, Gutierrez E, Gutierrez MG, Gwak HS, Haas A, Haber JE, Hadano S, Hagedorn M, Hahn DR, Halayko AJ, Hamacher-Brady A, Hamada K, Hamai A, Hamann A, Hamasaki M, Hamer I, Hamid Q, Hammond EM, Han F, Han W, Handa JT, Hanover JA, Hansen M, Harada M, Harhaji-Trajkovic L, Harper JW, Harrath AH, Harris AL, Harris J, Hasler U, Hasselblatt P, Hasui K, Hawley RG, Hawley TS, He C, He CY, He F, He G, He RR, He XH, He YW, He YY, Heath JK, Hebert MJ, Heinzen RA, Helgason GV, Hensel M, Henske EP, Her C, Herman PK, Hernandez A, Hernandez C, Hernandez-Tiedra S, Hetz C, Hiesinger PR, Higaki K, Hilfiker S, Hill BG, Hill JA, Hill WD, Hino K, Hofius D, Hofman P, Hoglinger GU, Hohfeld J, Holz MK, Hong Y, Hood DA, Hoozemans JJ, Hoppe T, Hsu C, Hsu CY, Hsu LC, Hu D, Hu G, Hu HM, Hu H, Hu MC, Hu YC, Hu ZW, Hua F, Hua Y, Huang C, Huang HL, Huang KH, Huang KY, Huang S, Huang S, Huang WP, Huang YR, Huang Y, Huang Y, Huber TB, Huebbe P, Huh WK, Hulmi JJ, Hur GM, Hurley JH, Husak Z, Hussain SN, Hussain S, Hwang JJ, Hwang S, Hwang TI, Ichihara A, Imai Y, Imbriano C, Inomata M, Into T, Iovane V, Iovanna JL, Iozzo RV, Ip NY, Irazoqui JE, Iribarren P, Isaka Y, Isakovic AJ, Ischiropoulos H, Isenberg JS, Ishaq M, Ishida H, Ishii I, Ishmael JE, Isidoro C, Isobe K, Isono E, Issazadeh-Navikas S, Itahana K, Itakura E, Ivanov AI, Iyer AK, Izquierdo JM, Izumi Y, Izzo V, Jaattela M, Jaber N, Jackson DJ, Jackson WT, Jacob TG, Jacques TS, Jagannath C, Jain A, Jana NR, Jang BK, Jani A, Janji B, Jannig PR, Jansson PJ, Jean S, Jendrach M, Jeon JH, Jessen N, Jeung EB, Jia K, Jia L, Jiang H, Jiang H, Jiang L, Jiang T, Jiang X, Jiang X, Jiang X, Jiang Y, Jiang Y, Jimenez A, Jin C, Jin H, Jin L, Jin M, Jin S, Jinwal UK, Jo EK, Johansen T, Johnson DE, Johnson GV, Johnson JD, Jonasch E, Jones C, Joosten LA, Jordan J, Joseph AM, Joseph B, Joubert AM, Ju D, Ju J, Juan HF, Juenemann K, Juhasz G, Jung HS, Jung JU, Jung YK, Jungbluth H, Justice MJ, Jutten B, Kaakoush NO, Kaarniranta K, Kaasik A, Kabuta T, Kaeffer B, Kagedal K, Kahana A, Kajimura S, Kakhlon O, Kalia M, Kalvakolanu DV, Kamada Y, Kambas K, Kaminskyy VO, Kampinga HH, Kandouz M, Kang C, Kang R, Kang TC, Kanki T, Kanneganti TD, Kanno H, Kanthasamy AG, Kantorow M, Kaparakis-Liaskos M, Kapuy O, Karantza V, Karim MR, Karmakar P, Kaser A, Kaushik S, Kawula T, Kaynar AM, Ke PY, Ke ZJ, Kehrl JH, Keller KE, Kemper JK, Kenworthy AK, Kepp O, Kern A, Kesari S, Kessel D, Ketteler R, Kettelhut Ido C, Khambu B, Khan MM, Khandelwal VK, Khare S, Kiang JG, Kiger AA, Kihara A, Kim AL, Kim CH, Kim DR, Kim DH, Kim EK, Kim HY, Kim HR, Kim JS, Kim JH, Kim JC, Kim JH, Kim KW, Kim MD, Kim MM, Kim PK, Kim SW, Kim SY, Kim YS, Kim Y, Kimchi A, Kimmelman AC, Kimura T, King JS, Kirkegaard K, Kirkin V, Kirshenbaum LA, Kishi S, Kitajima Y, Kitamoto K, Kitaoka Y, Kitazato K, Kley RA, Klimecki WT, Klinkenberg M, Klucken J, Knaevelsrud H, Knecht E, Knuppertz L, Ko JL, Kobayashi S, Koch JC, Koechlin-

Ramonatxo C, Koenig U, Koh YH, Kohler K, Kohlwein SD, Koike M, Komatsu M, Kominami E, Kong D, Kong HJ, Konstantakou EG, Kopp BT, Korcsmaros T, Korhonen L, Korolchuk VI, Koshkina NV, Kou Y, Koukourakis MI, Koumenis C, Kovacs AL, Kovacs T, Kovacs WJ, Koya D, Kraft C, Krainc D, Kramer H, Kravic-Stevovic T, Krek W, Kretz-Remy C, Krick R, Krishnamurthy M, Kriston-Vizi J, Kroemer G, Kruer MC, Kruger R, Ktistakis NT, Kuchitsu K, Kuhn C, Kumar AP, Kumar A, Kumar A, Kumar D, Kumar D, Kumar R, Kumar S, Kundu M, Kung HJ, Kuno A, Kuo SH, Kuret J, Kurz T, Kwok T, Kwon TK, Kwon YT, Kyrmizi I, La Spada AR, Lafont F, Lahm T, Lakkaraju A, Lam T, Lamark T, Lancel S, Landowski TH, Lane DJ, Lane JD, Lanzi C, Lapaquette P, Lapierre LR, Laporte J, Laukkarinen J, Laurie GW, Lavandero S, Lavie L, LaVoie MJ, Law BY, Law HK, Law KB, Layfield R, Lazo PA, Le Cam L, Le Roch KG, Le Stunff H, Leardkamolkarn V, Lecuit M, Lee BH, Lee CH, Lee EF, Lee GM, Lee HJ, Lee H, Lee JK, Lee J, Lee JH, Lee JH, Lee M, Lee MS, Lee PJ, Lee SW, Lee SJ, Lee SJ, Lee SY, Lee SH, Lee SS, Lee SJ, Lee S, Lee YR, Lee YJ, Lee YH, Leeuwenburgh C, Lefort S, Legouis R, Lei J, Lei QY, Leib DA, Leibowitz G, Lekli I, Lemaire SD, Lemasters JJ, Lemberg MK, Lemoine A, Leng S, Lenz G, Lenzi P, Lerman LO, Lettieri Barbato D, Leu JI, Leung HY, Levine B, Lewis PA, Lezoualc'h F, Li C, Li F, Li FJ, Li J, Li K, Li L, Li M, Li M, Li Q, Li R, Li S, Li W, Li W, Li X, Li Y, Lian J, Liang C, Liang Q, Liao Y, Liberal J, Liberski PP, Lie P, Lieberman AP, Lim HJ, Lim KL, Lim K, Lima RT, Lin CS, Lin CF, Lin F, Lin F, Lin FC, Lin K, Lin KH, Lin PH, Lin T, Lin WW, Lin YS, Lin Y, Linden R, Lindholm D, Lindqvist LM, Lingor P, Linkermann A, Liotta LA, Lipinski MM, Lira VA, Lisanti MP, Liton PB, Liu B, Liu C, Liu CF, Liu F, Liu HJ, Liu J, Liu JJ, Liu JL, Liu K, Liu L, Liu L, Liu Q, Liu RY, Liu S, Liu S, Liu W, Liu XD, Liu X, Liu XH, Liu X, Liu X, Liu X, Liu Y, Liu Y, Liu Z, Liu Z, Liuzzi JP, Lizard G, Ljujic M, Lodhi IJ, Logue SE, Lokeshwar BL, Long YC, Lonial S, Loos B, Lopez-Otin C, Lopez-Vicario C, Lorente M, Lorenzi PL, Lorincz P, Los M, Lotze MT, Lovat PE, Lu B, Lu B, Lu J, Lu Q, Lu SM, Lu S, Lu Y, Luciano F, Luckhart S, Lucocq JM, Ludovico P, Lugea A, Lukacs NW, Lum JJ, Lund AH, Luo H, Luo J, Luo S, Luparello C, Lyons T, Ma J, Ma Y, Ma Y, Ma Z, Machado J, Machado-Santelli GM, Macian F, MacIntosh GC, MacKeigan JP, Macleod KF, MacMicking JD, MacMillan-Crow LA, Madeo F, Madesh M, Madrigal-Matute J, Maeda A, Maeda T, Maegawa G, Maellaro E, Maes H, Magarinos M, Maiese K, Maiti TK, Maiuri L, Maiuri MC, Maki CG, Malli R, Malorni W, Maloyan A, Mami-Chouaib F, Man N, Mancias JD, Mandelkow EM, Mandell MA, Manfredi AA, Manie SN, Manzoni C, Mao K, Mao Z, Mao ZW, Marambaud P, Marconi AM, Marelja Z, Marfe G, Margeta M, Margittai E, Mari M, Mariani FV, Marin C, Marinelli S, Marino G, Markovic I, Marquez R, Martelli AM, Martens S, Martin KR, Martin SJ, Martin S, Martin-Acebes MA, Martin-Sanz P, Martinand-Mari C, Martinet W, Martinez J, Martinez-Lopez N, Martinez-Outschoorn U, Martinez-Velazquez M, Martinez-Vicente M, Martins WK, Mashima H, Mastrianni JA, Matarese G, Matarrese P, Mateo R, Matoba S, Matsumoto N, Matsushita T, Matsuura A, Matsuzawa T, Mattson MP, Matus S, Maugeri N, Mauvezin C, Mayer A, Maysinger D, Mazzolini GD, McBrayer MK, McCall K, McCormick C, McInerney GM, McIver SC, McKenna S, McMahon JJ, McNeish IA, Mechta-Grigoriou F, Medema JP, Medina DL, Megyeri K, Mehrpour M, Mehta JL, Mei Y, Meier UC, Meijer AJ, Melendez A, Melino G, Melino S, de Melo EJ, Mena MA, Meneghini MD, Menendez JA, Menezes R, Meng L, Meng LH, Meng S, Menghini R, Menko AS, Menna-Barreto RF, Menon MB, Meraz-Rios MA, Merla G, Merlini L, Merlot AM, Meryk A, Meschini S, Meyer JN, Mi MT, Miao CY, Micale L, Michaeli S, Michiels C, Migliaccio AR, Mihailidou AS, Mijaljica D, Mikoshiba K, Milan E, Miller-Fleming L, Mills GB, Mills IG, Minakaki G, Minassian BA, Ming XF, Minibayeva F, Minina EA, Mintern JD, Minucci S, Miranda-Vizuete A, Mitchell CH, Miyamoto S, Miyazawa K, Mizushima N, Mnich K, Mograbi B, Mohseni S, Moita LF, Molinari M, Molinari M, Moller AB, Mollereau B, Mollinedo F, Mongillo M, Monick MM, MontagnaroS, Montell C, Moore DJ, Moore MN, Mora-Rodriguez R, Moreira PI, Morel E, Morelli MB, Moreno S, Morgan MJ, Moris A, Moriyasu Y, Morrison JL, Morrison LA, Morselli E, Moscat J, Moseley PL, Mostowy S, Motori E, Mottet D, Mottram JC, Moussa CE, Mpakou VE, Mukhtar H, Mulcahy Levy JM, Muller S, Munoz-Moreno R, Munoz-Pinedo C, Munz C, Murphy ME, Murray JT, Murthy A, Mysorekar IU, Nabi IR, Nabissi M, Nader GA, Nagahara Y, Nagai Y, Nagata K, Nagelkerke A, Nagy P, Naidu SR, Nair S, Nakano H, Nakatogawa H, Nanjundan M, Napolitano G, Naqvi NI, Nardacci R, Narendra DP, Narita M, Nascimbeni AC, Natarajan R, Navegantes LC, Nawrocki ST, Nazarko TY, Nazarko VY, Neill T, Neri LM, Netea MG, Netea-Maier RT, Neves BM, Ney PA, Nezis IP, Nguyen HT, Nguyen HP, Nicot AS, Nilsen H, Nilsson P, Nishimura M, Nishino I, Niso-Santano M, Niu H, Nixon RA, Njar VC, Noda T, Noegel AA, Nolte EM, Norberg E, Norga KK, Noureini SK, Notomi S, Notterpek L, Nowikovsky K, Nukina N, Nurnberger T, O'Donnell VB, O'Donovan T, O'Dwyer PJ, Oehme I, Oeste CL, Ogawa M, Ogretmen B, Ogura Y, Oh YJ, Ohmuraya M, Ohshima T, Ojha R, Okamoto K, Okazaki T, Oliver FJ, Ollinger K, Olsson S, Orban DP, Ordonez P, Orhon I, Orosz L, O'Rourke EJ, Orozco H, Ortega AL, Ortona E, Osellame LD, Oshima J, Oshima S, Osiewacz HD, Otomo T, Otsu K, Ou JH, Outeiro TF, Ouyang DY, Ouyang H, Overholtzer M, Ozbun MA, Ozdinler PH, Ozpolat B, Pacelli C, Paganetti P, Page G, Pages G, Pagnini U, Pajak B, Pak SC, Pakos-Zebrucka K, Pakpour N, Palkova Z, Palladino F, Pallauf K, Pallet N, Palmieri M, Paludan SR, Palumbo C, Palumbo S, Pampliega O, Pan H, Pan W, Panaretakis T, Pandey A, Pantazopoulou A, Papackova Z, Papademetrio DL, Papassideri I, Papini A, Parajuli N, Pardo J, Parekh VV, Parenti G, Park JI, Park J, Park OK, Parker R, Parlato R, Parys JB, Parzych KR, Pasquet JM, Pasquier B, Pasumarthi KB, Patschan D, Patterson C, Pattingre S, Pattison S, Pause A, Pavenstadt H, Pavone F, Pedrozo Z, Pena FJ, Penalva MA, Pende M, Peng J, Penna F, Penninger JM, Pensalfini A, Pepe S, Pereira GJ, Pereira PC, Perez-de la Cruz V, Perez-Perez ME, Perez-Rodriguez D, Perez-Sala D, Perier C, Perl A, Perlmutter DH, Perrotta I, Pervaiz S, Pesonen M, Pessin JE, Peters GJ, Petersen M, Petrache I, Petrof BJ, Petrovski G, Phang JM, Piacentini M, Pierdominici M, Pierre P, Pierrefite-Carle V, Pietrocola F, Pimentel-Muinos FX, Pinar M, Pineda B, Pinkas-Kramarski R, Pinti M, Pinton P, Piperdi B, Piret JM, Platanias LC, Platta HW, Plowey ED, Poggeler S, Poirot M, Polcic P, Poletti A, Poon AH, Popelka H, Popova B, Poprawa I, Poulose SM, Poulton J, Powers SK, Powers T, Pozuelo-Rubio M, Prak K, Prange R, Prescott M, Priault M, Prince S, Proia RL, Proikas-Cezanne T, Prokisch H, Promponas VJ, Przyklenk K, Puertollano R, Pugazhenthi S, Puglielli L, Pujol A, Puyal J, Pyeon D, Qi X, Qian WB, Qin ZH, Qiu Y, Qu Z, Quadrilatero J, Quinn F, Raben N, Rabinowich H, Radogna F, Ragusa MJ, Rahmani M, Raina K, Ramanadham S, Ramesh R, Rami A, Randall-Demllo S, Randow F, Rao H, Rao VA, Rasmussen BB, Rasse TM, Ratovitski EA, Rautou PE, Ray SK, Razani B, Reed BH, Reggiori F, Rehm M, Reichert AS, Rein T, Reiner DJ, Reits E, Ren J, Ren X, Renna M, Reusch JE, Revuelta JL, Reyes L, Rezaie AR, Richards RI, Richardson DR, Richetta C, Riehle MA, Rihn BH, Rikihisa Y, Riley BE, Rimbach G, Rippo MR, Ritis K, Rizzi F, Rizzo E, Roach PJ, Robbins J, Roberge M, Roca G, Roccheri MC, Rocha S, Rodrigues CM, Rodriguez CI, de Cordoba SR, Rodriguez-Muela N, Roelofs J, Rogov VV, Rohn TT, Rohrer B, Romanelli D, Romani L, Romano PS, Roncero MI, Rosa JL, Rosello A, Rosen KV, Rosenstiel P, Rost-Roszkowska M, Roth KA, Roue G, Rouis M, Rouschop KM, Ruan DT, Ruano D, Rubinsztein DC, Rucker EB, 3rd, Rudich A, Rudolf E, Rudolf R, Ruegg MA, Ruiz-Roldan C, Ruparelia AA, Rusmini P, Russ DW, Russo GL, Russo G, Russo R, Rusten TE, Ryabovol V, Ryan KM, Ryter SW, Sabatini DM, Sacher M, Sachse C, Sack MN, Sadoshima J, Saftig P, Sagi-Eisenberg R, Sahni S, Saikumar P, Saito T, Saitoh T, Sakakura K, Sakoh-Nakatogawa M, Sakuraba Y, Salazar-Roa M, Salomoni P, Saluja AK, Salvaterra PM, Salvioli R, Samali A, Sanchez AM, Sanchez-Alcazar JA, Sanchez-Prieto R, Sandri M, Sanjuan MA, Santaguida S, Santambrogio L, Santoni G, Dos Santos CN, Saran S, Sardiello M, Sargent G, Sarkar P, Sarkar S, Sarrias MR, Sarwal MM, Sasakawa C, Sasaki M, Sass M, Sato K, Sato M, Satriano J, Savaraj N, Saveljeva S, Schaefer L, Schaible UE, Scharl M, Schatzl HM, Schekman R, Scheper W, Schiavi A, Schipper HM, Schmeisser H, Schmidt J, Schmitz I, Schneider BE, Schneider EM, Schneider JL, Schon EA, Schonenberger MJ, Schonthal AH, Schorderet DF, Schroder B, Schuck S, Schulze RJ, Schwarten M, Schwarz TL, Sciarretta S, Scotto K, Scovassi AI, Screaton RA, Screen M, Seca H, Sedej S, Segatori L, Segev N, Seglen PO, Segui-Simarro JM, Segura-Aguilar J, Seki E, Sell C, Seiliez I, Semenkovich CF, Semenza GL, Sen U, Serra AL, Serrano-Puebla A, Sesaki H, Setoguchi T, Settembre C, Shacka JJ, Shajahan-Haq AN, Shapiro IM, Sharma S, She H, Shen CK, Shen CC, Shen HM, Shen S, Shen W, Sheng R, Sheng X, Sheng ZH, Shepherd TG, Shi J, Shi Q, Shi Q, Shi Y, Shibutani S, Shibuya K, Shidoji Y, Shieh JJ, Shih CM, Shimada Y, Shimizu S, Shin DW, Shinohara ML, Shintani M, Shintani T, Shioi T, Shirabe K, Shiri-Sverdlov R, Shirihai O, Shore GC, Shu CW, Shukla D, Sibirny AA, Sica V, Sigurdson CJ, Sigurdsson EM, Sijwali PS, Sikorska B, Silveira WA, Silvente-Poirot S, Silverman GA, Simak J, Simmet T, Simon AK, Simon HU, Simone C, Simons M, Simonsen A, Singh R, Singh SV, Singh SK, Sinha D, Sinha S, Sinicrope FA, Sirko A, Sirohi K, Sishi BJ, Sittler A, Siu PM, Sivridis E, Skwarska A, Slack R, Slaninova I, Slavov N, Smaili SS, Smalley KS, Smith DR, Soenen SJ, Soleimanpour SA, Solhaug A, Somasundaram K, Son JH, Sonawane A, Song C, Song F, Song HK, Song JX, Song W, Soo KY, Sood AK, Soong TW, Soontornniyomkij V, Sorice M, Sotgia F, Soto-Pantoja DR, Sotthibundhu A, Sousa MJ, Spaink HP, Span PN, Spang A, Sparks JD, Speck PG, Spector SA, Spies CD, Springer W, Clair DS, Stacchiotti A, Staels B, Stang MT, Starczynowski DT, Starokadomskyy P, Steegborn C, Steele JW, Stefanis L, Steffan J, Stellrecht CM, Stenmark H, Stepkowski TM, Stern ST, Stevens C, Stockwell BR, Stoka V, Storchova Z, Stork B, Stratoulias V, Stravopodis DJ, Strnad P, Strohecker AM, Strom AL, Stromhaug P, Stulik J, Su YX, Su Z, Subauste CS, Subramaniam S, Sue CM, Suh SW, Sui X, Sukseree S, Sulzer D, Sun FL, Sun J, Sun J, Sun SY, Sun Y, Sun Y, Sun Y, Sundaramoorthy V, Sung J, Suzuki H, Suzuki K, Suzuki N, Suzuki T, Suzuki YJ, Swanson MS, Swanton C, Sward K, Swarup G, Sweeney ST, Sylvester PW, Szatmari Z, Szegezdi E, Szlosarek PW, Taegtmeyer H, Tafani M, Taillebourg E, Tait SW, Takacs-Vellai K, Takahashi Y, Takats S, Takemura G, Takigawa N, Talbot NJ, Tamagno E, Tamburini J, Tan CP, Tan L, Tan ML, Tan M, Tan YJ, Tanaka K, Tanaka M, Tang D, Tang D, Tang G, Tanida I, Tanji K, Tannous BA, Tapia JA, Tasset-Cuevas I, Tatar M, Tavassoly I, Tavernarakis N, Taylor A, Taylor GS, Taylor GA, Taylor JP, Taylor MJ, Tchetina EV, Tee AR, Teixeira-Clerc F, Telang S, Tencomnao T, Teng BB, Teng RJ, Terro F, Tettamanti G, Theiss AL, Theron AE, Thomas KJ, Thome MP, Thomes PG, Thorburn A, Thorner J, Thum T, Thumm M, Thurston TL, Tian L, Till A, Ting JP, Titorenko VI, Toker L, Toldo S, Tooze SA, Topisirovic I, Torgersen ML, Torosantucci L, Torriglia A, Torrisi MR, Tournier C, Towns R, Trajkovic V, Travassos LH, Triola G, Tripathi DN, Trisciuoglio D, Troncoso R, Trougakos IP, Truttmann AC, Tsai KJ, Tschan MP, Tseng YH, Tsukuba T, Tsung A, Tsvetkov AS, Tu S, Tuan HY, Tucci M, Tumbarello DA, Turk B, Turk V, Turner RF, Tveita AA, Tyagi SC, Ubukata M, Uchiyama Y, Udelnow A, Ueno T, Umekawa M, Umemiya-Shirafuji R, Underwood BR, Ungermann C, Ureshino RP, Ushioda R, Uversky VN, Uzcategui NL, Vaccari T, Vaccaro MI, Vachova L, Vakifahmetoglu-Norberg H, Valdor R, Valente EM, Vallette F, Valverde AM, Van den Berghe G, Van Den Bosch L, van den Brink GR, van der Goot FG, van der Klei IJ, van der Laan LJ, van Doorn WG, van Egmond M, van Golen KL, Van Kaer L, van Lookeren Campagne M, Vandenabeele P, Vandenberghe W, Vanhorebeek I, Varela-Nieto I, Vasconcelos MH, Vasko R, Vavvas DG, Vega-Naredo I, Velasco G, Velentzas AD, Velentzas PD, Vellai T, Vellenga E, Vendelbo MH, Venkatachalam K, Ventura N, Ventura S, Veras PS, Verdier M, Vertessy BG, Viale A, Vidal M, Vieira HL, Vierstra RD, Vigneswaran N, Vij N, Vila M, Villar M, Villar VH, Villarroya J, Vindis C, Viola G, Viscomi MT, Vitale G, Vogl DT, Voitsekhovskaja OV, von Haefen C, von Schwarzenberg K, Voth DE, Vouret-Craviari V, Vuori K, Vyas JM, Waeber C, Walker CL, Walker MJ, Walter J, Wan L, Wan X, Wang B, Wang C, Wang CY, Wang C, Wang C, Wang C, Wang D, Wang F, Wang F, Wang G, Wang HJ, Wang H, Wang HG, Wang H, Wang HD, Wang J, Wang J, Wang M, Wang MQ, Wang PY, Wang P, Wang RC, Wang S, Wang TF, Wang X, Wang XJ, Wang XW, Wang X, Wang X, Wang Y, Wang Y, Wang Y, Wang YJ, Wang Y, Wang Y, Wang YT, Wang Y, Wang ZN, Wappner P, Ward C, Ward DM, Warnes G, Watada H, Watanabe Y, Watase K, Weaver TE, Weekes CD, Wei J, Weide T, Weihl CC, Weindl G, Weis SN, Wen L, Wen X, Wen Y, Westermann B, Weyand CM, White AR, White E, Whitton JL, Whitworth AJ, Wiels J, Wild F, Wildenberg ME, Wileman T, Wilkinson DS, Wilkinson S, Willbold D, Williams C, Williams K, Williamson PR, Winklhofer KF, Witkin SS, Wohlgemuth SE, Wollert T, Wolvetang EJ, Wong E, Wong GW, Wong RW, Wong VK, Woodcock EA, Wright KL, Wu C, Wu D, Wu GS, Wu J, Wu J, Wu M, Wu M, Wu S, Wu WK, Wu Y, Wu Z, Xavier CP, Xavier RJ, Xia GX, Xia T, Xia W, Xia Y, Xiao H, Xiao J, Xiao S, Xiao W, Xie CM, Xie Z, Xie Z, Xilouri M, Xiong Y, Xu C, Xu C, Xu F, Xu H, Xu H, Xu J, Xu J, Xu J, Xu L, Xu X, Xu Y, Xu Y, Xu ZX, Xu Z, Xue Y, Yamada T, Yamamoto A, Yamanaka K, Yamashina S, Yamashiro S, Yan B, Yan X, Yan Z, Yanagi Y, Yang DS, Yang JM, Yang L, Yang M, Yang PM, Yang P, Yang Q, Yang W, Yang WY, Yang X, Yang Y, Yang Y, Yang Z, Yang Z, Yao MC, Yao PJ, Yao X, Yao Z, Yao Z, Yasui LS, Ye M, Yedvobnick B, Yeganeh B, Yeh ES, Yeyati PL, Yi F, Yi L, Yin XM, Yip CK, Yoo YM, Yoo YH, Yoon SY, Yoshida K, Yoshimori T, Young KH, Yu H, Yu JJ, Yu JT, Yu J, Yu L, Yu WH, Yu XF, Yu Z, Yuan J, Yuan ZM, Yue BY, Yue J, Yue Z, Zacks DN, Zacksenhaus E, Zaffaroni N, Zaglia T, Zakeri Z, Zecchini V, Zeng J, Zeng M, Zeng Q, Zervos AS, Zhang DD, Zhang F, Zhang G, Zhang GC, Zhang H, Zhang H, Zhang H, Zhang H, Zhang J, Zhang J, Zhang J, Zhang J, Zhang JP, Zhang L, Zhang L, Zhang L, Zhang L, Zhang MY, Zhang X, Zhang XD, Zhang Y, Zhang Y, Zhang Y, Zhang Y, Zhang Y, Zhao M, Zhao WL, Zhao X, Zhao YG, Zhao Y, Zhao Y, Zhao YX, Zhao Z, Zhao ZJ, Zheng D, Zheng XL, Zheng X, Zhivotovsky B, Zhong Q, Zhou GZ, Zhou G, Zhou H, Zhou SF, Zhou XJ, Zhu H, Zhu H, Zhu WG, Zhu W, Zhu XF, Zhu Y, Zhuang SM, Zhuang X, Ziparo E, Zois CE, Zoladek T, Zong WX, Zorzano A, Zughaier SM. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* 2016;**12**: 1–222.

103. Rubinsztein DC, Cuervo AM, Ravikumar B, Sarkar S, Korolchuk V, Kaushik S, Klionsky DJ. In search of an "autophagomometer". *Autophagy* 2009;**5**:585–589.
104. Mizushima N, Yamamoto A, Matsui M, Yoshimori T, Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell* 2004;**15**:1101–1111.
105. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, Tokuhisa T, Ohsumi Y, Yoshimori T. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol* 2001;**152**:657–668.
106. Kabeya Y, Mizushima N, Yamamoto A, Oshitani-Okamoto S, Ohsumi Y, Yoshimori T. LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J Cell Sci* 2004;**117**:2805–2812.
107. Kuma A, Matsui M, Mizushima N. LC3, an autophagosome marker, can be incorporated into protein aggregates independent of autophagy: caution in the interpretation of LC3 localization. *Autophagy* 2007;**3**:323–328.
108. Kimura S, Noda T, Yoshimori T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. *Autophagy* 2007;**3**:452–460.
109. Iwashita H, Sakurai HT, Nagahora N, Ishiyama M, Shioji K, Sasamoto K, Okuma K, Shimizu S, Ueno Y. Small fluorescent molecules for monitoring autophagic flux. *FEBS Lett* 2018;**592**:559–567.
110. Pietrocola F, Demont Y, Castoldi F, Enot D, Durand S, Semeraro M, Baracco EE, Pol J, Bravo-San Pedro JM, Bordenave C, Levesque S, Humeau J, Chery A, Metivier D, Madeo F, Maiuri MC, Kroemer G. Metabolic effects of fasting on human and mouse blood in vivo. *Autophagy* 2017;**13**:567–578.
111. Gupta SK, Foinquinos A, Thum S, Remke J, Zimmer K, Bauters C, de Groote P, Boon RA, de Windt LJ, Preissl S, Hein L, Batkai S, Pinet F, Thum T. Preclinical development of a microRNA-based therapy for elderly patients with myocardial infarction. *J Am Coll Cardiol* 2016;**68**:1557–1571.
112. Wang W, Chen Z, Billiar TR, Stang MT, Gao W. The carboxyl-terminal amino acids render pro-human LC3B migration similar to lipidated LC3B in SDS-PAGE. *PLoS One* 2013;**8**:e74222.
113. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 2000;**19**:5720–5728.
114. Barth S, Glick D, Macleod KF. Autophagy: assays and artifacts. *J Pathol* 2010;**221**: 117–124.
115. Zois CE, Giatromanolaki A, Sivridis E, Papaiakovou M, Kainulainen H, Koukourakis MI. "Autophagic flux" in normal mouse tissues: focus on endogenous LC3A processing. *Autophagy* 2011;**7**:1371–1378.
116. Fernandez AF. Autophagy and proteases: basic study of the autophagic flux by western blot. *Methods Mol Biol* 2018;**1731**:73–81.
117. Saito T, Nah J, Oka SI, Mukai R, Monden Y, Maejima Y, Ikeda Y, Sciarretta S, Liu T, Li H, Baljinnyam E, Fraidenraich D, Fritzky L, Zhai P, Ichinose S, Isobe M, Hsu CP, Kundu M, Sadoshima J. An alternative mitophagy pathway mediated by Rab9 protects the heart against ischemia. *J Clin Invest* 2019;**129**:802–819.
118. Tong M, Saito T, Zhai P, Oka SI, Mizushima W, Nakamura M, Ikeda S, Shirakabe A, Sadoshima J. Mitophagy is essential for maintaining cardiac function during high fat diet-induced diabetic cardiomyopathy. *Circ Res* 2019;**124**: 1360–1371.
119. Iwai-Kanai E, Yuan H, Huang C, Sayen MR, Perry-Garza CN, Kim L, Gottlieb RA. A method to measure cardiac autophagic flux in vivo. *Autophagy* 2008;**4**:322–329.
120. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet* 2009;**43**:67–93.
121. Sahani MH, Itakura E, Mizushima N. Expression of the autophagy substrate SQSTM1/p62 is restored during prolonged starvation depending on transcriptional upregulation and autophagy-derived amino acids. *Autophagy* 2014;**10**: 431–441.
122. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 2005;**171**:603–614.
123. Bauvy C, Meijer AJ, Codogno P. Assaying of autophagic protein degradation. *Methods Enzymol* 2009;**452**:47–61.
124. Wang J, Zhang J, Lee YM, Ng S, Shi Y, Hua ZC, Lin Q, Shen HM. Nonradioactive quantification of autophagic protein degradation with L-azidohomoalanine labeling. *Nat Protoc* 2017;**12**:279–288.
125. Hubbi ME, Hu H, Kshitiz Ahmed I, Levchenko A, Semenza GL. Chaperone-mediated autophagy targets hypoxia-inducible factor-1alpha (HIF-1alpha) for lysosomal degradation. *J Biol Chem* 2013;**288**:10703–10714.
126. Kaushik S, Cuervo AM. Methods to monitor chaperone-mediated autophagy. *Methods Enzymol* 2009;**452**:297–324.
127. Koga H, Martinez-Vicente M, Macian F, Verkhusha VV, Cuervo AM. A photoconvertible fluorescent reporter to track chaperone-mediated autophagy. *Nat Commun* 2011;**2**:386.
128. Kaushik S, Massey AC, Mizushima N, Cuervo AM. Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy. *Mol Biol Cell* 2008;**19**:2179–2192.
129. Kheloufi M, Boulanger CM, Codogno P, Rautou PE. Autosis occurs in the liver of patients with severe anorexia nervosa. *Hepatology* 2015;**62**:657–658.
130. Sun Y, Yao X, Zhang QJ, Zhu M, Liu ZP, Ci B, Xie Y, Carlson D, Rothermel BA, Sun Y, Levine B, Hill JA, Wolf SE, Minei JP, Zang QS. Beclin-1-dependent autophagy protects the heart during sepsis. *Circulation* 2018;**138**:2247.
131. Hariharan N, Zhai P, Sadoshima J. Oxidative stress stimulates autophagic flux during ischemia/reperfusion. *Antioxid Redox Signal* 2011;**14**:2179–2190.
132. Sinha RA, Farah BL, Singh BK, Siddique MM, Li Y, Wu Y, Ilkayeva OR, Gooding J, Ching J, Zhou J, Martinez L, Xie S, Bay BH, Summers SA, Newgard CB, Yen PM. Caffeine stimulates hepatic lipid metabolism by the autophagy-lysosomal pathway in mice. *Hepatology* 2014;**59**:1366–1380.
133. Kaizuka T, Morishita H, Hama Y, Tsukamoto S, Matsui T, Toyota Y, Kodama A, Ishihara T, Mizushima T, Mizushima N. An autophagic flux probe that releases an internal control. *Mol Cell* 2016;**64**:835–849.
134. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. *J Clin Invest* 2003;**112**: 1809–1820.
135. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, Kishi C, Kc W, Carrero JA, Hunt S, Stone CD, Brunt EM, Xavier RJ, Sleckman BP, Li E, Mizushima N, Stappenbeck TS, Virgin HW IV. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature* 2008;**456**: 259–263.
136. Fernandez AF, Lopez-Otin C. The functional and pathologic relevance of autophagy proteases. *J Clin Invest* 2015;**125**:33–41.
137. Yoshii SR, Kuma A, Akashi T, Hara T, Yamamoto A, Kurikawa Y, Itakura E, Tsukamoto S, Shitara H, Eishi Y, Mizushima N. Systemic analysis of Atg5-null mice rescued from neonatal lethality by transgenic ATG5 expression in neurons. *Dev Cell* 2016;**39**:116–130.
138. Karsli-Uzunbas G, Guo JY, Price S, Teng X, Laddha SV, Khor S, Kalaany NY, Jacks T, Chan CS, Rabinowitz JD, White E. Autophagy is required for glucose homeostasis and lung tumor maintenance. *Cancer Discov* 2014;**4**:914–927.
139. Toledo M, Batista-Gonzalez A, Merheb E, Aoun ML, Tarabra E, Feng D, Sarparanta J, Merlo P, Botre F, Schwartz GJ, Pessin JE, Singh R. Autophagy regulates the liver clock and glucose metabolism by degrading CRY1. *Cell Metab* 2018;**28**:268–281. e264.
140. Rocchi A, Yamamoto S, Ting T, Fan Y, Sadleir K, Wang Y, Zhang W, Huang S, Levine B, Vassar R, He C. A Becn1 mutation mediates hyperactive autophagic sequestration of amyloid oligomers and improved cognition in Alzheimer's disease. *PLoS Genet* 2017;**13**:e1006962.
141. Vega-Rubín-de-Celis S, Zou Z, Fernández ÁF, Ci B, Kim M, Xiao G, Xie Y, Levine B. Increased autophagy blocks HER2-mediated breast tumorigenesis. *Proc Natl Acad Sci USA* 2018;**115**:4176–4181.

142. Dolman NJ, Chambers KM, Mandavilli B, Batchelor RH, Janes MS. Tools and techniques to measure mitophagy using fluorescence microscopy. *Autophagy* 2013;**9**: 1653–1662.
143. Sun N, Yun J, Liu J, Malide D, Liu C, Rovira II, Holmstrom KM, Fergusson MM, Yoo YH, Combs CA, Finkel T. Measuring in vivo mitophagy. *Mol Cell* 2015;**60**: 685–696.
144. Katayama H, Kogure T, Mizushima N, Yoshimori T, Miyawaki A. A sensitive and quantitative technique for detecting autophagic events based on lysosomal delivery. *Chem Biol* 2011;**18**:1042–1052.
145. McWilliams TG, Prescott AR, Allen GF, Tamjar J, Munson MJ, Thomson C, Muqit MM, Ganley IG. mito-QC illuminates mitophagy and mitochondrial architecture in vivo. *J Cell Biol* 2016;**214**:333–345.
146. Sun N, Malide D, Liu J, Rovira II, Combs CA, Finkel T. A fluorescence-based imaging method to measure in vitro and in vivo mitophagy using mt-Keima. *Nat Protoc* 2017; **12**:1576–1587.
147. Berod A, Hartman BK, Pujol JF. Importance of fixation in immunohistochemistry: use of formaldehyde solutions at variable pH for the localization of tyrosine hydroxylase. *J Histochem Cytochem* 1981;**29**:844–850.
148. Basyuk E, Bertrand E, Journot L. Alkaline fixation drastically improves the signal of in situ hybridization. *Nucleic Acids Res* 2000;**28**:E46.
149. Shoji-Kawata S, Sumpter R, Leveno M, Campbell GR, Zou Z, Kinch L, Wilkins AD, Sun Q, Pallauf K, MacDuff D, Huerta C, Virgin HW, Helms JB, Eerland R, Tooze SA, Xavier R, Lenschow DJ, Yamamoto A, King D, Lichtarge O, Grishin NV, Spector SA, Kaloyanova DV, Levine B. Identification of a candidate therapeutic autophagy-inducing peptide. *Nature* 2013;**494**:201–206.
150. Terskikh A, Fradkov A, Ermakova G, Zaraisky A, Tan P, Kajava AV, Zhao X, Lukyanov S, Matz M, Kim S, Weissman I, Siebert P. “Fluorescent timer”: protein that changes color with time. *Science* 2000;**290**:1585–1588.
151. Ferree AW, Trudeau K, Zik E, Benador IY, Twig G, Gottlieb RA, Shirihai OS. MitoTimer probe reveals the impact of autophagy, fusion, and motility on subcellular distribution of young and old mitochondrial protein and on relative mitochondrial protein age. *Autophagy* 2013;**9**:1887–1896.
152. Hernandez G, Thornton C, Stotland A, Lui D, Sin J, Ramil J, Magee N, Andres A, Quarato G, Carreira RS, Sayen MR, Wolkowicz R, Gottlieb RA. MitoTimer: a novel tool for monitoring mitochondrial turnover. *Autophagy* 2013;**9**:1852–1861.
153. Stotland A, Gottlieb RA. alpha-MHC MitoTimer mouse: in vivo mitochondrial turnover model reveals remarkable mitochondrial heterogeneity in the heart. *J Mol Cell Cardiol* 2016;**90**:53–58.
154. Hansen MC, Palmer RJ Jr, Udsen C, White DC, Molin S. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology* 2001;**147**:1383–1391.
155. Kneen M, Farinas J, Li Y, Verkman AS. Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys J* 1998;**74**:1591–1599.
156. Cuervo AM, Dice JF. A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* 1996;**273**:501–503.
157. Fujiwara Y, Furuta A, Kikuchi H, Aizawa S, Hatanaka Y, Konya C, Uchida K, Yoshimura A, Tamai Y, Wada K, Kabuta T. Discovery of a novel type of autophagy targeting RNA. *Autophagy* 2013;**9**:403–409.
158. Dostanic I, Schultz JEJ, Lorenz JN, Lingrel JB. The alpha 1 isoform of Na, K-ATPase regulates cardiac contractility and functionally interacts and co-localizes with the Na/Ca exchanger in heart. *J Biol Chem* 2004;**279**:54053–54061.
159. Buss SJ, Muenz S, Riffel JH, Malekar P, Hagenmueller M, Weiss CS, Bea F, Bekeredjian R, Schinke-Braun M, Izumo S, Katus HA, Hardt SE. Beneficial effects of Mammalian target of rapamycin inhibition on left ventricular remodeling after myocardial infarction. *J Am Coll Cardiol* 2009;**54**: 2435–2446.
160. Flynn JM, O’Leary MN, Zambataro CA, Academia EC, Presley MP, Garrett BJ, Zykovich A, Mooney SD, Strong R, Rosen CJ, Kapahi P, Nelson MD, Kennedy BK, Melov S. Late-life rapamycin treatment reverses age-related heart dysfunction. *Aging Cell* 2013;**12**:851–862.
161. Kanamori H, Takemura G, Goto K, Tsujimoto A, Ogino A, Takeyama T, Kawaguchi T, Watanabe T, Morishita K, Kawasaki M, Mikami A, Fujiwara T, Fujiwara H, Seishima M, Minatoguchi S. Resveratrol reverses remodeling in hearts with large, old myocardial infarctions through enhanced autophagy-activating AMP kinase pathway. *Am J Pathol* 2013;**182**:701–713.
162. Wang B, Yang Q, Sun YY, Xing YF, Wang YB, Lu XT, Bai WW, Liu XQ, Zhao YX. Resveratrol-enhanced autophagic flux ameliorates myocardial oxidative stress injury in diabetic mice. *J Cell Mol Med* 2014;**18**:1599–1611.
163. Frati G, Vecchione C, Sciarretta S. Novel beneficial cardiovascular effects of natural activators of autophagy. *Circ Res* 2018;**123**:947–949.