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Mitochondria and Mitophagy: The Yin and Yang of Cell Death Control

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Abstract

Mitochondria are primarily responsible for providing the contracting cardiac myocyte with a continuous supply of ATP. However, mitochondria can rapidly change into death-promoting organelles. In response to changes in the intracellular environment, mitochondria become producers of excessive reactive oxygen species and release pro-death proteins, resulting in disrupted ATP synthesis and activation of cell death pathways. Interestingly, cells have developed a defense mechanism against aberrant mitochondria that can cause harm to the cell. This mechanism involves selective sequestration and subsequent degradation of the dysfunctional mitochondrion before it causes activation of cell death. Induction of mitochondrial autophagy, or mitophagy, results in selective clearance of damaged mitochondria in cells. In response to stress such as ischemia/reperfusion, pro-survival and pro-death pathways are concomitantly activated in cardiac myocytes. Thus, there is a delicate balance between life and death in the myocytes during stress, and the final outcome depends on the complex crosstalk between these pathways. Mitophagy functions as an early cardioprotective response, favoring adaptation to stress by removing damaged mitochondria. In contrast, increased oxidative stress and apoptotic proteases can inactivate mitophagy, allowing for the execution of cell death. Herein, we discuss the importance of mitochondria and mitophagy in cardiovascular health and disease, and provide a review of our current understanding of how these processes are regulated in the myocardium.

Keywords

mitochondria; autophagy; apoptosis; p53; Parkin; PINK1

Introduction

Mitochondria are primarily responsible for producing ATP via oxidative phosphorylation in the inner mitochondrial membrane. Due to the high energy demand of the heart, mitochondria make up at least 30% of the myocyte volume [1]. Mitochondria also play central roles in both necrosis and apoptosis, and can quickly change from a source of energy for sustaining contraction to an organelle that promotes cell death. In response to changes in the environment, ATP synthesis is disrupted, and mitochondria can become producers of excessive reactive oxygen species (ROS) and release proteins that participate in cell death pathways. Interestingly, cells have developed a defense mechanism against aberrant mitochondria, which can cause harm to the cell. This mechanism involves selective sequestration and subsequent degradation of the dysfunctional mitochondrion before it

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causes activation of cell death. This occurs through a process known as mitochondrial autophagy, or mitophagy.

Autophagy is an evolutionarily conserved process that is responsible for the degradation of components in the cytoplasm via the lysosomal pathway [2]. When the cell receives a signal to initiate autophagy, a membrane called the phagophore is formed (Figure 1). Initial phagophore formation, or nucleation, requires assembly of a complex consisting of BECLIN1, vacuolar protein sorting 34 (VPS34), and VPS15 [3]. Subsequent expansion of the membrane is mediated by two ubiquitin-like conjugation systems, LC3 and ATG12-ATG5, that promote assembly of the ATG16L complex and the conjugation of LC3 with phosphatidylethanolamine [4, 5]. The phagophore expands until its edges fuse around its target(s), forming a double-membrane structure called the autophagosome. Next, the autophagosome fuses with a lysosome and the contents are degraded by lysosomal enzymes [2].

Autophagy was initially believed to be a non-selective process whereby autophagosomes randomly engulf cytosolic material. However, it is now clear that autophagy specifically targets invading bacteria [6], protein aggregates [7], and organelles such as mitochondria [8] and ER [9]. Thus, autophagy constitutes a very important quality control mechanism in cells, particularly in post-mitotic cells such as cardiac myocytes.

Interestingly, mitochondria can activate both cell death and mitophagy, two opposing forces in the cell, in response to the same stimulus. These are highly regulated processes, and the delicate balance between the two determines whether a cell will live or die. Defining the role of mitochondria and mitophagy in various cardiovascular pathologies is currently an area of great interest. The importance of understanding how dysfunctional mitochondria and mitophagy contribute to cell survival and death in the myocardium during ischemia/reperfusion and heart failure is becoming increasingly apparent. This review highlights the role of mitochondria in cell death and mitophagy, and discusses our current understanding of how these processes are regulated in cells.

Mitochondria and Cell Death

Mitochondria are important regulators of cell death and respond to many different stress signals, including loss of growth factors, hypoxia, oxidative stress, and DNA damage. The switch to a cell death program is mediated by permeabilization of the outer mitochondrial membrane via BAX and/or BAK, or by opening of the mitochondrial permeability transition pore (mPTP) in the inner mitochondrial membrane (Figure 2). Permeabilization of the outer membrane by BAX/BAK results in the release of proapoptotic proteins such as cytochrome *c*, SMAC/DIABLO, apoptosis-inducing factor (AIF), and endonuclease G (EndoG) to activate apoptosis [10]. In contrast, opening of the mPTP causes rapid influx of solutes and water into the mitochondrial matrix, collapse of the proton gradient, disruption of ATP synthesis. This influx causes swelling of the inner membrane and eventual rupture of the outer membrane, culminating in necrotic cell death. Both forms of cell death are highly regulated by mitochondria, and have been implicated in loss of myocardial cells in pathologies such as I/R, cardiomyopathy, and congestive heart failure [11-14].

Regulation of Mitochondrial Permeabilization by BCL-2 Family Proteins

The BCL-2 family of proteins is at the center of mitochondrial apoptosis regulation. Anti-apoptotic members such as BCL-2 and BCL-X_L promote survival by inhibiting the function of the pro-apoptotic BCL-2 proteins. The pro-apoptotic members can be separated into two distinct subfamilies: the BH3-only proteins, which include BID, BNIP3, NIX/BNIP3L, and PUMA; and the effector proteins BAX and BAK [11]. The BH3-only proteins transduce stress

signals from the cytosol to the mitochondria to initiate cell death by binding to and neutralizing the anti-apoptotic BCL-2 proteins, thereby activating BAX and BAK [15, 16]. Some of the BH3-only proteins, such as tBID, can interact directly with BAX and BAK to induce their activation. Upon activation, BAX and BAK form pores in the outer mitochondrial membrane large enough to allow passage of cytotoxic proteins from the intermembrane space to the cytosol (Figure 2A) [17]. The regulation of apoptosis in the heart by BCL-2 proteins has been implicated in the pathogenesis of myocardial hypertrophy, infarction and heart failure [11]. Numerous studies have demonstrated a cardioprotective role for the anti-apoptotic BCL-2 proteins in response to various stressors, and transgenic mice overexpressing BCL-2 in the heart are resistant to I/R injury [18, 19]. Moreover, BAX is activated in myocytes in response to oxidative stress [20] and during ischemia [21]. Hearts from BAX-deficient mice have reduced mitochondrial damage and decreased infarct size after I/R compared to wild type mice, implicating BAX as a major player of mitochondrial dysfunction in I/R [22]. Among the BH3-only proteins, BID, PUMA, BNIP3, and NIX/BNIP3L have been implicated in cardiac myocyte death [23-26].

Mitochondria and the mPTP

The mPTP is a channel in the inner mitochondrial membrane that allows for passage of molecules up to 1.5 kDa [12]. Opening of the pore causes a collapse of the proton gradient and electrical potential across the inner mitochondrial membrane, leading to disruption of oxidative phosphorylation (Figure 2B). Opening of the mPTP also causes influx of water and subsequent swelling of the inner membrane. The outer mitochondrial membrane is unable to expand, resulting in rupture and release of pro-apoptotic proteins into the cytosol. The composition of the mPTP has remained elusive, and to date the only protein identified to be an essential component is cyclophilin D (CypD) [27, 28]. The mPTP is a major contributor of myocardial I/R injury, and inhibitors of the mPTP reduce infarct size in *ex vivo* I/R [29, 30]. Moreover, CypD-deficient mice are resistant to I/R injury [27, 28]. Interestingly, cells lacking CypD are still sensitive to apoptotic stimuli, suggesting that mPTP opening is not required for induction of apoptosis via the mitochondrial pathway [27].

Studies have also suggested the voltage-dependent anion channel (VDAC) and the adenine nucleotide transporter (ANT) as potential components of the mPTP. Interestingly, experiments in mice deficient for multiple isoforms of VDAC demonstrate that pore opening occurs in the absence of VDAC [31]. Similarly, mitochondria lacking ANT1/2 still undergo permeability transition, albeit at higher Ca^{2+} thresholds [32]. However, considering the critical role of the ANTs in ATP/ADP exchange, it is likely that a functional compensation by other mitochondrial carrier proteins counterbalances the lack of ANT. There's strong evidence that ANT and VDAC are important regulators of cell death. Studies have found that modulation of the levels of different ANTs can result in either cytoprotection or exacerbated cell death. For instance, overexpression of ANT1 causes apoptosis [33], whereas ANT2 inhibits mPTP opening [34]. Differential regulation of cell survival and death has also been reported for the different isoforms of VDAC. Tajeddine et al. reported that VDAC1 contributes to mitochondrial membrane permeabilization via activation of BAX [35]. In contrast, VDAC2 has been reported to inhibit apoptosis by sequestering BAK [36].

There exists substantial cross talk between the BCL-2 proteins and the mPTP. In the heart, BCL-2 has been reported to increase the calcium threshold for mPTP opening by blocking opening of the pore [37]. Additionally, Kitsis and colleagues recently made the discovery that *Bax/Bak/CypD* triple knockout mice fail to show further reduction in infarct size compared to *Bax/Bak* double knockout mice [38]. Moreover, the authors found that cells deficient only for *Bax/Bak* are also resistant to mPTP opening and necrotic cell death, [38] and that BAX can promote mPTP opening via a mechanism that is distinct from its ability to induce outer

mitochondrial membrane permeabilization. Thus, while apoptosis and necrosis are distinct pathways, there is substantial overlap of the two in both regulation and mechanism of action.

Regulation of ER Ca^{2+} Flux and Metabolism by BCL-2 Proteins

Release of Ca^{2+} from the ER via inositol triphosphate (IP3) receptors is another critical event for the initiation of apoptosis. Mitochondria are closely associated with ER, and a major portion of the Ca^{2+} that is released into the cytosol is absorbed by mitochondria [39]. This mitochondrial Ca^{2+} buffering both protects cells from the cytotoxic effects of Ca^{2+} and results in activation of several key enzymes in the mitochondrial matrix to enhance ATP production [39]. However, excess Ca^{2+} uptake by mitochondria leads to Ca^{2+} overload and opening of the mPTP [27]. The level of Ca^{2+} stored in the ER, and by extension released during stress, determines how much is subsequently taken up by mitochondria. BCL-2 proteins also localize to the ER and regulate Ca^{2+} homeostasis. BCL-2 and BCL-X_L repress pro-apoptotic Ca^{2+} signals from the ER by reducing ER Ca^{2+} stores, consequently reducing Ca^{2+} release during stress [40, 41]. Alternatively, BCL-2 can directly interact with IP3 receptors to inhibit IP3-mediated ER Ca^{2+} release [42]. In contrast, ER-localized BAX and BAK elevate the resting ER Ca^{2+} stores and trigger release of ER Ca^{2+} into the cytosol during stress [40, 43]. Moreover, the BH3-only protein NIX is localized to the ER/SR and is implicated with increasing Ca^{2+} release from ER/SR, leading to opening of the mPTP in cardiac myocytes [44]. Thus, pro-apoptotic BCL-2 proteins promote cell death both by directly causing mitochondrial permeability and indirectly by increasing ER calcium release for subsequent uptake by mitochondria.

Studies have demonstrated that anti-apoptotic BCL-2 family proteins can also influence cell survival by regulating mitochondrial bioenergetics. A pool of BCL-X_L localizes to the mitochondrial matrix, where it interacts with the β-subunit of the F_1F_0 ATP synthase and prevents proton leakage [45, 46]. Since proton leakage reduces available protons for the ATPase, decreasing this leak enhances the efficiency of mitochondrial ATP synthesis. This positive energetic change may increase resistance to stressors such as hypoxia. Similarly, Perciavalle et al. reported that Mcl-1 is localized to distinct mitochondrial locations where it exhibits differing functions [47]. Mcl-1 on the outer mitochondrial membrane has anti-apoptotic activity and maintains mitochondrial integrity, whereas Mcl-1 in the matrix is important in assembly of F_1F_0 ATP synthase oligomers.

Regulation of Autophagy by BCL-2 Family Proteins

BCL-2 and BCL-X_L are also negative regulators of autophagy via interaction with BECLIN1 [48] (Figure 1). Although BECLIN1 contains a BH3 domain, it does not function as a pro-apoptotic protein [49]. Instead, it is involved in autophagosome nucleation. He et al. recently reported that both fasting- and exercise-induced autophagy in the myocardium requires disruption of the BCL2 BECLIN1 complex, and knock-in mice expressing a mutant BCL-2 that is unable to dissociate from BECLIN1 fail to induce autophagy [50]. BCL-2 also inhibits autophagy by interacting with AMBRA1 (activating molecule in BECLIN1-regulated autophagy) [51]. AMBRA1 activates the BECLIN1-VPS34-VPS15 complex that is required for nucleation of phagophores[52]. During autophagy induction, BECLIN1 and AMBRA1 are released from BCL-2 to initiate the formation of the phagophore. The interaction between BECLIN1 and BCL-2/BCL-X_L can be disrupted by BH3-only proteins to activate autophagy [53, 54]. However, it is currently unknown what mechanism triggers the dissociation of AMBRA1 from BCL-2.

BCL-2 family proteins can also regulate autophagy via ER Ca^{2+} stores. Treatment of cells with intracellular Ca^{2+}-mobilizing agents results in increased autophagy, which is inhibited by ER-targeted BCL-2 [55]. Similarly, Brady et al. demonstrated that ER/SR-targeted BCL-2

negatively regulates starvation-mediated autophagy by depleting ER/SR Ca^{2+} content rather than via direct interaction with BECLIN1 in HL-1 myocytes [56].

BNIP3 and NIX/BNIP3L were originally identified as pro-apoptotic BH3-only proteins that cause cell death via permeabilization of the outer mitochondrial membrane [57-59]. Although it is now understood that they can activate both apoptosis and mitophagy, it is also evident that these are two distinct processes activated independently and in differing contexts. For instance, NIX/BNIP3L is essential for removal of mitochondria in reticulocytes by mitophagy [60, 61]. Similarly, BNIP3 is a potent inducer of mitophagy in cells including cardiac myocytes [8, 24]. In cells lacking BAX and BAK, overexpression of BNIP3 results in increased mitophagy in the absence of outer mitochondrial membrane permeabilization [62]. Interestingly, we found that disrupting BNIP3-mediated mitophagy has no effect on its pro-apoptotic activity [9]. This suggests that BNIP3 and NIX have the dual function of regulating both mitophagy [24, 60, 63] and cell death [57-59]. It is still unclear exactly under what conditions BNIP3 and NIX switch from mitophagy regulators to mitochondrial pro-death proteins.

Regulation of Cell Death and Autophagy by p53

The p53 tumor suppressor protein regulates both apoptosis and autophagy. The induction of cell death by p53 occurs via regulation of gene expression and by direct action at the mitochondria. Under normal conditions, p53 is maintained at low levels by the E3 ubiquitin ligase MDM2, which targets p53 for degradation by the proteasome [64]. In response to stress such as hypoxia or DNA damage, the levels of p53 increase in the cell. Nuclear p53 transactivates several pro-apoptotic genes, including *Bax*[65], *Bak*[65, 66], *Noxa*[67] and *Puma*[68], while it represses the transcription of anti-apoptotic *Bcl-2, Bcl-*X_L, and *Mcl-1*[66, 69, 70]. A portion of p53 also translocates to mitochondria, where it promotes mitochondrial membrane permeabilization by interacting with BCL-2 family proteins. p53 has been reported to directly bind to and activate BAX [71] and BAK 72, and to inhibit BCL-2/BCL-X_L[73]. Accumulating evidence indicates that p53 plays an important role in stress-induced apoptosis in the heart. Studies have found that p53 is upregulated in the heart in response to ischemia [74], oxidative stress [75], and anthracycline exposure [76]. Moreover, p53-deficient mice have reduced susceptibility to anthracycline-induced myocardial apoptosis and heart failure [77], and cardiac-specific deletion of *Mdm4*, a homolog of *Mdm2*, results in elevated levels of p53 and development of dilated cardiomyopathy [78].

p53 plays dual roles in regulating autophagy depending on the context and subcellular localization. Under normal conditions, cytoplasmic p53 suppresses the induction of autophagy. Tasdemir et al. initially reported that genetic deletion, knockdown, or inhibition of p53 induces autophagy in cells, suggesting that cytoplasmic p53 functions as an autophagy repressor [79]. In response to metabolic stress such as nutrient deprivation, nuclear p53 binds to the promoter region of multiple genes involved in inducing autophagy. Many products of these target genes, such as AMPK, tuberous sclerosis protein 2 and sestrin1/2 converge on the mTOR pathway [80, 81]. mTOR is a negative regulator of autophagy, and inhibition of this pathway results in the induction of autophagy. Zhang et al. initially discovered that p53 activates autophagy during glucose deprivation via inhibition of the mTOR pathway [82]. In contrast, during hypoxia, nuclear p53 reduces autophagy by repressing expression of BNIP3 [83]. Additionally, Hoshino et al. found that nuclear p53 attenuates autophagy and mitophagy in the ischemic heart, which results in accumulation of damaged mitochondria and increased myocardial damage [84]. Collectively, these studies suggest that p53 suppress autophagy under normal conditions, and that in response to stress, p53 can either induce or inhibit autophagy.

Regulation of Mitophagy by the PINK1/Parkin Pathway

Damage to mitochondria often results in activation of both mitophagy and mitochondrial apoptosis/permeabilization in the same cell. In an effort to prevent cell death, damaged mitochondria are sequestered by autophagosomes and degraded before apoptosis or necrosis can be triggered. Electron microscopy (EM) analysis of the infarct border zone reveals that 10% of autophagosomes contain mitochondria 8 hours after the myocardial infarction, while no mitochondria are found in autophagosomes in control mice [84]. EM also shows many autophagosomes containing mitochondria following *ex vivo* I/R [24]. The PTEN-induced putative kinase 1 (PINK1)/Parkin pathway is important in regulating mitophagy in cells. Mitophagy allows for the selective removal of only dysfunctional mitochondria by autophagosomes. The E3 ubiquitin ligase Parkin is predominantly cytosolic under basal conditions, but rapidly translocates to mitochondria upon loss of mitochondrial membrane potential ($\Delta\psi m$) (Figure 3) [85, 86]. Parkin then promotes ubiquitination of mitochondrial proteins, which serves as a signal for mitophagy [87]. To date, only four Parkin substrates have been identified on mitochondria; VDAC1 [87], Mitofusins1/2 [88, 89], and MIRO [90].

The serine/threonine kinase PINK1 plays a central role in communicating the collapse of the $\Delta\psi m$ to Parkin. PINK1 is found at very low levels on mitochondria with intact $\Delta\psi m$ because it is rapidly imported and cleaved by mitochondrial proteases, and then is degraded by the proteasome [91-93]. Upon collapse of the $\Delta\psi m$, the import and degradation of PINK1 is blocked, and PINK1 accumulates on the outer mitochondrial membrane. Studies have demonstrated that expression of PINK1 is necessary for the recruitment of Parkin to depolarized mitochondria [92, 94]. However, exactly how accumulation of PINK1 on the outer mitochondrial membrane results in recruitment and activation of Parkin is currently unclear. Based on experimental findings, three different models have been proposed. First, it has been suggested that PINK1 directly interacts with Parkin, thereby anchoring it to the mitochondria [95, 96]. Another model suggests that PINK1 directly phosphorylates Parkin, resulting in its activation [95, 97]. Alternatively, PINK1 may phosphorylate Parkin substrates on the mitochondria, thereby increasing their affinity for Parkin[90]. In contrast, other studies have found that Parkin does not require PINK1 for its function [98-100]. Additional studies are needed to elucidate the relationship between PINK1 and Parkin in mitophagy.

To date, very few PINK1 substrates have been identified, and only one of them is associated with the mitochondria. The atypical Rho GTPase MIRO plays an important role in the movement of mitochondria within cells by connecting mitochondria to microtubules [101]. Wang et al. reported that phosphorylation of MIRO by PINK1 activates proteasomal degradation of MIRO in a Parkin-dependent manner [90]. This supports the notion that the PINK1/Parkin pathway disrupts movement of damaged mitochondria in cells, thereby separating them from the pool of healthy mitochondria for mitophagy. Unlike neuronal cells, whose survival depends on efficient transport of mitochondria from the cell body to the axon terminal, mitochondria in cardiac myocytes do not display significant motility [102]. Interestingly, MIRO is highly expressed in heart and skeletal muscle [103], and it will be greatly beneficial to determine what role MIRO plays in PINK1/Parkin-mediated mitophagy in the myocardium.

PINK1 and Parkin are highly expressed in the heart, [104, 105] and recent studies have provided some insights into their functional role in the myocardium. Billia et al. reported that PINK1 protein levels are markedly reduced in end-stage human heart failure [104], and that PINK1-deficient mitochondria have reduced oxidative capacity, which correlates with the development of cardiac dysfunction and hypertrophy by two months of age [104]. Moreover, Parkin has been found to play an important role in clearing mitochondria in ischemic preconditioning (IPC), and mice deficient in Parkin are resistant to IPC [106]. We

have also found that BNIP3-mediated mitophagy is associated with the translocation of Parkin to mitochondria [107].

Selective Mitophagy is mediated via Autophagy Adaptors/Receptors

Previously, autophagy was thought to be an essentially non-selective pathway that accidentally sequestered material in the cytosol including mitochondria, but recent evidence suggests that autophagosomes carry selective cargoes. Until recently, it was unclear what signals or labels were used to target specific mitochondria for removal by the autophagy pathway. The recent identification of autophagy adaptor proteins and autophagy receptors on mitochondria has provided important new insights into this process. Currently, there is experimental evidence of at least two different mechanisms for selective mitophagy in mammalian cells (Figure 4).

Mitophagy via Autophagy Adaptors

One mechanism of mitochondrial clearance involves autophagy adaptors and ubiquitin, in which the ubiquitin is used as a signal for autophagic degradation of mitochondria [108, 109]. In this model, the dysfunctional mitochondrion is recognized by Parkin, which ubiquitinates specific protein substrates on the mitochondrion. The identification of the autophagy adaptor protein p62/SQSTM1 (hereafter referred to as p62) has provided important insights into the process of mitophagy [109]. The p62 protein binds to ubiquitinated proteins via its ubiquitin-associated (UBA) domain [110], and to LC3 on the phagophore via its LC3 Interacting Region (LIR) [109]. Thus, the recruitment and binding of p62 to ubiquitinated mitochondrial proteins docks the mitochondrion to the LC3-positive phagophore for engulfment (Figure 4A). In support of this model, it has been reported that p62 is recruited to mitochondria in a Parkin-dependent manner, and that knockdown of p62 substantially inhibits mitophagy [87, 111]. In contrast, another study found that depletion of p62 by RNAi has little effect on mitophagy in HeLa cells. The authors also found that p62 can be recruited to mitochondria by a mitochondrion-anchored ubiquitin fusion protein, but this does not induce substantial mitophagy. This suggests that p62 recruitment to mitochondria alone is not sufficient for mitophagy to ensue [112]. Therefore, p62 may not be responsible for Parkin-induced mitophagy, or there may be redundancy in autophagy adaptor proteins. NBR1 (neighbor of BRCA1 gene) contains both LIR and UBA motifs, and has been identified to act as an autophagy adaptor protein [108]. However, it is currently unknown if NBR1 plays a role in mitophagy.

AMBRA1 and Mitophagy

Recent evidence indicates that AMBRA1 plays a role in mitophagy. Upon induction of autophagy, AMBRA1 translocates to the ER and mitochondria, where it interacts with BECLIN1 to initiate nucleation. Strappazzon et al. reported that a pool of AMBRA1 is already docked at mitochondria by BCL-2 under normal conditions. This reserve of AMBRA1 is released from BCL-2 when autophagy is induced, allowing it to bind to BECLIN1 and initiate formation of the phagophore at the mitochondrion [51]. PARKIN also interacts with AMBRA1 at mitochondria to promote mitochondrial clearance, and depolarization of mitochondria increases the interaction between Parkin and AMBRA1 [113]. Interestingly, AMBRA1 is required for the clearance of mitochondria, but not for the translocation of Parkin. In contrast, AMBRA1-mediated mitophagy is dependent on Parkin, and overexpression of AMBRA1 in *Parkin*$^{-/-}$ cells fails to restore mitophagy levels. It has been hypothesized that local activation of the PI3K complex by AMBRA1 at the mitochondria allows for formation of new phagophores near or even directly around depolarized mitochondria. AMBRA1 recruitment to damaged mitochondria may therefore contribute to the efficient, spatially restricted, selective nature of mitophagy.

Currently, it is unclear whether these two different models of Parkin-mediated mitophagy are different or complimentary. Because LC3 only associates with phagophores after they have started to form [114], p62 can only recruit pre-existing phagophores to mitochondria and cannot induce the formation of new autophagosomes. In contrast, AMBRA1 stimulates new phagophore formation [52]. Thus, we believe that the two mechanisms have complimentary effects to allow for efficient mitophagy; AMBRA1 induces formation of new phagophores at the mitochondria, which, after incorporation of LC3, may be tethered to ubiquitinated mitochondria via p62.

NIX and BNIP3 as Autophagy Receptors on Mitochondria

There is also evidence that mitochondria can be cleared via an ubiquitin-independent pathway involving direct binding of ATG8 family proteins to autophagy receptors on the mitochondria (Figure 4B). In yeast, mitophagy is regulated by a single, ubiquitin-independent pathway that requires the autophagy receptor ATG32 [115, 116]. The ATG32 protein is an outer mitochondrial membrane protein that interacts with ATG8 via its LIR [115]. In mammalian cells, mitochondrial NIX/BNIP3L and BNIP3 have been identified as autophagy receptors for the selective clearance of mitochondria in cells [9, 117, 118]. We have found that BNIP3 promotes mitophagy in cells including cardiac myocytes [24, 119, 120]. NIX and BNIP3 target mitochondria for autophagy by directly binding to LC3/GABARAP on the autophagosome via their conserved LIR motifs [9, 117]. Interestingly, disrupting the interaction between LC3 and BNIP3 significantly decreases, but does not completely abrogate mitophagy [9], suggesting that BNIP3 is not the only autophagy receptor on the mitochondria. Similarly, disrupting the interaction between NIX and LC3/GABARAP causes only a partial reduction in mitophagy [117]. A study from Dorn's group confirmed that BNIP3 and NIX have overlapping functions as regulators of mitophagy in the adult myocardium. Long-term studies of *nix* and *bnip3* knockout mice revealed that these mice accumulate dysfunctional mitochondria in the heart with age. Interestingly, the accumulation occurs faster in *nix/bnip3* DKO mice [121], suggesting that BNIP3 and NIX have overlapping functions in regulating mitochondrial clearance in the adult heart. To date, only NIX and BNIP3 have been identified as mitophagy receptors in mammalian cells. However, it is likely that there exists a redundancy in the mitophagy pathway, and that multiple proteins can act as autophagy receptors to ensure the removal of aberrant mitochondria. On the autophagosome, only ATG8 family proteins have been identified to participate in the clearance of mitochondria and protein aggregates. However, it is possible that other autophagosomal proteins can participate in binding proteins on mitochondria to ensure docking and removal.

An important question is whether BNIP3/NIX and PINK1/Parkin participate in the same pathway. On this matter, studies have produced contradictory evidence. Parkin translocation is dependent on loss of $\Delta\psi m$, yet we have found that BNIP3 promotes mitophagy in BAX/BAK-deficient MEFs even when mitochondria retain their $\Delta\psi m$ [62]. In contrast, we have also found that Parkin translocates to mitochondria in response to BNIP3 overexpression in cardiac myocytes, and that Parkin-deficient myocytes have reduced autophagy in response to BNIP3 [107]. Furthermore, Ding et al. reported that CCCP-induced Parkin translocation is significantly reduced in NIX-deficient MEFs [111]. This suggests that NIX and BNIP3 may be important in recruiting Parkin to mitochondria.

Interplay Between Mitophagy and Mitochondrial Biogenesis

Not surprisingly, mitophagy is closely coupled to mitochondrial biogenesis. Studies have demonstrated that mitophagy has the capacity to clear most of the mitochondria in cells. In fact, cells overexpressing PINK1 or Parkin can degrade all their mitochondria within 24-96 h in response to treatment with mitochondrial uncouplers [86, 92]. It is therefore important for the cell to quickly replace the mitochondria that have been removed by mitophagy.

Mitochondria have a significant reserve capacity that can be utilized upon demand [122]. In response to modest mitophagy, myocytes can utilize their mitochondrial reserve to maintain energy production without affecting contractility. However, excessive mitophagy in the absence of mitochondrial biogenesis will result in the depletion of the bioenergetic reserve in the remaining mitochondria and subsequent cell death.

The transcriptional coactivator peroxisome-proliferator-activated receptor γ coactivator 1α (PGC-1α) is a master regulator of mitochondrial biogenesis [123]. PGC-1α is induced at birth in the mouse heart when there is a striking increase in mitochondrial biogenesis [123, 124]. Studies have found that fasting is associated with both increased mitophagy [125] and rapid upregulation of PGC-1α in heart tissues [123]. Additionally, Parkin has also been shown to be a central regulator of both mitophagy and mitochondrial biogenesis. Shin et al. recently identified PARIS (ZNF746), a KRAB and zinc finger protein, to be a Parkin substrate [126]. PARIS represses the expression of PGC-1α by binding to insulin response sequences in the PGC-1α promoter. Activation of Parkin promotes degradation of PARIS and subsequent activation of PGC-1α transcription. Although it has been shown that Parkin regulates mitophagy in myocytes [106, 107], it remains to be determined whether Parkin regulates mitochondrial biogenesis via PARIS in the heart.

Mitochondrial Dynamics are Integrated with Cell Death and Mitophagy

Mitochondrial fission and fusion are regulated by several different GTPases. Mitofusins 1 and 2 (MFN1 and MFN2) regulate fusion of the outer mitochondrial membrane, whereas optic atrophy protein 1 (OPA1) promotes fusion of the inner membrane [127, 128]. Mitochondrial fission is regulated by dynamin-related protein 1 (DRP1) and fission protein 1 (FIS1) [129, 130]. These proteins are highly expressed in the heart [131-134]. Deletion of *Drp1* is embryonic lethal, and myocytes in *Drp1* null embryos have reduced contractility [135]. Defects in DRP1 function are also associated with early infant mortality and cardiomyopathy [136, 137]. Knockdown of fusion proteins mitochondrial assembly regulatory factor (MARF) or OPA1 leads to the development of cardiomyopathy in *Drosophila* [138]. These studies indicate that functional mitochondrial dynamics are important for normal heart and mitochondrial function.

Importantly, mitochondrial dynamics influence cell death by mechanisms that are not fully understood, and it is unclear whether mitochondrial fragmentation is the cause or a consequence of the pathogenesis. Inhibition of mitochondrial fission attenuates disease progression in models of neurodegenerative and cardiovascular diseases [132, 139, 140]. Studies have found that excessive DRP1-mediated mitochondrial fission contributes to apoptotic cell death [141-143]. Mitochondrial fission occurs within the same time frame as activation of pro-apoptotic BAX and permeabilization of the mitochondrial outer membrane. Additionally, DRP1 co-localizes with BAX on the mitochondrial membrane at the onset of apoptosis [141]. However, fission is not required for BAX/BAK-dependent apoptosis, and inhibiting DRP1 only delays cell death [143, 144]. It has been found that inhibiting the fission machinery partially prevents cytochrome *c* release from mitochondria [143], and that cells with fragmented mitochondria are more sensitive to apoptosis [144]. This suggests that although DRP1-mediated fission is not required for apoptosis to proceed, it increases sensitivity to apoptotic stimuli possibly by enhancing cytochrome *c* release.

In addition, Piquereau et al. recently reported that cardiac mitochondria of $Opa1^{+/-}$ mice accumulate more calcium and present a delay in calcium-induced mPTP opening [145]. Opa1 down-regulation induces clear changes in mitochondrial morphology and alterations of the mitochondrial cristae. Unexpectedly, mitochondria appear larger in $Opa1^{+/-}$ myocytes. Similar findings have been reported in $Mfn2^{-/-}$ mice, in which MFN2 deficiency leads to

accumulation of large mitochondria in the myocardium [133]. In contrast, MFN1-deficient myocytes accumulate small spherical mitochondria, as has been previously reported in *Mfn1* null cell lines [146]. Although mitochondria in *Mfn1-/-* and *Mfn2-/-* hearts have opposing morphologies, they are both more resistant to mPTP opening [133146], suggesting that the process of fusion influences mPTP more than mitochondrial size.

Mitochondrial dynamics are closely integrated with mitophagy (Figure 3). Consistently, mitophagy is attenuated in cells with reduced mitochondrial fission, suggesting that fission is a prerequisite for mitophagy to occur [107, 147-149]. For instance, DRP1-deficient MEFs have significantly reduced Parkin-mediated mitophagy compared with wild-type MEFs [150]. We found that DRP1-mediated fission is a prerequisite for mitophagy by BNIP3 in cardiac myocytes [107]. It is currently unclear why mitochondrial fission must occur prior to mitophagy. One possibility could be that fission produces smaller mitochondrial fragments that can more easily be engulfed by autophagosomes. Mitochondria usually have an elongated shape and can be up to 5 μm long [151], whereas autophagosomes are spherical in shape with a diameter of about 1 μm [152]. Recent studies have connected the PINK1/Parkin mitophagy pathway with mitochondrial dynamics. MFN1 and MFN2 are substrates for Parkin [88, 150], although their ubiquitination does not serve as a signal for mitophagy. Instead, MFN1/2 ubiquitination leads to their proteasomal degradation prior to mitophagy [88, 150], indicating that ubiquitination of MFNs does not constitute a signal for mitophagy. Tanaka et al. also identified that p97 is required for proteasomal elimination of MFN1 and MFN2 [150]. p97 is an AAA+ ATPase that is involved in the retrotranslocation of ER membrane-spanning proteins after their ubiquitination [153]. Thus, the data suggest that degradation of MFN1/2 might serve to switch the balance of mitochondrial dynamics towards fission to facilitate mitophagy. The loss of MFNs may prevent damaged mitochondria from fusing with healthy mitochondria in the cell. Additional evidence that mitochondrial size plays a role in mitophagy came from studies published by Lippincott-Schwartz's and Scorrano's groups. They found that nutrient deprivation induces formation of hyperfused mitochondrial networks that protect mitochondria from elimination by autophagosomes [154, 155]. An alternative possibility is that fission segregates dysfunctional mitochondria prior to removal by autophagy. Shirinai and colleagues provided evidence that mitochondrial fission gives rise to two fragments with different $\Delta\psi m$ [149]. Mitochondrial fragments with low $\Delta\psi m$ are more likely to be targeted by autophagosomes, whereas fragments with high $\Delta\psi m$ have a higher probability of undergoing mitochondrial fusion.

Regulation of Cell Death and Autophagy by Reactive Oxygen Species (ROS)

Mitochondria are both a major source of and targets of ROS, which are by-products of mitochondrial electron transport activity [156]. Although significant amounts of ROS are generated by cardiac mitochondria, myocytes have developed a highly efficient antioxidant system that can neutralize ROS under normal conditions. However, damaged mitochondria can produce ROS at levels that exceed the capacity of the antioxidant system and can result in cell death. ROS can cause the oxidative modification of mitochondrial proteins, lipids, and mtDNA, resulting in mitochondrial dysfunction. ROS can also contribute to opening of the mPTP [12, 27]. Oxidative stress has been associated with loss of cells in both I/R injury [157, 158] and anthracycline-induced cardiomyopathy [159].

In addition, ROS play an important role in inducing autophagy in cells and during myocardial I/R [160]. Exactly how ROS regulate autophagy is unknown, although it is very possible that oxidative modification of transcription factors affects the levels of autophagy proteins. ROS can also directly regulate the formation of autophagosomes. ATG4 enables the conversion of LC3-I to lipidated LC3-II, its insertion into the autophagosome, and the

recycling of LC3-II after autophagosome-lysosome fusion. ATG4 is subject to oxidation and subsequent inactivation, which leads to accumulation of LC3-II and increased formation of autophagosomes [161]. Interestingly, increased oxidative/nitrosative stress can inhibit mitophagy by modification and inactivation of Parkin. Parkin contains multiple conserved cysteine residues that are important for maintaining its solubility [162], but are also susceptible to modification by oxidative and nitrosative stress that lead to Parkin inactivation and aggregation [163, 164]. Since cardiac mitochondria are a major source of ROS/RNS during stress, it is likely that aberrant modification of Parkin and subsequent inhibition of mitophagy will result.

Cross Talk Between Apoptosis and Mitophagy

During stress, pro-survival and pro-death processes are concomitantly activated in cells. The final outcome (life vs. death) depends on the balance between these pathways (Figure 5). Although it might be surprising that the cell is activating two opposing pathways, it is prudent to have multiple strategies in place for dealing with stress. The ability of a cell to repair itself and prevent unnecessary death is particularly important in a post-mitotic cell such as a myocyte that cannot be easily replaced. If exposure to modest stress results in damage to only a few mitochondria, the cell can easily clear those mitochondria via mitophagy. However, if the number of damaged mitochondria exceeds the capacity of mitophagy, or if mitophagy becomes inactivated, the cell is beyond rescue and apoptosis will become the dominant pathway to minimize extraneous tissue damage upon cell death.

Although there is overwhelming evidence that dysfunctional mitochondria activate mitophagy, several studies have found that mitochondrial stress and apoptosis inhibit induction of autophagy in cells. For instance, caspases have been reported to cleave BECLIN1 during apoptosis, thereby preventing the initiation of autophagy [165]. The resulting BECLIN1 fragment is unable to form a complex with VPS34, as is required for autophagy [166]. Instead, the BECLIN1 fragment translocates to mitochondria and enhances apoptosis. Atg5 has also been reported to be cleaved by calpain in response to apoptotic stimuli [167]. Similar to the BECLIN1 fragment, truncated Atg5 translocates from the cytosol to mitochondria, where it associates with BCL-X_L and triggers cytochrome *c* release and caspase activation.

AMBRA1 is also an important target during apoptosis. Pagliarini et al. found that AMBRA1 is subjected to cleavage by caspases and calpains, which results in inactivation of autophagy and promotion of cell death[168]. Interestingly, knockdown of AMBRA1 results in increased sensitivity to cell death, whereas overexpression of a caspase cleavage resistant form of AMBRA1 prolongs autophagy and reduces cell death. These findings suggest that when there is vast mitochondrial damage, such as that which occurs during a myocardial infarction, activation of apoptotic proteases will shut down autophagy/mitophagy and activate apoptosis to ensure cell death. Whether or not proteolytic cleavage of BECLIN1, Atg5, or AMBRA1 occurs in the myocardium in response to stress such as I/R is currently unknown and remains to be explored.

Conclusion

Understanding the interface between adaptation to stress and cell death is important for understanding the pathogenesis of cardiovascular disease. Basal levels of mitophagy are important for maintaining cellular homeostasis and protecting cells against accumulation of dysfunctional mitochondria. There is also cross talk between the autophagy and apoptotic pathways. During stress, there is concomitant activation of autophagy/mitophagy and apoptosis, where enhanced mitophagy is an early response to promote survival by removing

damaged mitochondria. With overwhelming mitochondrial damage, apoptosis becomes dominant, and inactivation of critical proteins of the autophagy pathway allows for cell death. Interestingly, enhanced levels of mitophagy can lead to excessive removal of mitochondria, loss of cardiac myocytes, and development of heart failure. It is important to clarify the differences between mitophagy pathways utilized in the degradation of damaged versus merely superfluous mitochondria. The manipulation of proteins that regulate mitochondrial integrity and mitophagy represents future therapeutic targets to preserve myocyte viability and prevent the development of heart disease. Therefore, it is important to gain insights into the mechanisms regulating the balance between survival and death, both under normal conditions and in the diseased myocardium.

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Non-standard Abbreviations and Acronyms

Ambra1	Activating molecule in beclin 1-regulated autophagy
AIF	Apoptosis Inducing Factor
ANT	Adenine nucleotide transporter
Atg5/7	Autophagy protein 5/7
Bnip3	BCL2/adenovirus E1B nineteen kDa protein-interacting protein 3
Bnip3L	BCL2/adenovirus E1B nineteen kDa protein-interacting protein 3-like
CypD	Cyclophilin D
Drp1	Dynamin-related protein 1
EndoG	Endonuclease G
Fis1	Mitochondrial fission 1
IP3	Inositol triphosphate

LAMP2	Lysosome-associated membrane protein 2
LC3	Microtubule-associated protein 1 light chain 3
Mfn1/2	Mitofusin-1/2
mPTP	Mitochondrial permeability transition
Opa1	Optic atrophy 1
PI3K	Phosphoinositide 3-kinase
PINK1	PTEN-induced putative kinase 1
ROS	Reactive oxygen species
VDAC	voltage-dependent anion channel

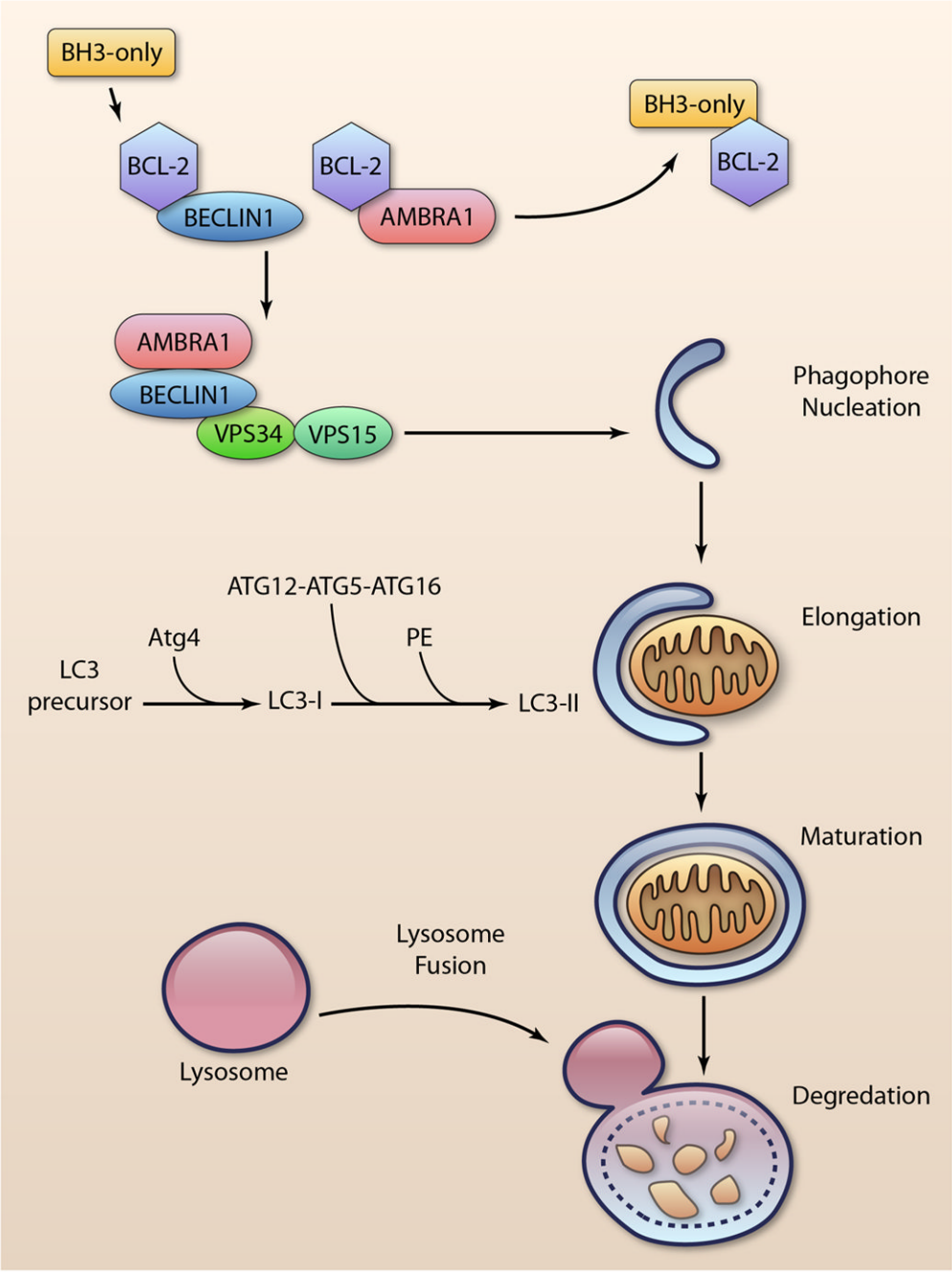

Figure 1.

Induction of autophagy. BCL-2/BCL-X_L prevents induction of autophagy by binding BECLIN1 and AMBRA1. Displacement by BH3-only proteins leads to activation of the BECLIN1-VPS34-VPS15 complex and phagophore nucleation. Elongation of the membrane requires two ubiquitin-like conjugation systems: ATG12-ATG5-ATG16L, and conjugation of LC3-I with phosphatidylethanolamine (PE) to form LC3-II. After maturation of the autophagosome, it fuses with the lysosome to degrade the cargo. (Illustration Credit: Ben Smith).

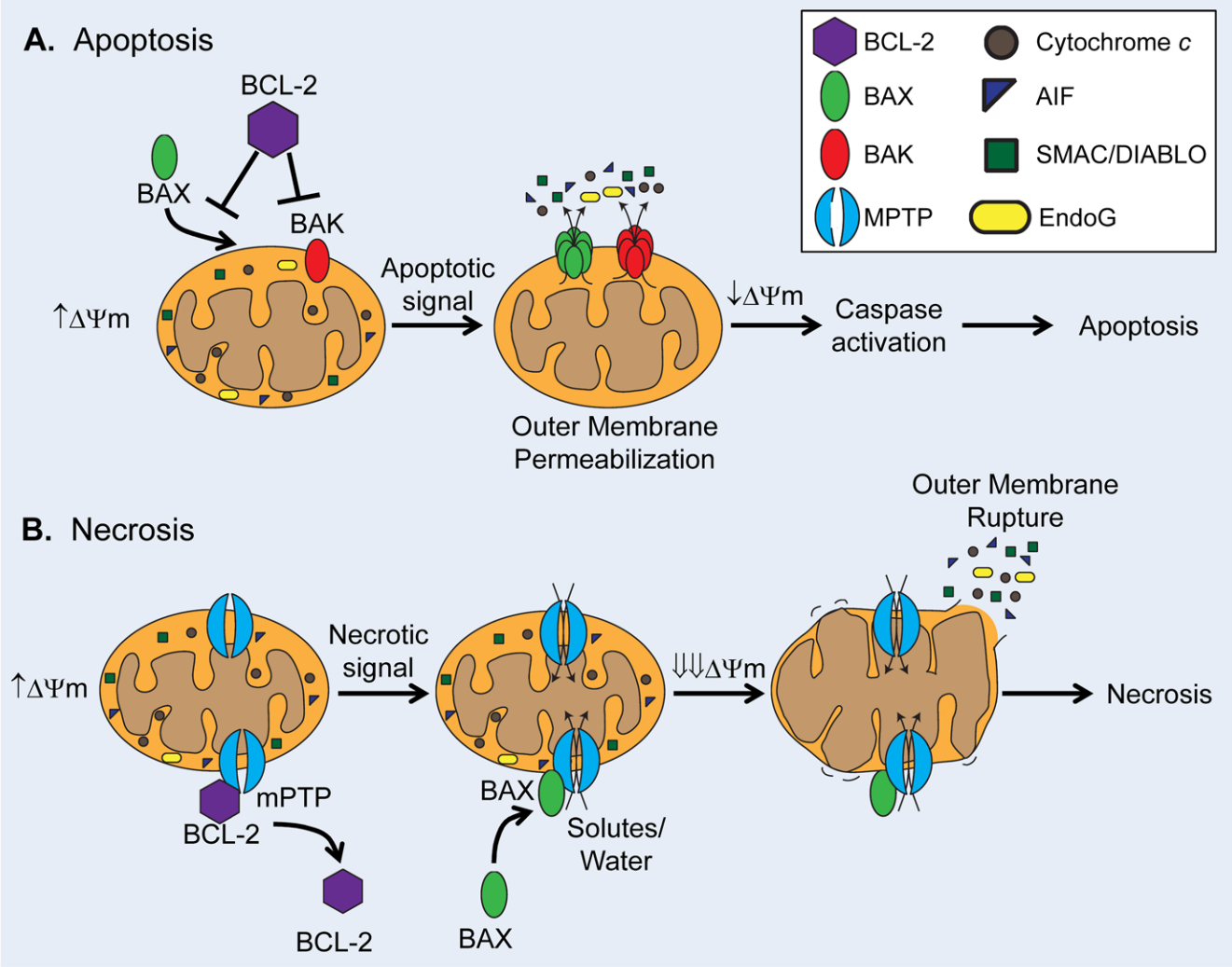

Figure 2.

Mechanisms of mitochondrial membrane permeabilization. **A.** Anti-apoptotic BCL-2 family proteins, such as BCL-2 and BCL-X_L, inhibit BAX/BAK-mediated outer mitochondrial membrane permeabilization. BAX/BAK activation causes release of cytotoxic proteins, slow loss of DYm, and culminates in apoptosis. **B.** Opening of the mPTP results in rapid influx of solutes and water which causes dissipation of the Dym, inner membrane swelling and subsequent outer membrane rupture. Destruction of the outer membrane releases cytotoxic proteins into the cytosol and leads to necrotic cell death. Anti-apoptotic BCL-2 proteins also prevent mPTP opening, while pro-apoptotic proteins such as Bax can enhance mPTP opening.

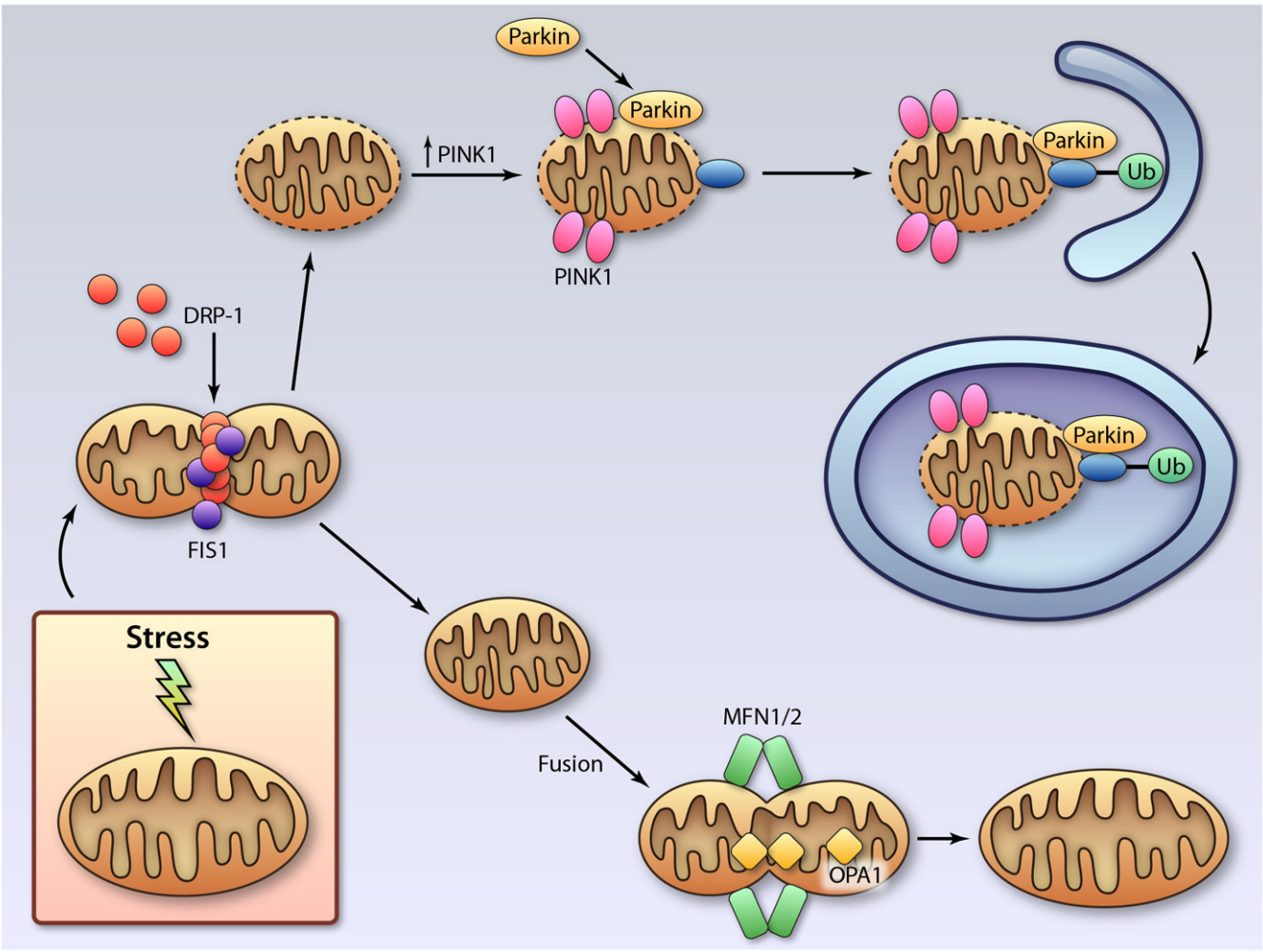

Figure 3.
Regulation of mitophagy. Damaged mitochondria undergo DRP1-mediated fission prior to mitophagy. Reduced mitochondrial membrane potential leads to accumulation of PINK1 and subsequent recruitment of the E3 ubiquitin ligase Parkin to mitochondria. Parkin promotes ubiquitination of proteins in the mitochondrial membrane, which targets the damaged mitochondrion for removal by an autophagosome. The healthy mitochondrial fragment will undergo fusion mediated by MFN1/2 and OPA1. (Illustration Credit: Ben Smith).

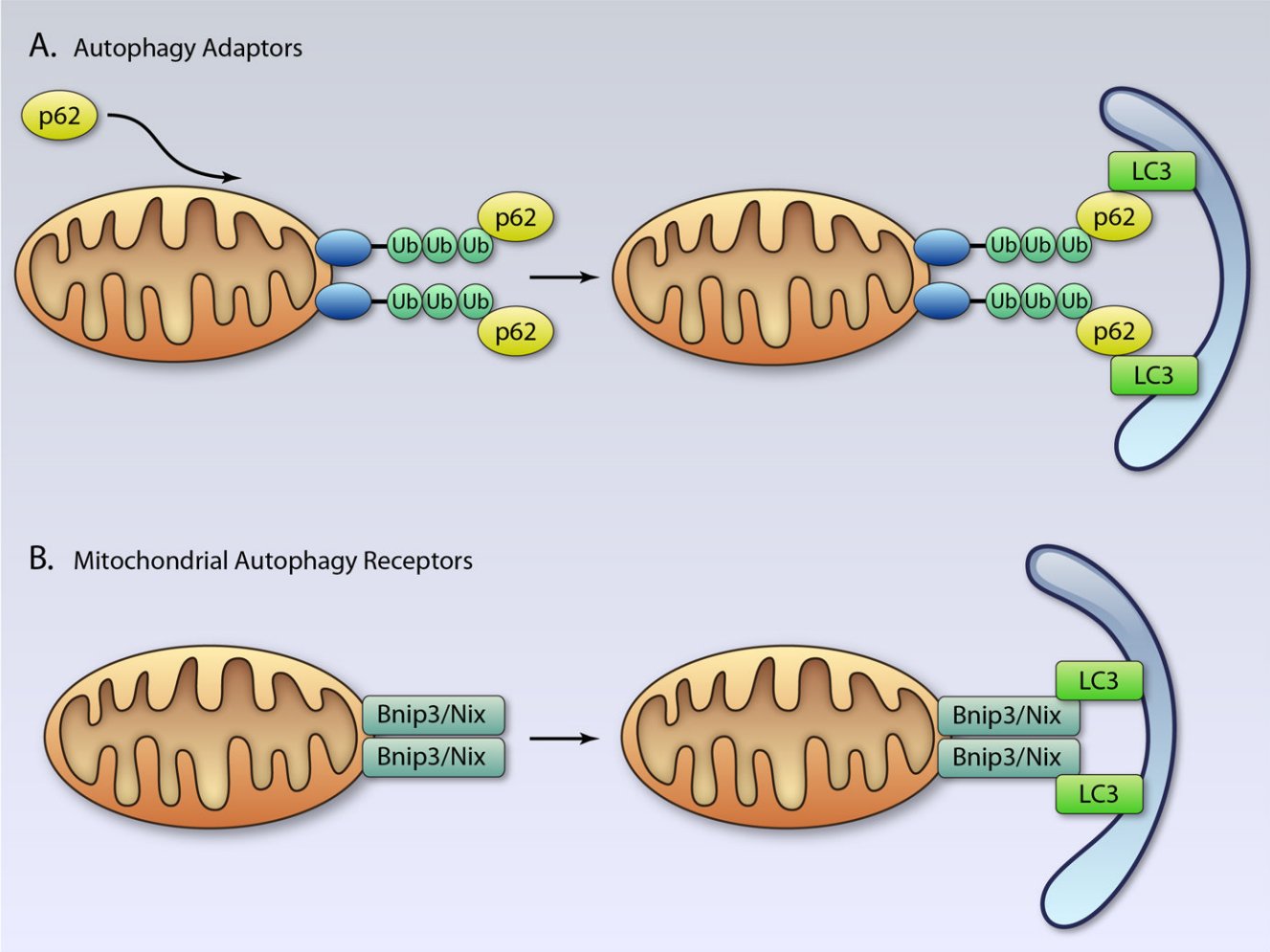

Figure 4.
Removal of mitochondria via autophagy adaptors or autophagy receptors. **A.** The p62 protein interacts with ubiquitinated proteins on the mitochondrion. The complex is then selectively sequestered by an autophagosome through the interaction between p62 and LC3. **B.** Autophagy receptors on the mitochondria such as Bnip3 and Nix interact directly with LC3 on the autophagosome. (Illustration Credit: Ben Smith).

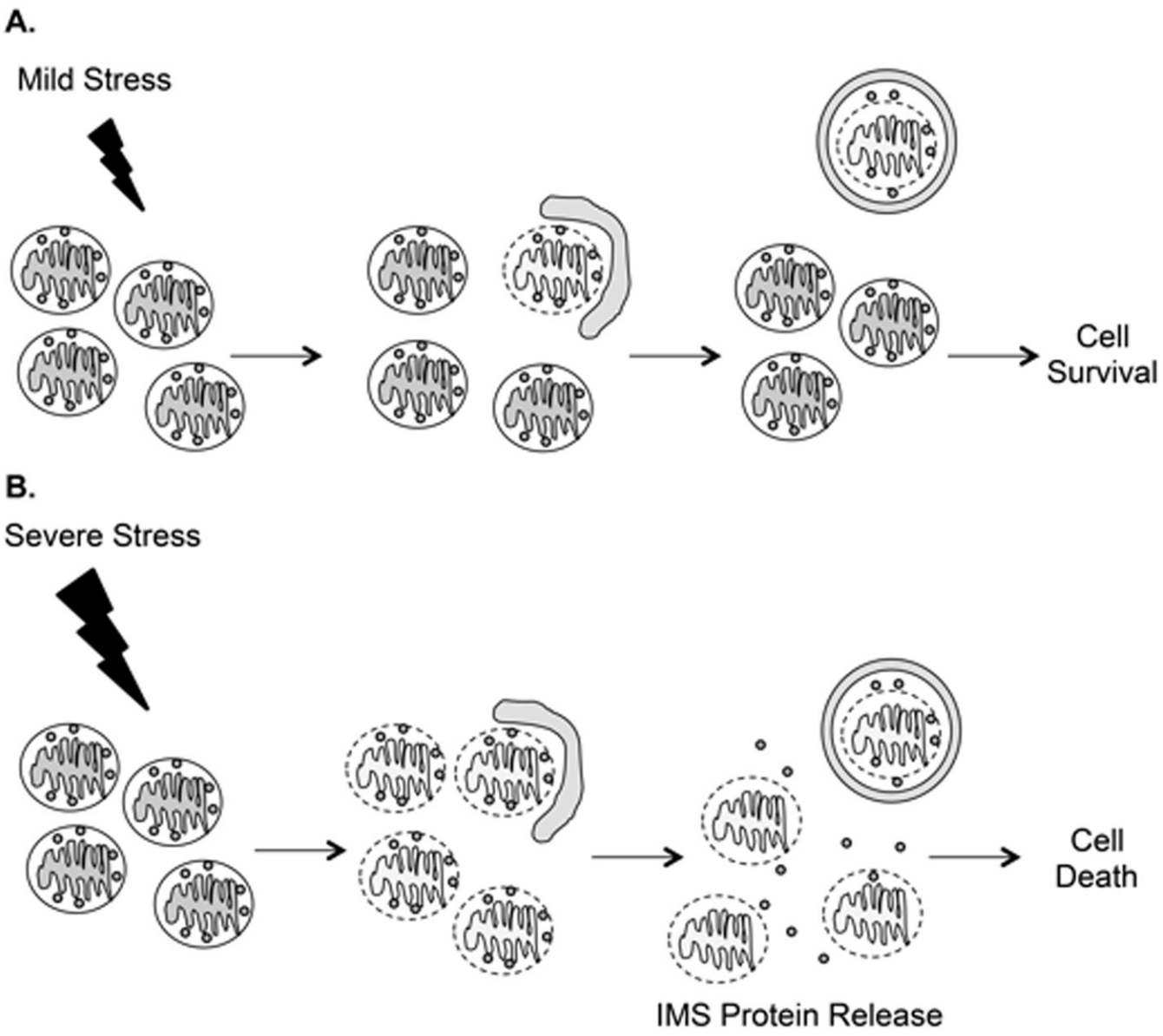

Figure 5.

Balance between life and death during stress. **A.** Mild stress causes damage to a few mitochondria, which are rapidly sequestered by autophagosomes. **B.** In response to severe stress, there is overwhelming mitochondrial damage that autophagosomes are unable to efficiently clear. These mitochondria will release pro-death proteins from the intermembrane space (IMS), such as cytochrome *c*, AIF, and SMAC/Diablo, that will activate cell death pathways.