

Review

Role of BNIP3 and NIX in cell death, autophagy, and mitophagy

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BNIP3 and NIX are proteins related to the BH3-only family, which induce both cell death and autophagy. Consistent with their ability to induce cell death, BNIP3 and NIX are implicated in the pathogenesis of cancer and heart disease. In tumor cells, BNIP3 and NIX are regulated by hypoxia, and the deregulation of BNIP3 or NIX expression is associated with tumor growth. In heart muscle, BNIP3 and NIX are regulated by hypoxia and G α q-dependent signaling, respectively, and their expression is associated with decreased myocardial function. Apart from their role in cell death, BNIP3 and NIX are also implicated in the induction of autophagy. In erythroid cells, NIX is required for a specialized type of autophagy that targets mitochondria for elimination (mitophagy). Similarly, BNIP3 regulates mitophagy in response to hypoxia. In this review, we will discuss possible mechanisms by which BNIP3 and NIX induce cell death and mitophagy. We will also consider the potential relationship between cell death pathways and autophagy in development and homeostasis.

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BNIP3 (BCL2 and adenovirus E1B 19-kDa-interacting protein 3) and BNIP3-like (BNIP3L), also known as NIX, are proteins with homology to BCL2 in the BH3 domain, which induce both cell death and autophagy. Although other proteins may induce cell death or autophagy more efficiently than BNIP3 or NIX, the ability of these proteins to do both makes them useful reagents to explore the relationship between these alternate cell fates. With this in mind, here we review the role of BNIP3 and NIX in cell death and autophagy, and discuss potential mechanisms by which they may function. In addition, we discuss a specialized type of autophagy that is relevant during development, namely mitophagy, in the erythroid lineage.

Discovery and Characterization of BNIP3

BNIP1, BNIP2, and BNIP3, were initially identified in a yeast two-hybrid screen as BCL2 and adenovirus E1B 19-kDa-interacting proteins.¹ E1B 19 kDa protein protects cells from death after adenovirus infection,^{2,3} and mutants of this protein that are defective for binding to BNIP proteins are also defective for cell death suppression.¹ The antiapoptotic protein BCL2 can functionally replace E1B 19 kDa protein in this context,^{4,5} and also directly interacts with BNIP proteins.¹ Furthermore, as is the case for E1B 19 kDa protein, mutants of BCL2 that are defective for binding to BNIP proteins are also defective in cell death suppression. These findings suggest that BNIP1, BNIP2, and BNIP3 are proapoptotic proteins that

are suppressed by E1B 19 kDa protein or BCL2. BNIP1 and BNIP2 localize to the nuclear envelope and endoplasmic reticulum, whereas BNIP3 localizes to mitochondria when overexpressed.¹ Thus, BNIP3 is a proapoptotic protein that may function through a mitochondrial pathway.

BNIP3 has death-inducing activity in cell lines, although its maximum effect is delayed relative to other proapoptotic proteins.^{6–8} BNIP3 has a putative BH3 domain (Figure 1) and C-terminal transmembrane domain.⁷ The BNIP3 BH3 domain differs from the consensus BCL2 family sequence, (L/V/M)¹XXXG⁵D⁶(D/E)⁷F⁸E⁹R¹⁰, at two evolutionarily conserved residues, W⁷ and W¹¹ (review by Aouacheria *et al.*¹¹). This domain, substituted for the corresponding sequence of BAX, confers proapoptotic activity, and the ability to heterodimerize with BCL-X_L.⁷ Furthermore, in stably transfected baby rat kidney cells undergoing p53-dependent cell death, the BNIP3 BH3 domain is required to reverse the antiapoptotic effect of BCL-X_L. On the other hand, mutation of the BNIP3 BH3 domain is associated with minimal loss of proapoptotic activity in transiently transfected MCF-7 breast carcinoma cells, and at least partial retention of the ability to bind BCL2 and BCL-X_L.^{7,8} By comparison, the C-terminal transmembrane domain of BNIP3 is essential for mitochondrial localization and proapoptotic activity.^{6–8} The BNIP3 transmembrane domain is required for interaction with BCL2, although either the transmembrane domain or the N-terminal 49 amino acids is sufficient for interaction with BCL-X_L. Interestingly, a cytochrome *b*₅ transmembrane domain substitution mutant of

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Abbreviations: Atg1, autophagy-related 1; BNIP3, BCL2 and adenovirus E1B 19 kDa-interacting protein 3; LC3, microtubule-associated protein 1 light chain 3; MPTP, mitochondrial permeability transition pore; NIX, NIP3-like protein X; ULK1, Unc51-like kinase

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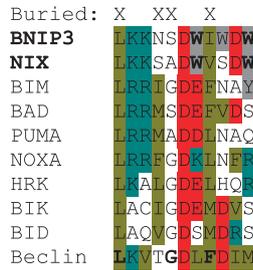


Figure 1 BH3 domain alignment. An alignment of the BH3 domains of human BNIP3, NIX, other BH3-only proteins, and Beclin-1 is shown. Hydrophobic nonpolar residues are highlighted in olive, hydrophobic polar residues in gray, negatively charged residues in red, and positively charged residues in teal. Conserved tryptophan residues in BNIP3 and NIX are shown in bold. BH3 residues that are partially or fully buried when bound to BCL-X_L are indicated by an 'X' at the top. Critical helical contact residues in Beclin-1 that disrupt the interaction with BCL-X_L (L, G),⁹ or BCL2 (F),¹⁰ when mutated are shown in bold. The first leucine residue in each row corresponds to position 1 in the review by Auouacheria *et al.*¹¹

BNIP3, targeted to the endoplasmic reticulum (ER), retains its ability to interact with BCL2 and BCL-X_L, and its proapoptotic activity.⁸ Thus, the BNIP3 transmembrane domain plays an important role in the interaction of BNIP3 with BCL2 or BCL-X_L, and in BNIP3-induced cell death, and the BNIP3 BH3 domain may mediate cell death in some conditions.

A putative BNIP3 homolog has been identified in *Caenorhabditis elegans* that is 21% identical with BNIP3 overall.^{12,13} Similar to mammalian BNIP3, ceBNIP3 induces death in cell lines, and interacts with CED-9 and CED-3. Also similar to BNIP3, the ceBNIP3 BH3 domain is not required for interaction with CED-9 or for induction of cell death, whereas the transmembrane domain is required for both.^{8,13} In addition, ceBNIP3-induced cell death is not prevented by caspase inhibitors. These features, and others that will be discussed, suggest that BNIP3 and ceBNIP3 cause an atypical form of cell death.

Discovery and Characterization of NIX

NIX was cloned from a human placenta cDNA library based on its homology (56% identical) to BNIP3.¹⁴ Stable NIX expression retards the growth of cancer cell lines, suggesting that NIX may be a tumor suppressor. Similar to BNIP3, NIX localizes to mitochondria, interacts with BCL2 and BCL-X_L, and is proapoptotic.¹⁵ Also similar to BNIP3, the C-terminal transmembrane domain of NIX, but not its BH3 domain, is essential for its proapoptotic activity. Furthermore, recombinant NIX protein induces loss of mitochondrial membrane potential ($\Delta\psi_m$) and cytochrome *c* release from isolated mitochondria *in vitro*. Ohi *et al.*¹⁶ identified NIX, while investigating adenovirus E1B 19-kDa-interacting proteins. They found that NIX localizes to the nuclear envelope, endoplasmic reticulum, and mitochondria, but in contrast to other investigators, did not find that NIX induced apoptosis. This lack of effect may be attributed to experimental factors, such as the use of an early time point after transfection, and a late indicator of cell death. By contrast, Chen *et al.*¹⁷ showed that NIX localizes to mitochondria, and is able to cause apoptosis when transiently expressed. Furthermore, they showed that BCL2 and BCL-X_L protect cells from NIX-induced

death, and that NIX is sensitive to proteasome-dependent degradation. Thus, NIX shares several key features with BNIP3, including an ability to interact with BCL2 and BCL-X_L, and to induce apoptosis through its C-terminal transmembrane domain; with regard to its proapoptotic activity, NIX appears to be similar to BNIP3.¹⁷

BNIP3, NIX, and Hypoxia-Induced Cell Death: Role in Cancer and Heart Disease

BNIP3 and NIX are implicated in hypoxia-induced tumor cell death. BNIP3 was identified in a subtractive hybridization screen in Chinese hamster ovary-K1 cells exposed to hypoxia, and hypoxia strongly induced BNIP3 mRNA.¹⁸ Furthermore, BNIP3 protein was induced by hypoxia in these cells, and the kinetics of induction correlated with cell death. The BNIP3 promoter has two HIF-1 α -binding sites, and the site at -234 relative to the translational start codon is required for transactivation by hypoxia and HIF-1 α . In another study, hypoxia induced BNIP3 expression in tumor cell lines, and BNIP3 was expressed in the perinecrotic areas of several epithelial cell carcinomas.¹⁹ In this study, BNIP3 was suppressed by Von Hippel-Lindau protein in a renal cell carcinoma cell line, consistent with its regulation through the HIF-1 α pathway. Hypoxia in tumors is a negative prognostic indicator; accordingly, deregulation of BNIP3 expression is associated with aggressive disease (reviewed by Burton and Gibson²⁰).

Regulation of NIX by hypoxia in tumor cell lines is also reported.^{18,19,21} In U2OS osteosarcoma cells, NIX is regulated transcriptionally and post-transcriptionally by hypoxia and p53.²¹ The transcriptional mechanism is postulated to involve HIF-1 α -dependent recruitment of CBP to the *Nix* gene, followed by recruitment of p53, whereas the post-transcriptional mechanism is not known. Experimental downregulation of NIX increases the growth of these cells in a tumor transplant model, suggesting that NIX may inhibit tumor growth in hypoxic conditions. In studies of human cancer, hypermethylation of the BNIP3 promoter was found in pancreatic cancer,²² and the *Nix* gene was infrequently mutated in a panel of primary breast and ovarian tumors.²³ Thus, BNIP3, and NIX are regulated by hypoxia in tumor cells, and their expression is associated with tumor cell death.

In heart muscle, there is evidence that BNIP3 and NIX have a role in pathological cell death. BNIP3 is induced by hypoxia in primary neonatal rat cardiomyocytes and in adult ventricular myocytes.²⁴⁻²⁶ Apart from the effect of hypoxia on BNIP3 expression, the combination of hypoxia and acidosis causes alkalai-resistant association of BNIP3 with mitochondria.²⁵ Hypoxia-induced cardiac myocyte cell death exhibits apoptotic features, such as DNA fragmentation and preservation of plasma membrane integrity,²⁵ however, the role of caspases is controversial. It is reported that BNIP3 expression causes cytochrome *c* release and myocyte death, which is blunted by caspase inhibitors,²⁶ but it is also reported that cytochrome *c* is not released, caspase-3 is inactive, and caspase inhibitors do not prevent cell death.²⁵ The role of caspases aside, it is generally agreed that BNIP3 causes mitochondrial depolarization in cardiac myocytes, which is blocked by mitochondrial permeability transition pore (MPTP) inhibitors. It is also reported that a C-terminal deletion mutant of BNIP3 lacking

its transmembrane domain functions as a dominant negative regulator of BNIP3-induced cell death.^{25,26} Notably, a role for BNIP3 in cardiac myocyte cell death has been confirmed *in vivo*; in mice subjected to ischemia-reperfusion, BNIP3 deficiency limits post-myocardial infarct ventricular remodeling by reducing peri-infarct apoptosis.²⁷ Conversely, high-level BNIP3 expression in the heart, driven by α -myosin heavy-chain regulatory sequences, significantly increases the frequency of apoptotic cells, and causes cardiomyopathy without ischemia.

NIX also has a role in heart disease, but with an important difference from BNIP3. NIX is regulated by cellular signals associated with cardiac hypertrophy, not hypoxia, in neonatal rat cardiac myocytes.²⁸ Specifically, NIX is regulated by $G_{\alpha q}$ signaling and protein kinase $C\alpha$ through an effect on Sp1 binding to the NIX promoter. As is the case for BNIP3, a role for NIX in cardiac myocyte apoptosis has been confirmed *in vivo*. High-level cardiac expression of NIX causes lethal perinatal cardiomyopathy, whereas expression of a naturally occurring C-terminally truncated variant of NIX, which is defective in mitochondrial localization, protects $G_{\alpha q}$ -overexpressing neonates from this condition.²⁹ In addition, NIX deficiency protects mice from $G_{\alpha q}$ -mediated and pressure-overload cardiomyopathy.³⁰ Thus, BNIP3 and NIX mediate parallel death pathways, which are regulated by cardiac ischemia and hypertrophy, respectively.

Mechanism of BNIP3- and NIX-induced Cell Death

The molecular mechanism through which BNIP3 and NIX induce cell death is not well understood. Vande Velde *et al.*³¹ first showed that BNIP3 mediates a non-apoptotic type of cell death. Specifically, they showed that overexpression of BNIP3 induces the death of 293T cells, in the absence of apaf-1, caspase-9, or caspase-3, and without cytochrome *c* release; cell death in this setting appears to be necrotic. Similar results were obtained for BNIP3-mediated cell death in cardiac myocytes, but only in the presence of acidosis and dependent on opening of the MPTP.²⁵ In isolated liver mitochondria, recombinant BNIP3 causes opening of the MPTP, and triggers cytochrome *c* release, through a transmembrane domain-dependent, BH3 domain-independent mechanism.³² Similar results were reported for NIX, but only at a 20-fold higher concentration of recombinant protein.¹⁵ Another group, using less NIX, failed to see an effect on the MPTP, but did observe cytochrome *c* release.³³ In murine embryonic fibroblasts, BAX or BAK is required for BNIP3-induced loss of $\Delta\psi_m$ and cytochrome *c* release.³⁴ In this study, inhibition of the MPTP was partially effective in preventing BNIP3-induced cell death, but did not prevent BAX or BAK activation.

Collectively, the data suggest that there is not a single mechanism through which BNIP3 and NIX induce cell death. Instead, there appears to be several, and the mechanism likely depends on the cell type and experimental conditions. One model, which fits most data is that upon activation BNIP3 inserts into the mitochondrial outer membrane, and causes opening of the MPTP, loss of $\Delta\psi_m$, generation of reactive oxygen species, and necrosis (Figure 2, left panel). BAX and BAK have a role in BNIP3-induced cell death,³⁴ and therefore

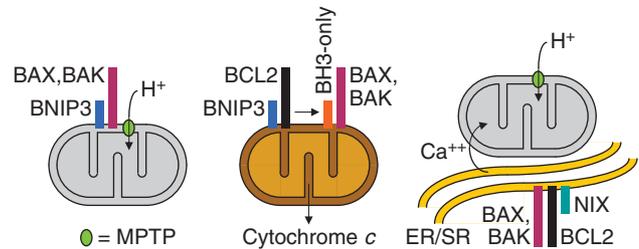


Figure 2 Potential mechanisms of BNIP3- and NIX-induced cell death. (Left panel) BNIP3 (blue box) or NIX (teal box), in concert with BAX or BAK (plum box), cause opening of the MPTP (lime oval), mitochondrial depolarization (represented as gray color), increased generation of reactive oxygen species, and cell death. (Center panel) BNIP3 or NIX bind BCL2 or BCL-X_L (black box), releasing BH3-only protein (orange box) to interact with BAX or BAK, causing cytochrome *c* release, caspase activation, and cell death. (Right panel) NIX binds to endoplasmic reticulum/sarcoplasmic reticulum (ER/SR), increasing ER/SR calcium (Ca⁺⁺) stores, causing mitochondrial calcium uptake, opening of the MPTP, and cell death

are included in this model; however, their contribution to opening of the MPTP by this mechanism, if any, is not well defined.

A second possibility is that BNIP3 and NIX function as typical BH3-only proteins (Figure 2, center panel); however, several lines of evidence suggest that this is not a major pathway of BNIP3- or NIX-induced cell death. First, compared with typical BH3-only proteins, BNIP3 and NIX are weak inducers of cell death.³⁵ Second, whereas typical BH3-only proteins trigger cell death through cytochrome *c* release, independent of the MPTP,³⁶ BNIP3- and NIX-induced cell death involves opening of the MPTP. Third, the proapoptotic activities of typical BH3-only proteins are highly dependent on their BH3 domains,³⁷ whereas the proapoptotic activities of BNIP3 and NIX are primarily determined by their transmembrane domains. Fourth, although typical BH3-only proteins cause apoptotic cell death, BNIP3- and NIX-induced cell death has necrotic features. We do not exclude the possibility that this mechanism may contribute to BNIP3- or NIX-induced cell death; however, it may be difficult to evaluate in the presence of the major MPTP-dependent pathway.

A recent study provides evidence for a third potential mechanism of BNIP3- or NIX-induced cell death (Figure 2, right panel).³⁸ BAX or BAK, and BCL2, have opposing effects on cell death, mediated at least in part through their effects on endoplasmic reticulum/sarcoplasmic reticulum (ER/SR) calcium stores, mitochondrial calcium uptake, and the MPTP.^{39–42} In this study, enforced expression of NIX was shown to increase, and loss of NIX was shown to decrease, ER/SR calcium stores in cardiac myocytes. Restoration of calcium stores by genetic ablation of an inhibitor of the ER/SR calcium-uptake pump reversed the beneficial effect of NIX deficiency on $G_{\alpha q}$ -mediated cardiomyopathy. Further, ER-targeted NIX caused mitochondrial depolarization and cell death. Whether this mechanism is relevant in non-cardiac cells remains to be determined.

Biophysical Properties of the BNIP3 Transmembrane Domain

BNIP3 and NIX could open the MPTP directly through an interaction with BAX or BAK, or a component of the MPTP,⁴³

or indirectly through increased mitochondrial calcium uptake. Alternatively, BNIP3 and NIX could form channels in the mitochondrial outer membrane, which activate the MPTP. BNIP3 and NIX undergo homodimerization in cells, which is resistant to denaturation by sodium dodecyl sulfate (SDS), or reducing conditions, and is mediated by their transmembrane domains.^{6,15} Given the importance of the transmembrane domains in BNIP3- and NIX-induced cell death, several studies have used biophysical methods to characterize this homotypic interaction. The BNIP3 transmembrane domain strongly self-associates in *Escherichia coli* membranes and SDS micelles.⁴⁴ Dimers form a right-handed parallel helix-helix structure with a continuous hydrophilic track that spans the lipid bilayer.^{45,46} Critical interfacial residues that mediate BNIP3 homodimerization are S¹⁷²H¹⁷³XXA¹⁷⁶XXL¹⁷⁹G¹⁸⁰XXI¹⁸³G¹⁸⁴ (numbers from human BNIP3). These residues are conserved between BNIP3 and NIX, include an AXXXGXXXG glycine zipper motif,⁴⁷ and participate in specific interactions, such as intermonomeric hydrogen bonds between polar residues at S172 and H173. It has been proposed that the residues S172 and H173 control an acid-sensitive proton channel in the mitochondrial outer membrane that can initiate cell death;⁴⁶ however, results with an H173A mutant of BNIP3 are inconsistent,^{48,49} and other dimerization-defective mutants of BNIP3 (L179S and G180E) fail to prevent BNIP3-induced cell death.⁸ It is possible that these mutants induce cell death, because they can still associate *in vivo*; regardless, additional studies are needed to establish a physiological role for BNIP3- or NIX-dependent channel formation.

Autophagy: An Alternate Cell Fate Determined by BNIP3 and NIX

Apart from the role of BNIP3 and NIX in cell death, an intriguing aspect of the biology of these proteins is their role in autophagy. Autophagy was initially described based on its ultrastructural features, in particular, double-membraned structures that surrounded cytoplasm and organelles in cells, known as autophagosomes.^{50,51} Autophagy is a catabolic program present in organisms as primitive as yeast, which is activated in response to starvation or changing nutrient conditions. Genetic studies in yeast have identified a number of proteins, and elucidated several biochemical pathways, that are essential for autophagy.⁵² One protein is the serine-threonine kinase, autophagy-related 1 (Atg1), which is required for preautophagosomal structure formation, and is negatively regulated by mTOR. Another protein is Atg6, which is a component of a class III PI3-K-containing complex. The mammalian homolog of Atg6, Beclin-1, has tumor suppressor activity.^{53,54} Growth of the preautophagosomal structure depends on the activity of two parallel ubiquitin-like conjugation pathways, which are both regulated by an E1-like enzyme Atg7. Apart from its role in the maintenance of cellular homeostasis, autophagy is required for differentiation in lower eukaryotes,⁵⁵ and there is a growing appreciation of its role in mammalian development.⁵⁶

Daido *et al.*⁵⁷ first described a role for BNIP3 in autophagy in the ceramide-induced autophagic death of malignant glioma cells. In these cells, ceramide induces an increase in BNIP3

expression, and BNIP3 causes mitochondrial depolarization and autophagy. This study was followed by another that showed a role for BNIP3 in arsenic trioxide-induced autophagic cell death.⁵⁸ In an ischemia-reperfusion model, BNIP3 induced autophagy, which protected HL-1 myocytes from cell death.⁵⁹ Similarly, hypoxia-induced BNIP3 increased autophagic death, but protected Saos2 cells from necrosis-type cell death.⁶⁰ Finally, in muscle wasting disorders, where autophagy is implicated in the pathogenesis, BNIP3 and NIX are upregulated, and the expression of either in skeletal muscle induces autophagosome formation.⁶¹ Thus, BNIP3 and NIX exhibit a dual nature; they induce cell death, and they also participate in the induction of autophagy. Induction of autophagy by BNIP3 or NIX has a protective effect in some settings, whereas in others it is associated with autophagic cell death. With regard to the latter, it is unclear if death is due to excess autophagy or to an independent death-inducing function of BNIP3 or NIX.

Role of NIX in Erythroid Development

NIX mRNA is expressed in many tissues at a fairly constant level,²⁸ however, along with the mammalian homologs of Atg1 and Atg8 (ULK1 and LC3), NIX is strongly upregulated during terminal erythroid differentiation.⁶²⁻⁶⁴ In contrast to the regulation of BNIP3 by hypoxia, or NIX by G α q-dependent signaling, the *cis*-acting sequences responsible for upregulation of NIX during erythroid differentiation have not been identified, nor has direct regulation of NIX by erythroid transcription factors been demonstrated. Possibly, NIX is induced by the master regulator of erythroid differentiation, GATA1. Another possibility is the AKT-regulated transcription factor FoxO3. FoxO3 activity is increased by starvation in muscle cells, binds LC3, *Bnip3*, and *Nix*, increases their expression, and induces autophagy.⁶¹ FoxO3 expression, nuclear localization, and transcriptional activity are induced during erythroid differentiation.^{65,66} Thus, an intriguing albeit untested model is that NIX and LC3 expression are induced by FoxO3.

Consistent with the erythroid-specific pattern of NIX expression, NIX-deficient mice exhibit defects in erythroid development. *Nix*^{-/-} mice have mild-to-moderate anemia, reticulocytosis, and an increase in splenic erythropoiesis.^{33,63,67} NIX and BCL-X_L are coordinately upregulated during erythroid differentiation,^{62,63} and it is suggested that the pro- and antiapoptotic balance between these proteins regulates erythrocyte production.³³ In general agreement with the notion that NIX deficiency confers a survival benefit, *Nix*^{-/-} erythroid progenitors show improved survival *in vitro* following erythropoietin deprivation or treatment with a calcium ionophore. On the other hand, the inability of NIX deficiency to rescue BCL-X_L deficiency (unpublished results), suggests that there is a proapoptotic factor besides NIX that causes cell death in the absence of BCL-X_L. Parenthetically, the rescue of BCL-X_L deficiency by combined deficiency of BAX and BAK,⁶³ indicates that this factor functions through the intrinsic apoptotic pathway.

Further analysis of NIX-deficient mice showed a defect in erythroid development that is not directly related to the regulation of death pathways. *Nix*^{-/-} reticulocytes have a

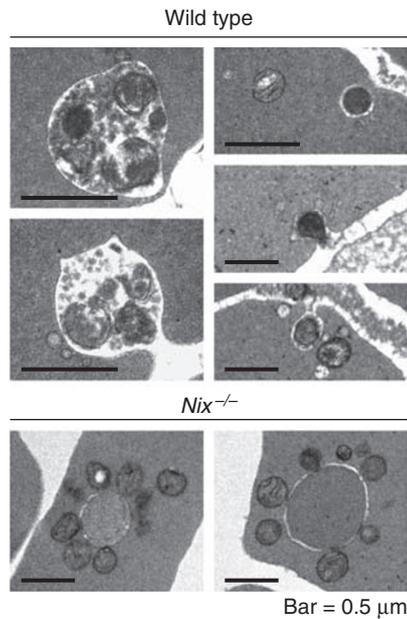


Figure 3 NIX is required for mitophagy in reticulocytes. (Top panel set) Mitochondria in wild-type reticulocytes undergoing exocytosis. Mitochondria in mature autophagic vacuoles (left two panels). Elimination of individual mitochondria (right three panels). (Bottom panel set) Mitochondria in *Nix*^{-/-} reticulocytes accumulate on the cytoplasmic face of autophagosomes, but are not eliminated. A 0.5 μm bar is shown for scale in all panels. Magnification is × 25 000 in all panels, except in the upper panel set the left two panels are × 75 000, and the upper right panel is × 50 000

defect in mitochondrial clearance (Figure 3).^{63,67} Nascent reticulocytes generated by the enucleation of late orthochromatic erythroblasts undergo maturation in the bone marrow and circulation. During this process, they lose surface area and volume,⁶⁸ undergo remodeling of the plasma membrane and cytoskeleton,⁶⁹ and eliminate all ribosomes and membrane-bound organelles, such as mitochondria.^{70,71} In *Nix*^{-/-} reticulocytes, these processes proceed normally with the exception of mitochondrial clearance. Ultrastructural studies show that mitochondria are cleared from reticulocytes through an autophagy-related process with the major difference that the contents of mature autophagic vacuoles are not recycled but are eliminated by exocytosis.^{70,72} Similar to wild-type reticulocytes undergoing maturation, autophagy is induced in *Nix*^{-/-} reticulocytes; however, the mitochondria are not eliminated. This failure to eliminate mitochondria indicates that they are not properly targeted to autophagosomes in the absence of NIX. There is also evidence that BNIP3 is required for mitophagy in response to hypoxia, suggesting a broader role for these proteins in mitochondrial elimination.⁷³ In the next section, we will consider three models for the mechanism of NIX-dependent mitophagy.

Mechanism of NIX-Dependent Mitophagy

The first and simplest model of NIX-dependent mitophagy we consider is that NIX triggers mitochondrial depolarization, which in turn causes mitochondrial autophagy and clearance (Figure 4, top row, left). This model is simplest in the sense

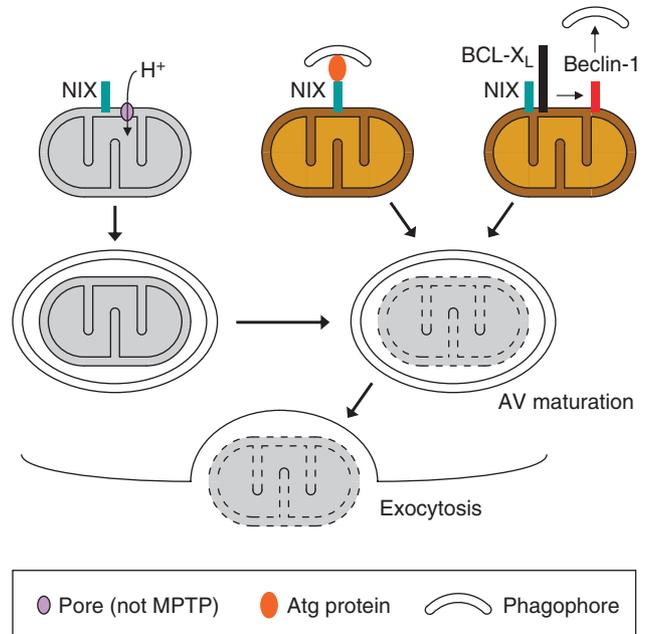


Figure 4 Potential mechanisms of NIX-dependent mitophagy in reticulocytes. (Top row, left panel) NIX (teal box) opens non-MPTP pore (lavender oval), causing mitochondrial depolarization (represented as gray color) and inducing mitophagy. (Top row, center panel) NIX recruits autophagy protein (orange oval), which supports formation of phagophore. (Top row, right panel) NIX binds BCL-X_L (black box), BCL-X_L releases Beclin-1 (red box), which activates autophagy and supports formation of phagophore. (Middle row) Completed autophagosome matures into autophagic vacuole (AV), and mitochondria is degraded. Depending on the mechanism, mitochondrial depolarization either occurs before this step or at this step. (Bottom row) A degraded mitochondria is eliminated by exocytosis. A legend is shown at the bottom

that there is already considerable evidence, reviewed herein, that BNIP3 and NIX can trigger mitochondrial depolarization, and that mitochondrial depolarization is sufficient to cause mitophagy.^{74,75} In support of this model, Sandoval *et al.*⁶⁷ show that reticulocyte mitochondria depolarize in culture over a period of 16 h, and that the mitochondrial clearance defect caused by NIX deficiency can be rescued by compounds that cause mitochondrial depolarization. However, one caveat in interpreting these results is the lack of evidence that NIX directly triggers mitochondrial depolarization in reticulocytes. As illustrated in Figure 4 (second row, right), it is equally plausible that NIX-dependent mitochondrial depolarization occurs after autophagosome formation,⁷⁶ but before elimination.⁷⁷ Studies of mitochondrial clearance in autophagy-defective reticulocytes should help resolve this question.

Another problem with the idea that NIX first triggers mitochondrial depolarization is that mitophagy in reticulocytes occurs independent of BAX and BAK.⁶³ In this regard, ABT-737, which rescues the NIX defect,⁶⁷ requires BAX or BAK to induce mitochondrial depolarization (unpublished results) and cell death;⁷⁸ therefore, its mechanism of action is likely different from NIX. Arguably, NIX may still cause mitochondrial depolarization through a BAX- or BAK-independent pathway; however, this remains speculative, primarily as mitochondrial clearance also occurs independent of the

MPTP.⁶³ This independence from BAX, BAK, and the MPTP suggests that the mechanism of NIX-dependent mitophagy is distinct from any of the proposed mechanisms of BNIP3- or NIX-induced cell death (Figure 2).

A second model of NIX-dependent mitophagy assumes a novel function of NIX, namely an ability to recruit autophagy components, independent of an ability to trigger mitochondrial depolarization (Figure 4, top row, center). As discussed earlier, enforced expression of BNIP3 or NIX induces autophagy in different conditions,^{57–61} which suggests that the ability to induce autophagy may be an intrinsic property of these proteins. Importantly, BNIP3 and NIX are not homologs of any of the canonical autophagy proteins identified in yeast,⁵² and NIX deficiency does not generally impair autophagy in erythroid cells, but instead interferes with the targeting of mitochondria to autophagosomes.^{63,67} A simple model consistent with these findings is that NIX functions as an adaptor protein and recruits components of the autophagy machinery to mitochondria. Consistent with this suggestion, preliminary studies indicate that NIX directly interacts with LC3 (I Dikic, personal communication).

A third model of NIX-dependent mitophagy is derived from a recently proposed model of crosstalk between autophagy and cell death pathways. Both BNIP3 and Beclin-1 were initially identified as BCL2- or BCL-X_L-interacting proteins.^{1,79} Beclin-1 is part of a class III PI3-K-containing complex that regulates autophagy induction,⁵² and BCL2 inhibits autophagy by binding to Beclin-1.¹⁰ Structural and mutational studies show that this interaction is mediated through a BH3-like domain in Beclin-1 (Figure 1).^{9,80} BH3-only proteins or mimetics can induce autophagy by competing with Beclin-1 for binding to BCL2 or BCL-X_L.⁸¹ Thus, it is possible that BNIP3 or NIX compete with Beclin-1 for binding to BCL-X_L, and that increased NIX expression during erythroid differentiation disrupts pre-existing BCL-X_L-Beclin-1 complexes, thereby releasing Beclin-1 and activating autophagy (Figure 4, top row, right). However, there are two caveats with this model. First, as already noted, autophagy is not generally impaired in NIX-deficient reticulocytes, suggesting that the core autophagy machinery is intact, and that there are other routes of autophagy activation in erythroid cells. In this regard, NIX may only be required to activate autophagy near the mitochondrial outer membrane, where it is located. Second, NIX must exhibit specificity for BCL-X_L-Beclin-1 complexes. Otherwise, if NIX were to disrupt BCL-X_L interactions with typical BH3-only proteins, it could lead to cell death. The same is true for Beclin-1, and at least for Beclin-1 there is a preliminary report that its overexpression does not induce cell death.⁸²

Conclusions and Perspective

BNIP3 and NIX share certain features with the BH3-only protein subgroup of the BCL2 family, such as sequence homology in the BH3 domain, residence in the mitochondrial outer membrane, and the ability to interact with BCL2 and BCL-X_L. Consequently, initial studies of these proteins focused on their role in cell death. Intriguingly, these studies found that BNIP3 and NIX do not function similar to typical BH3-only proteins. BNIP3 and NIX are weak inducers of cell death that cause loss of mitochondrial $\Delta\psi_m$, opening of the

MPTP, and variably release of cytochrome *c*. In addition, the transmembrane domains, but not the BH3 domains, of BNIP3 and NIX, play a major role in the induction of cell death. Subsequent studies showed that BNIP3 and NIX induce an atypical form of cell death, and that they also induce autophagy.

A central question is whether the ability of BNIP3 and NIX to induce cell death and autophagy are related or independent functions. As discussed in an earlier section, they may be mechanistically related; namely, the death-inducing function of BNIP3 and NIX, which is mediated through mitochondrial depolarization, may also serve as the event that initiates autophagy. Alternatively, they may be mechanistically independent, but functionally related. In that case, mitochondrial depolarization and autophagy-inducing functions may localize to different domains of BNIP3 and NIX. Finally, they may be completely independent functions. How might mitochondrial depolarization and autophagy be functionally related? In some respects, they are opposing functions. Mitochondrial depolarization eventually leads to compromise of the mitochondrial outer membrane, and this leads to the release or activation of destructive enzymes. Autophagy-generated membranes serve to compartmentalize these enzymes, and perform a protective function. Together, these properties may allow for limited subcellular destruction, which in turn may be important in cellular remodeling, homeostasis, and development. However, if the destructive process were to exceed autophagy-dependent containment, cell death would result.

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