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Review

Mitochondrial autophagy: Origins, significance, and role of BNIP3 and NIX☆

Paul A. Ney *

Department of Cell & Molecular Biology, Lindsley F. Kimball Research Institute, New York Blood Center, 310 East 67 Street, New York, NY 10065-6275, USA

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ABSTRACT

Mitochondrial autophagy (mitophagy) is a core cellular activity. In this review, we consider mitophagy and related cellular processes and discuss their significance for human disease. Strong parallels exist between mitophagy and xenophagy employed in host defense. These mechanisms converge on receptors in the innate immune system in clinically relevant scenarios. Mitophagy is part of a cellular quality control mechanism, which is implicated in degenerative disease, especially neurodegenerative disease. Furthermore, mitophagy is an aspect of cellular remodeling, which is employed during development. BNIP3 and NIX are related multi-functional outer mitochondrial membrane proteins. BNIP3 regulates mitophagy during hypoxia, whereas NIX is required for mitophagy during development of the erythroid lineage. Recent advances in the field of BNIP3- and NIX-mediated mitophagy are discussed. This article is part of a Special Issue entitled: Mitophagy.

1. Introduction

The ability of cells to manage their native and foreign subcellular contents is important for their health; when this process breaks down, cellular dysfunction ensues, which leads to disease. The principal strategy employed by cells is to isolate, destroy, and eliminate unwanted cargo. Whether the cargo is a membrane-bound organelle, precipitated protein aggregate, or an intracellular pathogen, the solution is the same: erect a barrier, direct destructive agents into the newly-formed compartment, and recycle or remove the debris. The process of confinement (see below) is critical; it determines what will be saved or destroyed and consequently determines the cellular outcome.

Mitochondria are a particularly relevant cargo and the focus of this review. Mitochondrial health must be monitored carefully, because mitochondria are hardwired to the major metabolic pathways of the cell, and, due to their exceptional origin [1], are prone to cause cell death and a variety of other problems. Here, after a general discussion of mitophagy, we will review and discuss two related outer mitochondrial membrane (OMM) proteins, BNIP3 and BNIP3L (NIX). BNIP3 and NIX impact an array of mitochondrial and extramitochondrial functions. Through our study of these proteins, we hope to gain a better appreciation of the relationship between mitochondria and cells. We also expect to gain insight into the rules of isolation membrane construction, and how they apply to various cargo. These are worthwhile objectives, because of the importance of these processes for human health, aging, and a broad range of human disease.

2. Selective autophagy: evolving concepts

Macroautophagy is the process of isolation membrane production, sealing, and degradation through the lysosomal pathway. For a recent review of macroautophagy in mammalian cells, please see Yang and Klionsky [2]. In macroautophagy, isolation membrane construction is considered to be nonselective; formally, this means the contents of sealed autophagosomes are on the average the same as the contents of the whole cell. By contrast, selective autophagy is the process whereby isolation membranes are erected around "selected" subcellular cargo. The evidence, initially, suggested that selective autophagy was closely related to macroautophagy; the only difference appeared to be an additional step targeting isolation membranes to cargo. However, current studies show that components of the canonical macroautophagy pathways, as defined in yeast, are often dispensable for selective autophagy. Whether this reflects the greater complexity (and redundancy) of macroautophagy in mammalian cells or differences specific to selective autophagy is not clear.

Most subcellular structures are targets of selective autophagy, including mitochondria, golgi, endoplasmic reticulum (ER), peroxisomes, ribosomes, the midbody, lipid droplets, and glycogen storage granules

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* Tel.: +1 212 570 3434.
E-mail address: pney@nybloodcenter.org.

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(reviewed in [3]). Ubiquitination of cargo is a critical step in selective autophagy. The prevailing model is that cargo-bound receptors recruit LC3 through an LC3-interacting region (LIR), bridging cargo with preformed, autophagy-generated membrane. In this model, receptors are either integral to the cargo, or recruited to the cargo via ubiquitination. A scaffold protein, which recruits additional autophagy-related proteins, may also be involved [4]. For example, in selective mitophagy in yeast, an OMM protein Atg32 serves as an autophagy receptor, and Atg11 as a scaffold protein. Atg32 mediates mitochondrial clearance in response to changing metabolic conditions [5–7].

Recent studies have revealed that LIR motifs are present not only in receptors, but in canonical autophagy-related proteins, such as Atg1/ULK1, Atg3, and Atg4B [3]. LIR motifs are also found in endosomal trafficking proteins, such as Rab guanosine triphosphatase-activating proteins [8]. The implication of these findings is that LC3 recruitment to cargo is not only the final objective, but also an intermediate step in the recruitment of additional autophagy-related proteins. Another study shows that two ubiquitin-binding receptors, p62 and NBR1, are recruited to sites of autophagosome formation independent of LC3 [9]. p62 and NBR1 colocalize with autophagy-related proteins, independent of class III phosphatidylinositol 3-kinase, suggesting that they may be able to directly recruit these factors to ubiquitinated cargo. Consistent with this model, ULK1 and Atg9A are recruited to depolarized mitochondria, followed by other autophagy factors, independent of LC3 [10]. LC3 is still required in this model for the efficient incorporation of cargo into the developing phagophore. The main distinction between the two models is the role of the LIR–LC3 interaction in the recruitment of upstream autophagy factors, such as ULK1. Perhaps LC3 is not required to recruit upstream factors for phagophore initiation, but is required for phagophore growth and cargo incorporation.

3. Origins of mitophagy: host defense

3.1. Bacterial autophagy (xenophagy)

Of all its functions, the first purpose of selective autophagy was probably host defense. As primitive cells acquired the ability to invade one another, they directed their defenses internally, which necessitated developing the means to isolate cargo. Intracellular pathogens usually gain entry to cells by ingestion, where they reside in a single-membraned, endosomal structure known as the phagosome. Fusion with a lysosome creates a phagolysosome, in which ingested bacteria are destroyed. Bacteria that escape the phagosome and enter the cytoplasm are tagged by ubiquitin, which in turn engages the autophagy machinery. Ubiquitinated *Salmonella* are targeted by the bi-functional receptor Optineurin through its ubiquitin binding domain. Optineurin in turn recruits the autophagy-related protein LC3, which promotes phagophore elongation and isolation of the pathogen [11]. Rather than ubiquitinate cargo directly, another study finds that disruption of *Salmonella*-laden endosomes is followed by the ubiquitination of host proteins [12]. Core autophagy complexes are recruited initially, in this instance, followed by LC3. The advantage of targeting host-proteins is presumably to minimize the effect of microbial diversity. *Mycobacterium marinum* can infect host macrophages, like its close relative *M. tuberculosis*. In a race against time, the host seeks to isolate the mycobacterium inside an autophagosome-like structure, before it sheds its cell wall, develops an actin tail, and escapes [13]. Notably, Atg5 and LC3 are not required for this process. In another variation, *E. coli*, killed yeast, or particles that engage Toll-like receptors (TLR) during the process of ingestion, cause LC3 recruitment to phagosomes [14]. In contrast with the previous examples, in this instance LC3 recruitment is not associated with the formation of a demonstrable isolation member; rather, the association of LC3 with the phagosome promotes lysosomal fusion and phagosome maturation. Thus, in the realm of host defense alone, selective autophagy exhibits significant mechanistic diversity.

3.2. The host response to free mitochondria: echoes of an ancient conflict

Mitochondria are thought to be the evolutionary descendants of an α-proteobacterial endosymbiont [1]. Consistent with this common origin, there are similarities in the relationships between cells and microbes, and cells and mitochondria. One curious similarity is that both microbes and mitochondria are able to move between cells in a productive manner. The endosymbiont Wolbachia supplies its filarial host with heme, as do mitochondria in eukaryotic cells [15]. Wolbachia reach the female germline of *B. malayi* larvae by cell–cell invasion [16]. Similarly, mitochondria from human marrow nonhematopoietic stem-progenitor cells or skin fibroblasts can rescue aerobic respiration in A549 rho^0 cells, which requires direct cell–cell contact [17]. Further, mitochondria from bone marrow-derived stromal cells can rescue alveolar bioenergetics in an lipopolysaccharide (LPS)-induced model of acute lung injury [18]. Mitochondrial transfer in this model, occurs through nanotubes and microvesicles, and depends on connexin.

As opposed to the potentially beneficial effects of cell–cell mitochondrial transfer, mitochondrial release into the extracellular space is mostly detrimental. Activated platelets release free mitochondria and mitochondria in microvesicles [19]. These serve as a substrate for the bacteria-specific phospholipase A2-IIA, releasing inflammatory mediators; they also interact with neutrophils in vivo, triggering neutrophil adhesion to endothelium. Notably, levels of extracellular mitochondria in blood products correlates with adverse reactions upon transfusion. Similar to platelets, mast cells stimulated to degranulate, release mitochondrial components [20].

Necrosis leading to release of free mitochondria into the extracellular space is a clinically important scenario. Necrosis causes loss of plasma membrane integrity and leakage of cell contents, including mitochondria, into the extracellular space. Necroptosis, a TNF-α-induced form of programmed necrosis, causes the release of whole mitochondria, which are engulfed by macrophages, causing cytokine production (including TNF-α, in a potential feedback loop) [21]. Interestingly, in this model, mitochondrial clearance precedes overt membrane permeabilization, suggesting that mitochondrial elimination may be an aspect of necroptosis. Necrosis secondary to trauma in humans causes the release of mitochondrial formyl peptides and DNA into the circulation and sepsis-like systemic inflammatory response syndrome [22]. Mitochondrial formyl peptides and DNA activate neutrophils through the formyl peptide receptor and TLR9, respectively. Similarly, in an animal model of focal hepatic necrosis induced by thermal injury, mitochondrial formyl peptide acts as a chemoattractant at the final step of a multi-step cascade that recruits neutrophils to sites of sterile inflammation [23].

Compartmentalization of dysfunctional mitochondria in cells that are stressed or damaged, but not committed to undergo necrosis, is important in limiting the inflammatory response. Treatment of cells with pathogen-associated molecular pattern molecules, such as LPS and ATP, causes mitochondrial dysfunction, increases production of reactive oxygen species (ROS) and activates the inflammasome [24]. This in turn activates the mitochondrial permeability transition (MPT), which causes leakage of mitochondrial DNA (mtDNA) into the cytosol, and activates caspase-1. Autophagy limits these effects, as LC3B-deficient mice showed enhanced caspase-1 activation and increased mortality in sepsis models. This principle applies to different types of cellular stress. In the heart, in the setting of increased pressure afterload, DNase II deficiency is associated with myocarditis and dilated cardiomyopathy [25]. In this model, the accumulation of undigested mitochondria in autolysosomes once again causes leakage of mtDNA into the cytosol where it activates a TLR9-dependent inflammatory response leading to cardiomyopathy.

Thus, it is important to compartmentalize dysfunctional mitochondria because they can cause cell death, but also because they are a source of damage-associated molecular patterns, which can engage pro-inflammatory innate immune mechanisms with adverse consequences (Fig. 1).

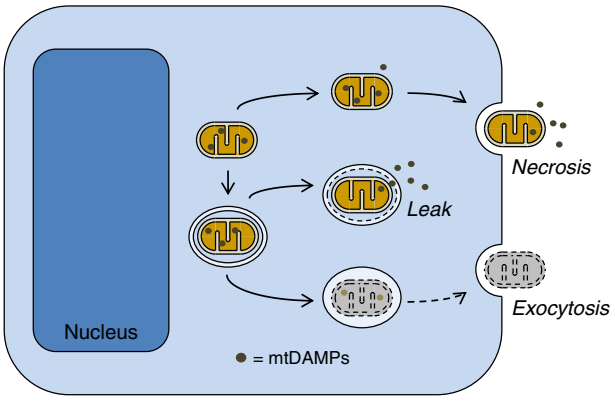

Fig. 1. Mode of mitochondrial elimination affects mitochondrial damage-associated molecular pattern (mtDAMP) release and activation of the innate immune system. (Top) Necrosis causes loss of plasma membrane integrity and allows mitochondria and mtDAMPs access to the extracellular space. (Middle) If mitophagy is impaired or inadequate, mtDAMPs can leak into the cytosol where they activate innate immune receptors. (Bottom) If mitophagy is effective, mitochondria and mtDAMPs are degraded, dampening the immune response. Degraded mitochondria may remain in the cell or be cleared (dashed arrow). The small brown circles are mtDAMPS, such as mtDNA.

4. Mitophagy

4.1. Clearance of depolarized mitochondria

Mitochondria are the principal site of oxidative phosphorylation and energy production in animal cells. They are also the principal site of ROS production. ROS are normal signaling intermediates, but in excess can have a detrimental effect on cellular health. Declining mitochondrial function has been linked to aging and a broad range of human disease [26]. The strongest evidence linking defective mitochondrial elimination to human disease comes from the Parkinson's disease (PD) field. First, there is evidence of mitochondrial abnormalities in PD [27]. Second, mitochondrial toxins elicit a PD-like syndrome [28]. Third, genes have been identified that are frequently mutated in juvenile-onset PD [29,30]. Two of these, PTEN-induced kinase-1 (PINK1) and Parkin, have been shown to mediate the clearance of depolarized mitochondria from cells [31,32]. Other PD-associated genes have also been implicated in mitochondrial quality control (reviewed in [33]). The mechanism of Parkin-dependent mitochondrial clearance is covered elsewhere in this special issue, but is covered briefly here.

Genetically, PINK1 and Parkin are on the same pathway [34,35]. PINK1 is a serine–threonine kinase, whereas Parkin is an E3 ubiquitin ligase. Normally, PINK1 is imported into mitochondria, cleaved by the inner membrane protease Presenilin-associated Rhomboid-like (PARL), released into the cytosol, and degraded by the proteasome [36]. Upon mitochondrial depolarization, PINK1 is no longer imported, leading to its stabilization on the OMM [32]. PINK1 expression on the OMM recruits Parkin to mitochondria [32,37–39]. The mechanism involves PINK1-mediated phosphorylation of ubiquitin Ser65 and the equivalent serine residue in the ubiquitin-like domain of Parkin, leading to Parkin activation and recruitment (reviewed in [40]). A role for MFN2 has also been proposed [41]. Identified OMM targets of Parkin ligase activity include MFN1, VDAC1, and MIRO [37,42]. MFN1 is extracted from the OMM by a ubiquitin-dependent chaperone and degraded by the proteasome. Depletion of MFN1 favors mitochondrial fission, which promotes mitophagy. Ubiquitination of VDAC1 and other OMM proteins provides a ligand for the recruitment of ubiquitin-binding receptors and the autophagy machinery, as discussed previously. Proteomic analysis of Parkin-dependent ubiquitination has shown that Parkin causes extensive OMM remodeling [43,44].

The inciting event for Parkin-mediated mitochondrial clearance in these models is mitochondrial depolarization. Depolarization is chemically induced with the protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) in these experiments and affects the majority of the mitochondria in the cells. By contrast, mitochondrial quality control in vivo is applied to individual mitochondria. Damage to individual mitochondrial has been modeled by photodamage [45]. Whereas Parkin-mediated mitochondrial clearance requires core autophagy proteins, photodamage-induced mitochondrial elimination appears to be Beclin-1 independent. Thus, more than one mechanism exists for the clearance of depolarized mitochondria. Naturally occurring depolarization may be a consequence of activation of the MPT, or an indirect effect of mitochondrial outer membrane permeabilization (MOMP). Induction of MOMP in neurons causes clearance of the entire cohort of mitochondria, conditional on caspase inactivation [46]. Whereas MOMP was once thought to be binary, incomplete MOMP has been described [47]. In cells undergoing incomplete MOMP, depolarized mitochondria are cleared, leaving healthy mitochondria to repopulate the cell.

4.2. Clearance of functioning mitochondria

Mitochondrial depolarization is a potent stimulus for clearance; however, there are other signals that lead to the elimination of still-functioning, polarized mitochondria. These signals may be the first line of defense, since it is presumably desirable to remove dysfunctional mitochondria before they depolarize. Alternatively, they may also provide a mechanism for regulating mitochondrial content in the face of changing metabolic conditions. Notably, the metabolic milieu can be dominant, blocking mitochondrial elimination when it might otherwise occur.

Some of the strongest evidence for metabolic regulation of mitochondrial content comes from the yeast model. First, it should be noted that mitochondrial depolarization triggers mitophagy in yeast as it does in mammalian cells [48,49]; although, curiously, CCCP does not [50]. Switching yeast from rich medium to nitrogen starvation condition induces mitophagy when the carbon source is glucose (i.e., fermentable), but not lactate, which requires mitochondria for metabolism [7]. The difference in conditions is mitophagy specific, as macroautophagy is induced in both. Conversely, when yeast grown in lactate medium reach stationary phase, mitophagy is induced. It would be interesting to know the metabolic signal for mitophagy under these conditions, but it has not yet been discovered.

Starvation-induced autophagy in mammalian cells is thought to be nonselective; however, this may depend on cell type. In hepatocytes, starvation and glucagon treatment cause mitophagy and the elimination of polarized mitochondria (although they undergo MPT-dependent depolarization inside autophagosomes) [45]. The mechanism depends on classical autophagy (i.e., Beclin-1/Vps34). The means by which mitochondria are targeted is not known, but possibly relates to the source of the autophagy membrane, as one potential source, mitochondria-associated membrane, is in close proximity. Interestingly, the opposite phenomenon has also been described, namely mitochondrial fusion in response to starvation, sparing mitochondria from autophagy [51,52]. The latter studies were done in murine embryonic fibroblasts (MEF), which may respond differently to starvation than hepatocytes.

There is considerable interest in the mechanism linking energy sensing to mitophagy. A key insight in this regard is that the intracellular energy sensor adenosine monophosphate-activated protein kinase (AMPK) phosphorylates and activates ULK1, the upstream autophagy regulator and mammalian homologue of Atg1 [53]. Along the same line, oxidative phosphorylation promotes mitophagy [54]. This correlates with recruitment of the upstream mTOR activator Ras homologue enriched in brain (Rheb) to mitochondria, where it interacts with an OMM protein (NIX, see following discussion). Whereas a closely related protein is known to bind Rheb, inhibiting mTOR activation [55], in this setting, there is no effect on mTOR activity. The effect of Rheb is to stabilize the interaction between NIX and LC3, thereby promoting mitophagy. Another interesting observation is that iron chelation

generates a strong mitophagy response [56]. Mitochondria in deferiprone-treated cells retain their membrane potential, but exhibit a dramatic decrease in oxygen consumption. The signal inducing mitophagy in this setting is not known; however, it is independent of ROS, PINK1, and Parkin. Similar to the dominant effect of metabolism in yeast, mitophagy in this model, as well as in neurons [57], is decreased in respiratory conditions. Hypoxia is another type of metabolic stress, which stimulates autophagy via AMPK [58]. Hypoxia regulates mitophagy through BCL-X_L by derepressing the activity of the phosphatase PGAM5 toward the receptor FUNDC1 [59,60].

4.3. Programmed mitochondrial clearance

By definition, programmed mitochondrial clearance refers to mitochondrial elimination in the context of a developmental program. There are three well established models of developmentally regulated mitochondrial elimination, the sperm and early zygote, the lens of the eye, and the nascent reticulocyte (precursor to the erythrocyte). In an early hint of the mechanisms of selective autophagy, bovine and nonhuman primate sperm were shown to be ubiquitinated in the male reproductive tract and eliminated prior to the third embryonic cleavage [61]. Notably, sperm mitochondria from interspecies hybrids were not ubiquitinated and persisted beyond the third embryonic division. In *C. elegans* sperm, which lack a flagellum, mitochondria are packed around the nucleus. *C. elegans* sperm are not ubiquitinated; however, a polyubiquitination signal appears around paternal pronuclear DNA in early embryos [62,63]. This signal does not colocalize with mitochondria, but rather with specialized vesicular structures (membranous organelles, MO) nearby. Both mitochondria and MO are found in autophagosomes, but only MO are ubiquitinated. Thus, mitochondria are "swept up" along with MO by the autophagy process. Autophagy is required for elimination of paternal mitochondria in this model; these persist in autophagy-deficient embryos, but do not increase in number. Prefertilization elimination of mtDNA occurs in flagellated *Drosophila* sperm through the actions of Endonuclease G and a cellular remodeling process during individualization [64]. Postfertilization *Drosophila* mitochondrial derivatives devoid of mtDNA are ubiquitinated and cleared by a mixed endocytic/autophagic mechanism [65]. Reminiscent of LC3-associated phagocytosis, mentioned earlier, this mechanism involves Atg8 and the *Drosophila* p62 ortholog, but not the autophagy initiation complexes [66]. In mice, sperm mtDNA is eliminated prior to fertilization [67]. Atg5 does not play a role in post-fertilization processing of sperm mitochondria in mice, arguing against post-fertilization mitochondrial clearance in this setting, or in favor of a noncanonical autophagy-related mechanism.

Mitochondria and other organelles are cleared from the central lens fiber cells during development to create an "organelle-free zone". Not surprisingly, failure to create an organelle-free zone is associated with cataracts. Studies implicate an autophagy-related process in organelle clearance in these cells, negatively regulated by MAPK/JNK signaling through mTOR [68]. In a recurring theme, Atg5 is not required for organelle clearance from lens fiber cells, which is suggestive of a noncanonical process [69]. Interestingly, Atg5 deficiency is still associated with cataract formation due to the accumulation of p62-positive inclusions [70]. The third example of programmed mitochondrial clearance in mammalian cells is in the precursor cell of erythrocytes (known as reticulocytes), which is discussed later.

5. BNIP3 and NIX: multifunctional outer mitochondrial membrane proteins

BNIP3 is one of three proteins identified in a screen for adenovirus E1B-19 K-interacting proteins, which exhibited pro-death activity [71]. BNIP3 and the closely related protein NIX (approximately 50% identical amino acids) are single-pass, transmembrane proteins, located in the OMM (reviewed in [72]) (Fig. 2). The transmembrane domains of BNIP3 and NIX are glycine zippers, which form detergent-resistant homodimers [73]. The majority of both proteins is cytoplasmic, with only 10 amino acids protruding into the intermembrane space. BNIP3 and NIX contain an atypical BCL2-homology 3 (BH3) domain, which is functional under some circumstances. Additionally, the cytoplasmic domain contains a LIR motif and a novel short linear motif (SLiM). Bioinformatic analysis predicts that the cytoplasmic domain is disordered, but has regions of secondary structure [74]. The LIR is predicted to form a β-strand and the SLiM an α-helix centered on a triplet of hydrophobic amino acids.

The BH3 domains of BNIP3 and NIX impart some pro-death activity, but the majority is mediated by the transmembrane domain. An important insight into the mechanism was provided by the demonstration that BNIP3 causes cell death without cytochrome *c* release or caspase activation [75]. In this study, BNIP3 was shown to activate the MPT, which was associated with increased ROS production and excessive autophagy. Cell death was inhibited by cyclosporin A and bongkrekic acid, suggesting it is mediated by the MPT.

Added to isolated mitochondria, BNIP3 causes cytochrome *c* release, depolarization, and swelling [76]. NIX, by contrast, causes cytochrome *c* release but does not activate the MPT [77]. This represents an exception to the overall functional similarity between the two proteins. Thus, BNIP3 can directly activate the MPT; however, the BNIP3-induced pore has nonclassical features. BNIP3 activates BAX and BAK, classical mediators of apoptosis [78]. BNIP3-induced cytochrome *c* release and mitochondrial depolarization are both dependent on the presence of BAX or BAK, and cell death is only partially blocked by inhibitors of the MPT. BNIP3 induces permeabilization of the inner and outer mitochondrial membranes, which is independent of cyclophilin D, but involves disruption of Opa1 complexes and remodeling of the inner mitochondrial membrane [79,80]. The BNIP3 BH3 domain is required for BNIP3-induced cell death, but is dispensable for mitochondrial fragmentation [79]. Thus, enforced expression of BNIP3 creates a novel BAX/BAK-dependent pore in mitochondria, with mixed features of the MPT and MOMP, which disrupts the inner and outer mitochondrial membranes, and mediates cell death. Interestingly, BNIP3 has been identified as a mediator of TNF-induced necroptosis [81].

BNIP3 and NIX are implicated in cardiomyocyte cell death in different settings. BNIP3 is a HIF-1α target induced by hypoxia [82], which mediates cell death after ischemia–reperfusion. By contrast, NIX is regulated by Gαq signaling, which is associated with cardiac pressure overload [83]. NIX causes death by two pathways. NIX targeted to mitochondria triggers BAX/BAK-dependent MOMP and apoptosis, whereas NIX targeted to the ER–SR causes necrosis [84,85]. In the latter instance, NIX increases ER–SR calcium stores, which are released to mitochondria, activating the classic MPT.

BNIP3 and NIX are linked to the induction of autophagy, and there are several potential mechanisms. First, by causing mitochondrial dysfunction, BNIP3 or NIX may increase production of ROS, which can activate autophagy [86]. Second, competition by BNIP3 or NIX for binding to BCL2 (or a related protein) could liberate Beclin-1 from BCL2 complexes and activate autophagy [87–89]. Third, BNIP3 binds to and inhibits Rheb [55]. Rheb is an upstream activator of mammalian target of rapamycin (mTOR); therefore, BNIP3 may activate autophagy by repressing mTOR. Thus, BNIP3 and NIX regulate two basic cellular functions, cell death and

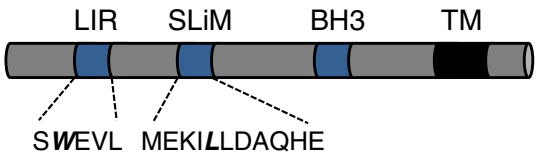

Fig. 2. Schematic diagram of NIX domains. Shown are the LC3-interacting region (LIR), an essential short linear motif (SLiM), BCL2 homology domain 3 (BH3), and the transmembrane domain (TM). Amino acid sequence is shown below the LIR and SLiM with critical residues in bold italic.

autophagy. In the next section, we address the role of NIX in programmed mitochondrial clearance.

6. Role of NIX in programmed mitochondrial clearance: an update

As mentioned previously, the clearance of mitochondria from nascent reticulocytes is now a widely accepted model for the study of programmed mitochondrial elimination. NIX interested us, because it is coordinately upregulated with an antiapoptotic protein (BCL-X_L) during erythroid development [90,91]. This suggested it might have a noncanonical role in development (in addition to its prodeath function). We discovered that a substantial proportion of erythrocytes in the circulation of NIX-deficient mice had abnormally retained mitochondria, and that NIX-deficient reticulocytes exhibited defective mitochondrial clearance in culture [91,92]. Ultrastructural studies showed a striking picture of mitochondria juxtaposed with phagophores and arrayed against the cytoplasmic face of immature autophagosomes. Based on the ultrastructural appearance of wild type and NIX-deficient reticulocytes, we concluded that the cellular function of NIX is the recruitment of membranes to individual mitochondria (Fig. 3). We also concluded that this is a necessary intermediate step for fusion with autophagosomes or the plasma membrane. Our subsequent studies and those of others have focused on the molecular mechanism of NIX-dependent membrane recruitment to the mitochondria.

In light of NIX's ability to cause mitochondrial dysfunction, we considered whether NIX-mediated mitochondrial depolarization was the stimulus for clearance. Along this line, chemically-induced mitochondrial depolarization can rescue mitochondrial clearance in NIX-deficient reticulocytes [92]. However, BAX-, BAK-deficient mice did not have a defect in mitochondrial clearance [91]. BAX and BAK are essential downstream effectors of classical MOMP [93,94]; therefore, this process is not involved. Also, BIM- and PUMA-deficient mice had no defect in clearance, indicating that NIX does not sensitize mitochondria to BH3-only protein-mediated depolarization. Further, BCL-X_L is not co-required with NIX [91], nor does it prevent clearance in NIX's absence [74], although there is evidence that it enhances BNIP3-mediated mitochondrial clearance in Hela cells [95]. Finally, *Bak* disruption is the only genetic mutation to date, shown to suppress the NIX phenotype [74]. The mechanism is unknown, but may involve a change in mitochondrial dynamics or susceptibility to the MPT [96,97]. Regarding the chemical rescue of mitochondrial clearance in NIX-deficient reticulocytes [92], the mechanism appears to be different than that of NIX [98]. Curiously, NIX has been shown to facilitate chemically-induced depolarization, autophagy induction, and Parkin translocation [99]. Otherwise, NIX has not been strongly linked with Parkin-mediated mitophagy.

Experiments employing chemical inhibitors or cyclophilin D-deficient MEFs suggest that the classical MPT is also not required for clearance [91,100]. Although BNIP3 induces an atypical MPT-like leak pathway, NIX has not been shown to have the same effect. The possibility that NIX can cause mitochondrial depolarization through an undefined leak pathway was examined in autophagy-deficient reticulocytes. Mitochondrial clearance is somewhat impaired in Atg7-deficient reticulocytes [98,101,102]. After three days in culture, mitochondria in Atg7-deficient reticulocytes remained polarized, whereas those in wild type reticulocytes rapidly depolarized and were cleared. This result suggests that mitochondrial depolarization in reticulocytes is a consequence, rather than a cause, of membrane recruitment and autophagosome formation. Although these studies suggest that NIX does not cause frank mitochondrial depolarization in reticulocytes, we do not discount the possibility that there is a subtle but relevant effect of NIX on mitochondrial membrane potential ($\Delta\Psi_m$). A quantitative decrease in $\Delta\Psi_m$ is sufficient to induce mitophagy [103], and BNIP3 promotes the degradation of proteins involved in oxidative phosphorylation [104].

Regarding NIX's mechanism, another issue is the role of autophagy. Autophagy is upregulated by the transcription factors GATA1 and FOXO3 during erythroid development [105,106], and loss of NIX does not change the pattern [91]. Thus, NIX is not an upstream regulator of canonical autophagy in erythroid cells. On the other hand, BNIP3 and NIX regulate hypoxia-induced mitophagy [107], which has been attributed to an effect on Beclin1–Bcl2 complexes [87]. Given the ultrastructural demonstration of robust phagophore and autophagosome formation during the process, and biochemical evidence of activation of autophagy, we were surprised to find that Atg7 deficiency had only a modest effect on mitochondrial clearance in reticulocytes (despite inactivation of Atg7-regulated pathways) [98,101,102]. Consistent with this result, Atg5 is also dispensable for mitochondrial clearance [69,108]. By contrast, deficiency of ULK1, the mammalian upstream initiator of autophagy, causes a significant defect in clearance (more severe than Atg7 but less so than NIX) [109]. Further, deficiency of AMPK, an upstream activator of ULK1, also causes a defect in clearance [110]. Based on ultrastructure, mitochondrial clearance is carried out by an autophagy-related process, even in the absence of Atg7 [98]; therefore, these studies suggest the existence of an ULK1-dependent, Atg5/Atg7-independent, autophagy-related process. Alternative autophagy has been described in etoposide-treated, Atg5-deficient MEFs, and attributed at least in part to Rab9-dependent fusion of *trans*-Golgi-derived vesicles [101]. The extent to which this pathway overlaps the one in reticulocytes remains to be determined.

A screen for autophagy receptors revealed that NIX interacts with GABARAP and LC3 through its LIR motif [111,112]. The strength of the interaction was much less than that of LC3 and p62 (60-fold), but was

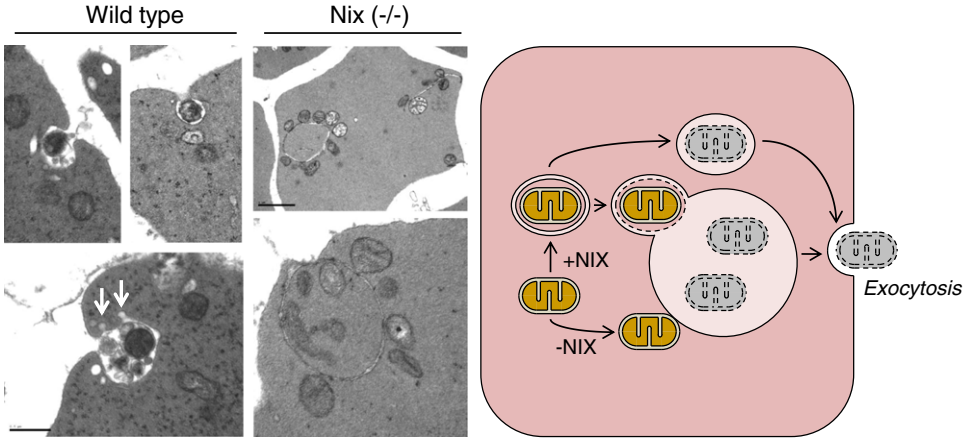

Fig. 3. Mitochondrial clearance in reticulocytes. NIX is required to form isolation membranes around mitochondria; mitochondrial clearance is blocked at the step of fusion with cellular membranes in the absence of NIX. In the bottom wild type panel, the arrows point to vesicles that appear to fuse with the autophagic vacuole and are possibly involved in vacuolar maturation.

demonstrable. In reticulocytes, mutation of NIX's LIR had a modest but measurable effect on mitochondrial clearance. This result was not too surprising, given the relatively modest effect of Atg7 deficiency on mitochondrial clearance. Whereas it was initially reported that LC3 co-localizes with mitochondria in reticulocytes [92], a recent study found poor colocalization in reticulocytes derived from the fetal liver [108]; potentially this reflects differences in the developmental stage. Together, these studies cast some doubt on the importance of NIX-mediated LC3 recruitment for mitochondrial clearance in reticulocytes. On the other hand, there may be settings where this interaction is important, such as hypoxia- or depolarization-induced, BNIP3-mediated, mitophagy. In this regard, phosphorylation of serine residues flanking the BNIP3 LIR regulate its interaction with LC3 and mitophagy [95].

Although the role of LC3 in NIX-mediated mitochondrial clearance is open to debate, the aforementioned studies established an adaptor mode of NIX's function. Our laboratory pursued this concept in a structure–function analysis in mice [74]. Apart from NIX's transmembrane domain, which is required for proper subcellular targeting, we found that almost all of NIX's activity localizes to a novel short linear motif (SLiM) in NIX's cytoplasmic domain. The SLiM is perfectly conserved in BNIP3 (which also rescues clearance in NIX-deficient reticulocytes). It is approximately 11 amino acids comprising a triplet of hydrophobic amino acid residues at its center and flanking charged residues. Mutation of the central leucine residue in the hydrophobic triplet abolishes all NIX's activity. It is predicted to form an α-helix. From these results, we infer that NIX's SLiM interacts with a hydrophobic pocket in another protein. The identity of the putative interacting protein is not known, but should be revealing of NIX's function.

One facet of BNIP3's and NIX's function, which is understudied, is the role of dimer formation. Mutants of BNIP3's transmembrane domain that preserve mitochondrial localization, but disrupt dimerization fail to induce autophagy [113]. Our preliminary studies similarly show that disruption of NIX's glycine zipper interferes with mitochondrial clearance in reticulocytes (P. Ney, unpublished result). Potentially, NIX's adaptor function requires dimerization.

7. BNIP3 and NIX in disease

At the risk of oversimplification, BNIP3 and NIX can potentially cause disease by two mechanisms. Stress-induced overexpression of either protein can directly lead to mitochondrial dysfunction and cell death. Alternatively, impairment of BNIP3 or NIX function can lead to defective mitochondrial quality control, which in turn leads to mitochondrial dysfunction and cell death. The best characterized models of BNIP3 and NIX-caused disease to date are in the heart. BNIP3 is regulated by hypoxia. Experiments, conducted in the Dorn laboratory have shown that enforced expression of BNIP3 causes cardiomyopathy, and that BNIP3 deficiency limits post-infarct remodeling and improves cardiac performance [114]. NIX is regulated by Gαq signaling, which is associated with cardiac hypertrophy. Enforced expression of NIX causes lethal perinatal cardiomyopathy in mice, whereas NIX deficiency protects mice from Gαq-mediated and pressure overload cardiomyopathy [115,116]. Mice with germline deletion of BNIP3 and conditional cardiac deletion of NIX develop normally, but by 30 weeks develop massive cardiac enlargement and decreased left ventricular ejection fraction [117]. Mitochondria in the hearts of these mice are increased in number, and show marked variation in size and internal structure. Moreover, young BNIP3/NIX-deficient mice subjected to aortic banding rapidly develop heart failure.

Humans and mice heterozygous for the pancreatic duodenal homeobox (PDX1) transcription factor develop diabetes. Diabetes in this setting is attributed to programmed β cell death. Enforced expression of NIX in MIN6 insulinoma and pancreatic islet β cells induces programmed cell death of a mixed apoptotic and necrotic type [118]. Remarkably, inactivating *Nix* in *Pdx1* heterozygous mice rescued pancreatic islet architecture, β cell mass, and insulin secretion, and eliminated reactive hyperglycemia after glucose challenge.

Starvation induces BNIP3 expression in the liver, consistent with a role in hepatic function [119]. BNIP3 deficiency is associated with the accumulation of dysfunctional mitochondria in the liver, which is associated with an abnormal metabolic profile. BNIP3-deficient liver cells have an elevated ATP content and reduced AMPK activity leading to a defect in hepatic glucose output in response to fasting. Further, lipid synthesis is increased and β-oxidation of fatty acids is reduced. In the absence of BNIP3, there are elevated ROS, inflammation, and features of steatohepatitis. Thus, loss of BNIP3 leads to mitochondrial defects in the liver, which impair metabolic homeostasis.

BNIP3 is utilized in epithelial cells by *Shigella*, although to an uncertain end [120]. *Shigella* infection in nonmyeloid (epithelial) cells triggers BNIP3-mediated mitochondrial depolarization and necrosis, which is opposed by prosurvival pathways simultaneously activated by the bacteria. Thus, *Shigella* causes programmed necrosis in epithelial cells via BNIP3, which was not known previously.

8. Conclusions

Selective autophagy is a basic cellular function, with a likely origin in host defense. It has evolved in multi-cellular organisms into a mechanism of cellular remodeling, which is summoned in the setting of quality control in long-lived cells, stress, and differentiation. Meanwhile, it has retained its original purpose. Based on a rapidly expanding number of studies, we know that there is significant overlap in the mechanisms of selective autophagy as it applies to the elimination of bacteria and other types of cargo. Understanding the similarities and differences will provide a mechanistic foundation for linking diverse human diseases.

Selective autophagy in higher eukaryotes involves a complex assortment of regulatory mechanisms, which utilize various components of the core autophagy machinery. At present, the relationship between the core autophagy machinery and the different types of selective autophagy is unclear; there is no apparent pattern to the utilization of the core components. Some types of selective autophagy proceed independent of upstream regulators, whereas others dispense with the terminal ubiquitin-like conjugation pathways. BNIP3 and NIX appear to fall into the latter category. One of the key questions to be addressed is the role of LC3. Does it function to "zip up" cargo into the autophagosome? Does it recruit the upstream autophagy machinery to sites of phagophore production? Does it always need to be lipidated to function? A recent study suggests, possibly not [121]. Another key question is the role of ubiquitination. With regard to selective autophagy, is it necessary to ubiquitinate specific substrates, or does it work through mass action? There is evidence that one of the functions of ubiquitination is to recruit receptors, but what is their purpose: to recruit autophagy complexes, membrane, cytoskeleton, or all of these? Elucidation of BNIP3's and NIX's mechanism of action will provide answers to some of these questions.

BNIP3 and NIX can affect mitochondrial function in surprisingly many ways. Whether these effects are part of a continuum or whether they occur in different settings remains to be determined. Ongoing studies of BNIP3 and NIX should provide additional insights into the relationship between mitochondria and cells, and the ways in which this relationship can be manipulated to treat human disease.

Transparency document

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

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