ANTIOXIDANTS & REDOX SIGNALING
Volume 14, Number 10, 2011

DOI: 10.1089/ars.2010.3772

FORUM REVIEW ARTICLE

Mechanisms and Biology of B-Cell Leukemia/Lymphoma 2/Adenovirus E1B Interacting Protein 3 and Nip-Like Protein X

Ji Zhang and Paul A. Ney

Abstract

B-cell leukemia/lymphoma 2 (BCL-2)/adenovirus E1B interacting protein 3 (BNIP3) and Nip-like protein X (NIX) are atypical BCL-2 homology domain 3-only proteins involved in cell death, autophagy, and programmed mitochondrial clearance. BNIP3 and NIX cause cell death by targeting mitochondria, directly through BCL-2-associated X protein- or BCL-2-antagonist/killer-dependent mechanisms, or indirectly through an effect on calcium stores in the endoplasmic reticulum. BNIP3 and NIX also induce autophagy through an effect on mitochondrial reactive oxygen species production, or by releasing Beclin 1 from inhibitory interactions with antiapoptotic BCL-2 family proteins. BNIP3 downregulates mitochondrial mass in hypoxic cells, whereas NIX is required for mitochondrial elimination during erythroid development. BNIP3 and NIX have an emerging role in human health. Cell death mediated by BNIP3 and NIX is implicated in heart disease and ischemic injury. Cancer progression is linked to loss of the prodeath function of BNIP3, but also to induction of its prosurvival activity. Finally, BNIP3 and NIX are implicated in mitochondrial quality control, which is important in aging and degenerative disease. Elucidation of the mechanisms by which BNIP3 and NIX regulate cell death, autophagy, and mitochondrial clearance may lead to treatments for these conditions. *Antioxid. Redox Signal.* 14, 1959–1969.

Introduction

Viruses are adept at manipulating a broad range of host cellular processes. Along this line, the study of viruses and virally encoded proteins has yielded insights into the regulation of cell death. Adenovirus E1B 19 kDa protein (E1B-19K) suppresses the death of adenovirus-infected cells, allowing an increase in virus production. E1B-19K is a viral B-cell leukemia/lymphoma 2 (BCL-2) homolog that binds exposed BCL-2 homology domain 3 (BH3) domains, such as those of BCL-2-associated X protein (BAX), BCL-2-antagonist/killer (BAK), and BCL-2-interacting killer, and inhibits their activity [reviewed by Cuconati and White (16)]. In addition to BAX, BAK, BCL-2-interacting killer, lamin A, and lamin C, three novel E1B-19K-interacting proteins (BNIP 1–3) were identified by yeast two-hybrid screen (8). The ability of these proteins to bind mutants of E1B-19K was found to correlate with the ability of these mutants to suppress the death of adenovirus-infected cells, suggesting that they regulate cell death pathways. Of these, BNIP1 is a target membrane-associated soluble NSF attachment protein receptor protein, involved in membrane trafficking (71); BNIP2 is a Rho-GAP-related GTPase (60); and BNIP3 is an atypical BCL-2 family protein, located in the mitochondrial outer membrane (14, 86, 106). Here we review recent advances in our understanding of BNIP3, and the closely related protein BNIP3L (Nip-like protein X [NIX]).

BNIP3- and NIX-Dependent Cell Death

Role of BNIP3 and NIX domains in cell death

BNIP3 has a carboxy-terminal transmembrane domain that directs BNIP3 to mitochondria (Fig. 1) (8, 14, 106). E1B-19K lacks a transmembrane domain, and localizes to the nuclear envelope-endoplasmic reticulum (ER) region. Coexpression of BNIP3 and E1B-19K directs BNIP3 to the nuclear envelope-ER, which may play a role in E1B-19K-mediated repression of BNIP3 activity. BNIP3 also has the motif $L^{1}KKNSD^{6}W^{7}IWDW^{11}$ (106), which is related to the BH3 domain of the BCL-2 family. The hydrophobic residue at position 1 and aspartic acid at position 6 are highly conserved in other BH3 domains. Further, computational threading programs align the BH3 domain of BNIP3 with that of BH3 interacting death domain agonist (3). On the other hand, this motif has tryptophan residues at positions 7 and 11, which is not typical of BH3 domains (3), and it is not evolutionarily

Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee.

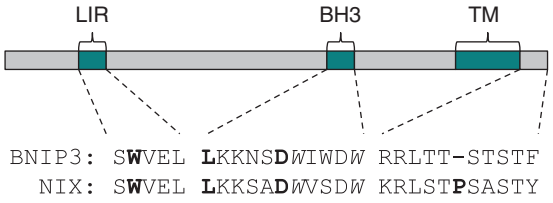

FIG. 1. Domains of B-cell leukemia/lymphoma 2 (BCL-2)/adenovirus E1B interacting protein 3 (BNIP3) and Nip-like protein X (NIX). Known functional domains of BNIP3 and NIX are shown (*teal boxes*). The LC3-interaction region (LIR) contains a critical tryptophan residue (bold), and is identical between BNIP3 and NIX. An atypical BCL-2 homology domain 3 (BH3) contains highly conserved leucine and aspartic acid residues at positions 1 and 6 (bold), and nonconserved tryptophan residues at positions 7 and 11 (italic). The carboxy-terminal transmembrane domain (TM) is indicated. Also shown are amino acid residues at the extreme carboxy terminus, which reside in the mitochondrial intermembrane space. These residues have a role in BNIP3-induced mitochondrial depolarization (see Fig. 2 and text for details). NIX differs from BNIP3 by the insertion of a proline residue (bold) in this region. (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

conserved (105). Thus, BNIP3 appears to be an atypical member of the BH3-only subfamily of BCL-2-related proteins. Different results have been obtained regarding interactions between the BNIP3 BH3 domain and antiapoptotic BCL-2-related proteins. Some have found that the BNIP3 BH3 domain is required for an interaction between BNIP3 and E1B-19K or BCL-2-like 1 (BCL-X_L) (106), whereas others have found that the BH3 domain is dispensable, and the interaction depends on the amino terminus of BNIP3 or its transmembrane domain (86). Regardless, if BNIP3 interacts with BCL-2 or BCL-X_L, it could have a proapoptotic effect. In this respect, initial studies showed that enforced expression of BNIP3 causes cell death, which is delayed compared with other proapoptotic BCL-2-related proteins (14, 86, 106). Later studies found subtle or absent death-promoting activity (6, 81, 98). Evidence suggests that some death-promoting activity is conveyed by the BNIP3 BH3 domain. First, it can substitute for the BH3 domain of BAX in apoptosis assays (106). Second, it is required to reverse the antiapoptotic effect of BCL-X_L in a p53-dependent model of cell death (106). On the other hand, deletion of the BNIP3 BH3 domain has a modest effect on death, and coexpression of BCL-2 and BNIP3 delays but does not prevent this outcome (14, 86, 106). Taken together, the evidence suggests that the BH3 domain of BNIP3 has a minor role in its death-promoting activity.

NIX is a BNIP3-related protein, identified by high-throughput cDNA sequencing (65), *in silico* approaches (13, 104), and a yeast two-hybrid screen employing E1B-19K as bait (as for BNIP3 itself) (78). Human NIX is 56% identical with human BNIP3, and 97% identical with murine NIX (13). Like BNIP3, NIX has a carboxy-terminal transmembrane domain, and localizes to mitochondria (13, 40, 104). Also, NIX causes cell death when overexpressed in most but not all cell lines (13, 40, 78, 104). Mutation of the NIX BH3 domain causes partial loss of its death-promoting activity (40). NIX interacts with E1B-19K, through its middle region (104), although more weakly than the interaction between BNIP3 and E1B-19K (78). There are reports that NIX can interact with BCL-2 and BCL-X_L (40, 104), but these are inconsistent (78), and coexpression of NIX with BCL-2 or BCL-X_L diminishes, but does not eliminate, its death-promoting activity (13, 40). In sum, NIX and BNIP3 have similar activities.

In contrast to the BH3 domain, the transmembrane domains of BNIP3 and NIX are essential for their death-promoting activity. Deletion of this domain causes the mislocalization of BNIP3 and NIX to the cytoplasm, and renders the proteins incompetent to induce cell death (14, 40, 86). This may be relevant *in vivo*, since a naturally occurring splice variant of NIX in the mouse heart, sNIX, which encodes a carboxy-terminal truncated protein, localizes to the cytoplasm and inhibits the proapoptotic activity of NIX (107). Importantly, the death-inducing activity of BNIP3 is preserved following the substitution of its transmembrane domain for that of BCL-2 or cytochrome b_5 (86). These sequences direct BNIP3 to the mitochondria or ER, respectively. Thus, either location is permissive for BNIP3-mediated cell death.

BNIP3 and NIX can trigger mitochondrial outer membrane permeabilization (MOMP), cytochrome *c* release, caspase activation, and cell death. However, they can also cause cell death by other means. Vande Velde *et al.* showed that BNIP3 causes cell death without cytochrome *c* release or caspase activation (100). In this study, expression of BNIP3 was associated with the mitochondrial permeability transition (MPT). The MPT is a calcium-activated state change associated with the passive diffusion of solutes <1500 daltons across the mitochondrial membranes, and leading to mitochondrial depolarization and swelling (37). The precise structure of the MPT pore is not known, but it includes cyclophilin D, and it is inhibited by cyclosporin A or bongkrekic acid. Consistent with activation of the MPT, BNIP3 causes an increase in mitochondrial membrane permeability and reactive oxygen species (ROS) generation, and mitochondrial depolarization, which are inhibited by cyclosporin A and bongkrekic acid (100). BNIP3-expressing cells also exhibit an increase in plasma membrane permeability and delayed DNA fragmentation, suggesting that they undergo necrotic cell death. In addition, ultrastructurally, there is evidence of excessive autophagy.

Role of BNIP3 and NIX in cardiomyocyte cell death

Study of primary cardiomyocytes exposed to hypoxia or mechanical stress has yielded additional insights into the mechanisms of BNIP3- and NIX-dependent cell death. Bruick showed that BNIP3 is induced by hypoxia in cell lines through a HIF response element in its proximal promoter (9). Of note, BNIP3 and NIX are implicated in cardiomyocyte cell death. In one study, BNIP3 was induced in cardiomyocytes by hypoxia or heart failure, and caused caspase-dependent cell death (87). In another, metabolic acidosis was required in addition to BNIP3, and was associated with BNIP3 integration into the mitochondrial outer membrane and caspase-independent cell death (51). In both cases, cell death was associated with the MPT, and was prevented by inhibitors of the MPT pore. NIX is also an important effector of cardiomyocyte cell death (15, 23). Mitochondria-directed NIX-ActA fusion protein causes BAX/BAK-dependent MOMP, and caspase-dependent cell death. ER-sarcoplasmic reticulum (SR)-directed NIX-cytochrome b_5 fusion protein causes cyclophilin D-dependent opening of the MPT pore, mitochondrial

depolarization, and caspase-independent cell death. Mitochondrial depolarization causes mitochondrial swelling, and disruption of the mitochondrial inner membrane; this in turn causes release of noncaspase cell death mediators, such as apoptosis-inducing factor and endonuclease G. A minor proportion of NIX is associated with the ER-SR fraction in cardiomyocytes under normal conditions; however, this increases following transverse aortic banding (23). Thus, NIX translocates to the ER-SR under stress, and causes cell death. As previously shown for BAX and BAK (77, 92), NIX increases ER-SR calcium stores and mitochondrial calcium uptake, which activates the MPT. In the absence of NIX, ER-SR calcium stores decrease, which has a protective effect on cardiac function. Restoration of ER-SR calcium stores reverses this effect, showing that ER-SR calcium stores and NIX-mediated cell death are causally linked.

Mechanisms of BNIP3- and NIX-dependent cell death

Several mechanisms are needed to account for all the death-inducing activities attributed to BNIP3 and NIX. First, BNIP3 and NIX may engage antiapoptotic BCL-2 family members located in the mitochondrial outer membrane to trigger BAX/BAK-dependent MOMP, and caspase-dependent cell death (Fig. 2A). BNIP3 and NIX are not direct activators of BAX and BAK (43); therefore, they must act through other BH3-only proteins. Second, BNIP3 and NIX may modulate ER-SR calcium stores, which activates the cyclophilin D-dependent MPT, and supports caspase-independent cell death (Fig. 2B).

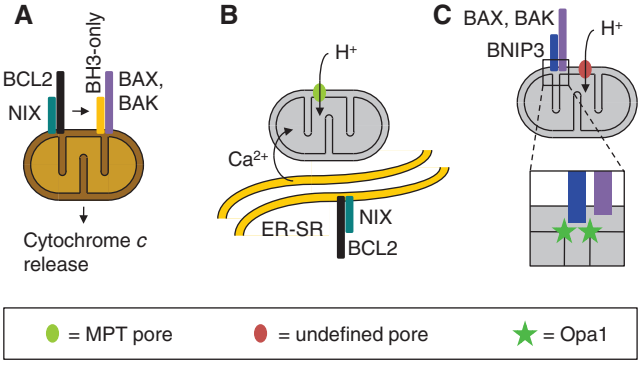

FIG. 2. Mechanisms of BNIP3- and NIX-induced cell death. (A) Proapoptotic BH3-only proteins are held in check by multidomain antiapoptotic proteins, such as BCL-2. NIX (or BNIP3) can potentially disrupt these complexes, releasing BH3-only proteins (*arrow*), and activating BCL-2-associated X protein (BAX) or BCL-2-antagonist/killer (BAK). BAX or BAK activation causes cytochrome *c* release and caspase-dependent cell death. **(B)** NIX (or BNIP3) and BCL-2 modulate endoplasmic reticulum-sarcoplasmic reticulum (ER-SR) calcium stores. Increased ER-SR calcium stores cause increased mitochondrial calcium uptake, which in turn activates the mitochondrial permeability transition (MPT). Activation of the MPT causes mitochondrial depolarization (shown in grey), and the eventual release of caspase-dependent and -independent mediators of cell death. **(C)** Physical interaction between the carboxy-terminal end of BNIP3 and Opa1 (*inset*) causes disruption of Opa1 complexes and mitochondrial inner membrane remodeling. This process, which is dependent on BAX or BAK, causes opening of an undefined pore, and mitochondrial depolarization.

Specific features of BNIP3 or NIX-induced cell death depend on whether one or both mechanisms are activated, and on factors such as the cell type or the nature of the stress. However, this is not the complete picture, since BNIP3 may also function through a novel mitochondrial leak pathway (Fig. 2C). Recombinant BNIP3, added to isolated liver mitochondria, causes cytochrome *c* release, depolarization, and mitochondrial swelling (45). In contrast to the other two mechanisms, these effects require the carboxy-terminal sequences of BNIP3, located in the intermembrane space. Further, in an exception to the overall similarity between BNIP3 and NIX, recombinant NIX causes cytochrome *c* release, but does not elicit depolarization or mitochondrial swelling (21). Two recent studies suggest that the mechanism may involve a direct interaction between the BNIP3 carboxy terminus and the inner mitochondrial membrane-associated, dynamin-related protein Opa1 (Fig. 2C, inset) (56, 84). Opa1 complexes control the shape of mitochondrial cristae, and cytochrome *c* release during apoptosis (30). The interaction between BNIP3 and Opa1 disrupts Opa1 complexes, and this is sufficient to cause mitochondrial fragmentation, but not cell death, which additionally requires the BNIP3 BH3 domain, and BAX or BAK (56). The interaction with Opa1 causes remodeling and permeabilization of the mitochondrial inner membrane; however, in contrast to the classical MPT, these changes are BAX/BAK dependent and cyclophilin D independent (56, 84).

The transmembrane domain is required for mitochondrial localization of BNIP3 and NIX, but it may have an additional role in cell death mediated by these proteins. BNIP3 and NIX form SDS-resistant homodimers through their transmembrane domains (40, 96), which raises the possibility that they can form a pore in the mitochondrial outer membrane, leading to mitochondrial dysfunction. Along this line, the structure of BNIP3 transmembrane domain dimers in lipid micelles has been solved, and they form an acid-sensitive pore with a Ser-His node at its entrance (7, 97). However, NIX also forms SDS-resistant dimers, but does not activate the MPT in isolated mitochondria. Further, while some studies have found that dimerization-defective mutants of BNIP3 cannot induce cell death (52), others have found that they still can cause cell death (29, 86). Another possibility is that the transmembrane domain may mediate interactions with other proteins. Indeed, replacement of the BNIP3 transmembrane domain with that of BCL-2 or Omp25 disrupts the interaction between BNIP3 and Opa1 (56). The transmembrane domains of BNIP3 and NIX are highly related, but not identical, and their carboxy-terminal ends differ by the presence of a proline residue in NIX (Fig. 1). These differences may be important, independent of an effect on dimerization. Thus, BNIP3 and NIX potentially cause cell death by several distinct mechanisms. In future studies, it will be important to account for all mechanisms, and to include controls that allow their discrimination.

BNIP3- and NIX-Dependent Autophagy

Autophagy is a lysosomal degradative pathway, which is stimulated by starvation, and serves to maintain cellular homeostasis (103). Genetic experiments in yeast have led to identification of the core autophagy machinery (102). BNIP3 and NIX are not part of this core machinery; however, they are implicated in the induction of autophagy. As previously noted, BNIP3 expression is associated with opening of the

MPT pore, and ultrastructural changes consistent with autophagy (100). Further, BNIP3 expression is associated with the ceramide- and arsenic trioxide-induced autophagic death of malignant glioma cells (17, 42). Since these initial studies linking BNIP3 with autophagic cell death, considerable attention has been focused on the induction of BNIP3 by hypoxia, and the role of BNIP3-induced autophagy as a cell death or survival mechanism. In one study, of glioma and breast cancer cells exposed to hypoxia, autophagic cell death was mediated by BNIP3 (4). Along the same line, BNIP3 was shown to be derepressed in the absence of RB1, and to cause autophagic cell death in hypoxic fetal liver (98). In a study of HL-1 myocytes, BNIP3 induced autophagy; however, in this case mitochondria were targeted, and autophagy was protective against BNIP3-induced mitochondrial dysfunction (36). Finally, induction of autophagy by BNIP3 and NIX had a prosurvival effect in primary fibroblasts, and breast and prostate cancer cell lines exposed to hypoxia (6). Thus, BNIP3-induced autophagy is protective, but if unchecked can contribute to cell death.

The mechanism whereby BNIP3 and NIX induce autophagy is uncertain, although several ideas have been advanced. The first is that BNIP3 and NIX can cause mitochondrial depolarization and dysfunction (Fig. 3A). This increases production of ROS, which induce autophagy (20) [reviewed in (89)]. The second is that BNIP3 and NIX can disrupt repressive Beclin 1-BCL-2 (or BCL-X_L) complexes, liberating Beclin 1, and activating autophagy (Fig. 3B) (6, 61, 83). Consistent with this latter model, addition of peptides derived from the BH3 domains of BNIP3 and NIX to normoxic cells causes an increase in autophagy and disruption of these complexes (6). BNIP3 binds and inhibits Rheb, an upstream activator of mammalian target of rapamycin; thus, a third possibility is that BNIP3 and NIX activate autophagy by inhibiting mammalian target of rapamycin activity (58). All of these models require additional genetic testing *in vivo*. It should also be noted that hypoxia induces autophagy through AMPK, independent of BNIP3 and NIX (82), and that hypoxia is not the only pathway that causes induction of BNIP3 and autophagy. With regard to the latter point, the AKT-regulated transcription factor FoxO3 binds *Bnip3*, *Bnip3l*, and *LC3* in skeletal muscle, activates their expression, and causes induction of autophagy (62).

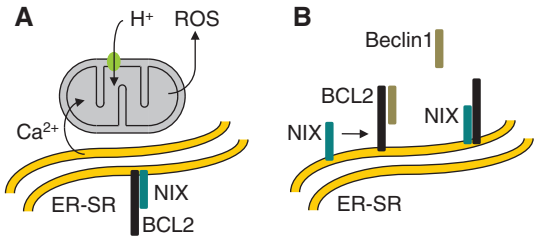

FIG. 3. Mechanisms of BNIP3- and NIX-induced autophagy. (A) Mitochondrial depolarization (shown in grey) causes mitochondrial dysfunction, and increased production of reactive oxygen species (ROS), which upregulates autophagy. This can be due to an indirect effect of NIX or BNIP3 on the MPT, as shown, or a direct effect of BNIP3 on mitochondria (as illustrated in Fig. 2). **(B)** The autophagy protein Beclin 1 is bound in an inactive form at the ER by multidomain antiapoptotic proteins. BH3-only proteins, such as NIX and BNIP3, can disrupt these complexes releasing beclin 1 (*arrow*), and upregulating autophagy.

BNIP3- and NIX-Dependent Mitophagy

Role of NIX in erythroid development

Apart from the propensity of BNIP3 and NIX to cause mitochondrial dysfunction, recent observations indicate that they also regulate the opposing process of mitochondrial clearance. Mitochondrial clearance is a type of quality control, which is thought to be important for cellular homeostasis in long-lived postmitotic cells, such as neurons and cardiomyocytes. Mitochondrial clearance may also be involved in the regulation of mitochondrial mass in response to stress. In this regard, BNIP3 is involved in decreasing the mitochondrial mass in hypoxic murine embryonic fibroblasts (108). Recently, insights into the mechanism of mitochondrial clearance have come from the study of erythroid cell differentiation. NIX is upregulated during erythroid development, in parallel with BCL-X_L (1, 91). In keeping with its proapoptotic role, erythroid cells that lack NIX are resistant to erythropoietin deprivation-induced death (21). In addition, they are developmentally abnormal. NIX-deficient mice are anemic as a consequence of defective reticulocyte maturation (88, 91). Reticulocytes are newly formed erythrocytes generated by the enucleation of late-stage erythroblasts in the bone marrow. Nascent reticulocytes undergo cellular remodeling, which includes elimination of the entire cohort of mitochondria (34, 50). In the absence of NIX, a substantial proportion (30%–50%) of circulating erythrocytes retain their mitochondria (88, 91). Further, NIX-deficient reticulocytes exhibit defective mitochondrial clearance *in vitro*. Thus, NIX has a central role in mitochondrial clearance in erythroid cells.

Role of NIX in mitochondrial clearance in reticulocytes

Ultrastructural examination of mature erythroblasts shows that mitochondria are cleared by an autophagy-related process (38). Considering the previous discussion, this raises the possibility that the role of NIX in mitochondrial clearance, in erythroid cells, is to activate autophagy. However, this does not appear to be the case, since autophagy is preserved in NIX-deficient reticulocytes, as demonstrated by ultrastructural and biochemical approaches (91). In cultured nascent reticulocytes from $Nix^{+/+}$ mice, mitochondria are surrounded by autophagy-like double membranes, which fuse with the plasma membrane, or with autophagosomes, resulting in mitochondrial elimination by exocytosis (Fig. 4). Autophagosome maturation in this setting appears to be mediated by fusion with endocytic vesicles (110). Mitochondria inside autophagosomes still have their outer mitochondrial membrane, suggesting that this does not undergo direct fusion with autophagosomal membranes. By contrast, in reticulocytes from $Nix^{-/-}$ mice, most mitochondria are located in the cytoplasm, or are in contact with the cytoplasmic face of autophagosomes. These observations suggest that the main role of NIX is to recruit membranes to mitochondria, and that this is required for subsequent fusion events (Fig. 5).

Two models of BNIP3- and NIX-dependent mitochondrial clearance

Current evidence supports two models of mitochondrial clearance, which are not mutually exclusive. The first is that mitochondrial depolarization causes mitochondrial clearance. Depolarization can be induced experimentally by proto-

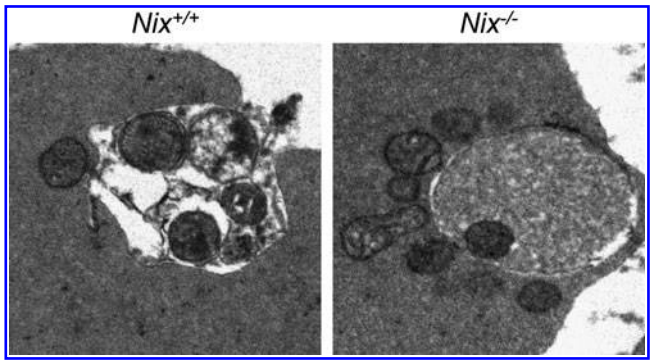

FIG. 4. NIX is required for mitochondrial clearance in reticulocytes. Ultrastructural appearance of mitochondrial elimination in newly formed reticulocytes in culture. In the presence of NIX ($Nix^{+/+}$ *panel*), mitochondria are directed to degradative vacuoles, and eliminated by exocytosis. In the absence of NIX ($Nix^{-/-}$ *panel*), most mitochondria are found in the cytoplasm or in contact with the cytoplasmic face of double-membraned autophagosomes.

nophores, like FCCP, and by photodamage, which activates the MPT (55). In both cases, depolarization leads to clearance (44, 72). This model fits well with the concept of mitochondrial autophagy as a quality-control mechanism, since depolarized mitochondria produce ROS, which are detrimental to cells. Supportive evidence for this model comes from the study of Parkinson's disease. Parkin and PINK1 are two proteins that are frequently mutated in autosomal recessive Parkinson's disease (19, 47). Studies show that mitochondrial depolarization stabilizes PINK1, which recruits the E3 ligase Parkin, and that Parkin ubiquitinates substrates, such as VDAC1, in the mitochondrial outer membrane (20, 32, 73). This leads to recruitment of the adaptor proteins p62 and HDAC6. p62 binds ubiquitin and the autophagy protein LC3 through its LC3-interaction region (LIR). This suggests a mechanism for recruitment of the autophagy machinery to mitochondria. However, the need for p62 is questionable, since it is required for mitochondrial clustering, but not clearance (74). HDAC6 binds ubiquitin and dynein motors, and mediates mitochondrial aggregation, which is required for efficient clearance (57). Of greater physiological relevance than chemically induced depolarization, starvation can also activate the MPT, and cause depolarization (12, 26). Interestingly, although activation of the MPT is required for starvation-induced mitochondrial clearance, it is not required for LC3-containing membrane recruitment to mitochondria (44). Mitochondrial fission also gives rise to depolarized mitochondria, which are eliminated by autophagy (99).

BNIP3 causes mitochondrial depolarization and clearance (5, 36, 85, 108). Similarly, it has been suggested that mitochondrial clearance in reticulocytes is caused by NIX-mediated depolarization (Fig. 6A) (88). In support of this idea, mitochondria depolarize before elimination (109), and mitochondrial clearance in NIX-deficient reticulocytes is rescued by FCCP or the BH3-mimetic ABT-737 (88). On the other hand, mitochondrial clearance in reticulocytes does not require BAX or BAK, nor activation of the MPT (91). Likewise, BNIP3-mediated mitochondrial clearance does not require the MPT (85). Further, NIX does not activate the MPT (except indirectly *via* the ER, but ER is not abundant in reticulocytes) (21). Finally, mitochondria in autophagy-defective reticulocytes exhibit impaired depolarization, indicating that depolarization is at least partially a consequence of autophagosome formation (110). Together, these findings suggest that mitochondrial clearance in reticulocytes is not primarily driven by NIX-dependent mitochondrial depolarization. The ability of protonophores and chemical inducers of MOMP to rescue mitochondrial clearance in NIX-deficient reticulocytes may reflect activation of a latent depolarization-driven pathway.

The second model is that adaptor proteins directly recruit components of the autophagy or vesicular trafficking ma-

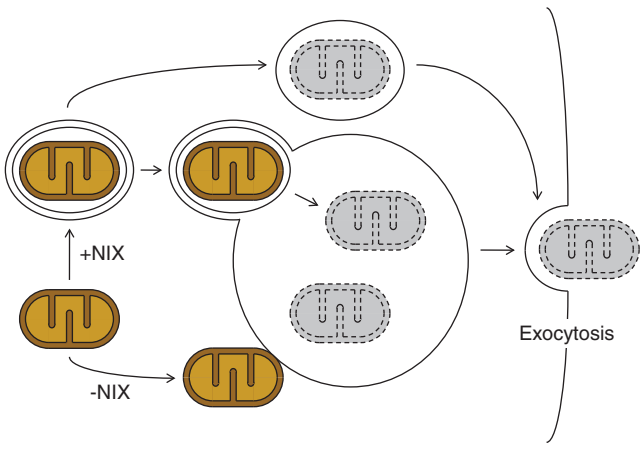

FIG. 5. Role of NIX in mitochondrial clearance in reticulocytes. In the presence of NIX, double membranes are recruited to mitochondria, which allows their subsequent fusion with autophagosomes, or the plasma membrane. In the absence of NIX, membranes are not recruited, and mitochondria are unable to fuse with these structures.

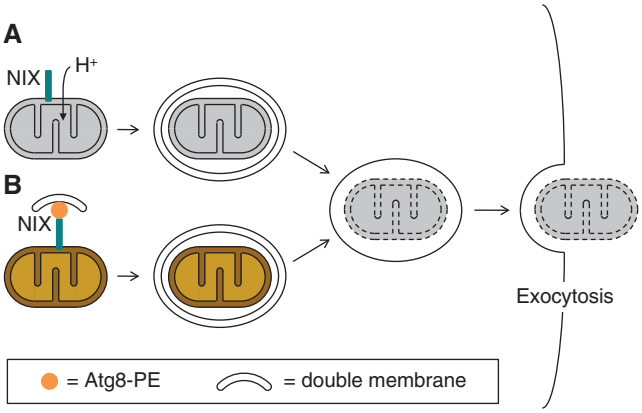

FIG. 6. Two models of NIX-dependent mitochondrial clearance in reticulocytes. (A) In the first model, NIX causes mitochondrial depolarization, which leads to membrane recruitment. A caveat with this model is evidence is lacking that NIX can directly act on mitochondria to cause depolarization. **(B)** In the second model, NIX functions as an adaptor for the recruitment of autophagy or membrane trafficking proteins, which recruit membranes. In both models, once mitochondria are completely surrounded by double membrane, they undergo degradation and elimination.

chinery to mitochondria (Fig. 6B). As discussed above for PINK1 and Parkin, depolarization can cause changes in the mitochondrial outer membrane that lead to the recruitment of adaptors and autophagy proteins; hence, the two models are not mutually exclusive. However, mitochondrial depolarization is not essential for autophagy membrane recruitment (44). Two recent studies showed that the amino terminus of NIX interacts with several different mammalian Atg8 homologs, and causes their recruitment to mitochondria (76, 90). Schwarten *et al.*, employing phage display, found that GABARAP interacts NIX. Similarly, Novak *et al.*, using a yeast two-hybrid screen, found that GABARAP-L1 and LC3 are NIX interacting proteins. Both groups found that the interaction is mediated through a "WVEL" LIR near the amino terminus of NIX (Fig. 1). The LIR is a common motif in adaptor proteins that link cargo (*e.g.*, ubiquitinated protein aggregates) to the autophagy machinery (46). It is also present in an adaptor, Atg32, which is involved in mitochondrial clearance in yeast (41, 80). Importantly, mutation of a critical tryptophan residue in this motif impairs mitochondrial clearance in reticulocytes (76). Further, colocalization of GABARAP-L1 and NIX with mitochondria in CCCP-treated murine embryonic fibroblasts depends on an intact LIR in NIX. Thus, in reticulocytes, NIX links mitochondria to the autophagy machinery, and this contributes to mitochondrial clearance.

Role of autophagy in mitochondrial clearance in reticulocytes

The ability of NIX to recruit Atg8 homologs to mitochondria raises the question of the role of autophagy in mitochondrial clearance. Two pathways that are essential for autophagy in yeast are the Atg12–Atg5 and Atg8–phosphatidylethanolamine (PE) ubiquitin-like conjugation pathways (39, 67). The products of these pathways, Atg12–Atg5 and Atg8–PE, are required for the expansion of autophagosomal membranes. To address the role of autophagy in mitochondrial clearance in mammals, several groups have studied mice in which these pathways have been disrupted. Initially, it was noted that erythrocytes in the circulation of $Atg5^{-/-}$ neonates did not contain mitochondria, suggesting that Atg5 was dispensable for mitochondrial clearance (64); however, the effect of Atg5 deficiency on autophagy in these cells was not examined. Subsequently, our laboratory generated $Atg7^{-/-}$ fetal liver transplanted mice (110). Atg7 is an E1 enzyme that is required for the activity of both ubiquitin-like conjugation pathways. Circulating erythrocytes in $Atg7^{-/-}$ transplant recipients did not exhibit retained mitochondria; nonetheless, mitochondrial clearance in Atg7-deficient reticulocytes was defective. Importantly, Atg12–Atg5 and Atg8–PE conjugates were not detected in $Atg7^{-/-}$ fetal liver cells, indicating that both ubiquitin-like conjugation pathways were inactive. A similar study of $Atg7^{fl/fl}$; Tg(*Vav-Cre*) mice, which delete Atg7 in the hematopoietic lineage, identified a moderate mitochondrial retention defect (15% of circulating CD71-negative mature erythrocytes) (68). For reasons that are not entirely clear, these mice develop severe, progressive anemia. Thus, mitochondrial clearance is partially impaired in the absence of Atg7, arguing that it is regulated by both autophagy-dependent and -independent mechanisms.

Atg1 is a central regulator of autophagy that functions as a switch between the cytoplasm-to-vacuole targeting and autophagy pathways in yeast. In mammals, there are two Atg1 homologs, Unc-51 like kinase 1 (ULK1) and ULK2. ULK1 is upregulated during erythroid differentiation, and is the major Atg1 homolog in these cells (53). To study the role of ULK1 in autophagy, Kundu *et al.* generated $Ulk1^{-/-}$ mice. ULK1-deficient erythrocytes exhibit abnormal mitochondrial retention. Interestingly, this defect is more severe than the one caused by the absence of Atg7 (but less severe than that caused by loss of NIX). This result is potentially explained by the recent discovery of an alternative autophagy pathway that is regulated by ULK1, and redundant with Atg5 and Atg7 (75).

Biological Role of BNIP3 and NIX *In Vivo*

The lifespan of certain postmitotic cells, such as those of the brain or heart, equals that of the whole organism, whereas the lifespan of mitochondria is measured in days. Clearance of effete and poorly functioning mitochondria is presumed to be critical for the maintenance of cellular homeostasis. Given that $Bnip3^{-/-}$ and $Nix^{-/-}$ mice are viable and exhibit specific phenotypes (21, 88, 91), it is reasonable to ask whether BNIP3 or NIX have a general role in this process. In this regard, the absence of a generalized defect in these mice may be explained by functional redundancy between these closely related proteins. Indeed, we have found that enforced expression of BNIP3 rescues mitochondrial clearance in NIX-deficient reticulocytes (unpublished results). Further, mice with combined deficiency of NIX and BNIP3 in the heart were recently described. The hearts in these mice exhibited increased mitochondria, loss of subcellular organization, and mitochondrial size heterogeneity and degeneration (25). This result is consistent with a general role for BNIP3 and NIX in mitochondrial quality control. The absence of a more severe defect may also be explained by the presence of other redundant mechanisms. An interesting speculation is that BNIP3 and NIX are part of a mechanism for the routine elimination of minimally damaged or dysfunctional mitochondria, whereas the ubiquitination machinery, involving E3 ligases such as Parkin, provides a second line of defense for the elimination of severely damaged, depolarized mitochondria.

BNIP3 is induced by hypoxia (9, 18, 95), and can cause cell death during ischemia (35, 51, 87). A series of elegant experiments from the Dorn laboratory has elucidated the role of BNIP3 and NIX in heart disease in mice. In these studies, enforced expression of BNIP3 caused cardiomyopathy in nonischemic mice, whereas BNIP3 deficiency limited post-infarction ventricular remodeling and improved cardiac performance (22). The basic helix-loop-helix transcription factor p8 negatively regulates FOXO3 activity, BNIP3, and basal autophagy. Consistent with an observed increase in BNIP3 expression, p8-deficient mice developed left ventricular dilation and posterior wall thinning (48). NIX is induced in the setting of hypoxia, by p53 and CBP (28); however, in cardiomyocytes NIX is primarily regulated by Gαq signaling, which is associated with cardiac hypertrophy (31). Enforced expression of NIX causes lethal perinatal cardiomyopathy in mice, whereas NIX deficiency protects mice from Gαq-mediated and pressure overload cardiomyopathy (24, 107). Collectively, these studies suggest that BNIP3 and NIX contribute to the pathophysiology of heart disease.

BNIP3 and NIX in Human Disease

There are reports of BNIP3 and NIX deregulation in cancer, including pancreas, prostate, colon, liver, breast, lung, brain, and blood (10, 11, 27, 33, 49, 69, 70, 79, 93, 94). Deregulation of BNIP3 is correlated with tumor grade, metastases, and poor prognosis. Methylation of the *Bnip3* promoter is associated with decreased BNIP3 expression (11, 69, 70, 79), and nuclear sequestration interferes with its proapoptotic activity (10). Not only are BNIP3 and NIX subject to epigenetic silencing, but also *Nix* is located in the 8p21 genomic region that exhibits frequent loss of heterozygosity in human cancer; however, except for a small series of hepatocellular carcinoma cases, there is little evidence for *Nix* mutation or deletion in cancer (54, 59, 101). Consistent with the notion that there is selective pressure against BNIP3 and NIX expression in cancer, deficiency of either protein promotes the growth of breast or osteosarcoma cell lines, respectively, in mice (28, 63). BNIP3 and NIX are also upregulated in the setting of ischemic injury, such as glaucoma, stroke, hemorrhagic shock, and myocardial infarction (2, 22, 66, 111, 112). Collectively, these studies make a case that BNIP3 and NIX are involved in disease in various clinical settings. In some situations it appears that they cause disease by promoting cell death, whereas in others they may be prosurvival.

Conclusions

That BNIP3 and NIX have many different activities makes them intriguing. They cause cell death, but weakly so or in a delayed manner. They induce autophagy and they are mediators of mitochondrial clearance. A unifying theme underlying these various activities is they all involve mitochondria. BNIP3 and NIX function upstream of mitochondria, by several different mechanisms, to cause mitochondrial dysfunction and the release of prodeath molecules. Mitochondria are also important intermediaries of BNIP3- and NIX-induced autophagy. Finally, mitochondria are targeted for elimination by BNIP3 and NIX during development and under stress. By comparison, classical BH3-only proteins are strong inducers of cell death, but do not have other activities. In this regard, study of BNIP3 and NIX may provide a more nuanced picture of the relationship between mitochondria and cells, which is an important question in cell biology. Our preliminary analyses of NIX suggest that some of these properties may be separable, and this area appears open to examination. A greater appreciation of the mechanisms by which BNIP3 and NIX operate may also yield unexpected insights, for example, insights into the process whereby cells eliminate intracellular pathogens. Indeed, although some of the activities of BNIP3 and NIX are modest, there is increasing evidence for their involvement in human disease. In the settings of ischemic injury and heart disease, expression of BNIP3 and NIX appears to be maladaptive, and contributes to an overall decline in function. The role of BNIP3 and NIX in cancer is more complex, as they may be prodeath or prosurvival, depending on the context. Still, BNIP3 and NIX are deregulated in a broad range of common cancers, suggesting that they have an impact upon cancer progression. In this review, we have discussed a variety of BNIP3- and NIX-dependent mechanisms. In the future, the challenge will be to fully elucidate these mechanisms, and to determine their role in human disease.

Acknowledgments

The authors acknowledge the support of the National Institutes of Health (R21 DK074519 to P.A.N.), and the American, Lebanese, and Syrian Associated Charities (ALSAC). We also acknowledge the technical support provided by Melanie Loyd and Mindy Randall. The authors also wish to thank their wives, Jie Jiang and Mary Braza, without whose support these studies would not have been possible.

References

1. Aerbajinai W, Giattina M, Lee YT, Raffeld M, and Miller JL. The proapoptotic factor Nix is coexpressed with Bcl-xL during terminal erythroid differentiation. *Blood* 102: 712–717, 2003.
2. Althaus J, Bernaudin M, Petit E, Toutain J, Touzani O, and Rami A. Expression of the gene encoding the pro-apoptotic BNIP3 protein and stimulation of hypoxia-inducible factor-1alpha (HIF-1alpha) protein following focal cerebral ischemia in rats. *Neurochem Int* 48: 687–695, 2006.
3. Aouacheria A, Brunet F, and Gouy M. Phylogenomics of life-or-death switches in multicellular animals: Bcl-2, BH3-Only, and BNip families of apoptotic regulators. *Mol Biol Evol* 22: 2395–2416, 2005.
4. Azad MB, Chen Y, Henson ES, Cizeau J, Millan-Ward E, Israels SJ, and Gibson SB. Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. *Autophagy* 4: 195–204, 2008.
5. Band M, Joel A, Hernandez A, and Avivi A. Hypoxia-induced BNIP3 expression and mitophagy: *in vivo* comparison of the rat and the hypoxia-tolerant mole rat, Spalax ehrenbergi. *FASEB J* 23: 2327–2335, 2009.
6. Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, and Mazure NM. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Mol Cell Biol* 29: 2570–2581, 2009.
7. Bocharov EV, Pustovalova YE, Pavlov KV, Volynsky PE, Goncharuk MV, Ermolyuk YS, Karpunin DV, Schulga AA, Kirpichnikov MP, Efremov RG, Maslennikov IV, and Arseniev AS. Unique dimeric structure of BNip3 transmembrane domain suggests membrane permeabilization as a cell death trigger. *J Biol Chem* 282: 16256–16266, 2007.
8. Boyd JM, Malstrom S, Subramanian T, Venkatesh LK, Schaeper U, Elangovan B, Sa-Eipper C, and Chinnadurai G. Adenovirus E1B 19 kDa and Bcl-2 proteins interact with a common set of cellular proteins. *Cell* 79: 341–351, 1994.
9. Bruick RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. *Proc Natl Acad Sci U S A* 97: 9082–9087, 2000.
10. Burton TR, Henson ES, Baijal P, Eisenstat DD, and Gibson SB. The pro-cell death Bcl-2 family member, BNIP3, is localized to the nucleus of human glial cells: implications for glioblastoma multiforme tumor cell survival under hypoxia. *Int J Cancer* 118: 1660–1669, 2006.
11. Calvisi DF, Ladu S, Gorden A, Farina M, Lee JS, Conner EA, Schroeder I, Factor VM, and Thorgeirsson SS. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. *J Clin Invest* 117: 2713–2722, 2007.
12. Carreira RS, Lee Y, Ghochani M, Gustafsson AB, and Gottlieb RA. Cyclophilin D is required for mitochondrial

removal by autophagy in cardiac cells. *Autophagy* 6: 462–472, 2010.
13. Chen G, Cizeau J, Vande VC, Park JH, Bozek G, Bolton J, Shi L, Dubik D, and Greenberg A. Nix and Nip3 form a subfamily of pro-apoptotic mitochondrial proteins. *J Biol Chem* 274: 7–10, 1999.
14. Chen G, Ray R, Dubik D, Shi L, Cizeau J, Bleackley RC, Saxena S, Gietz RD, and Greenberg AH. The E1B 19K/Bcl-2-binding protein Nip3 is a dimeric mitochondrial protein that activates apoptosis. *J Exp Med* 186: 1975–1983, 1997.
15. Chen Y, Lewis W, Diwan A, Cheng EH, Matkovich SJ, and Dorn GW. Dual autonomous mitochondrial cell death pathways are activated by Nix/BNip3L and induce cardiomyopathy. *Proc Natl Acad Sci U S A* 107: 9035–9042, 2010.
16. Cuconati A and White E. Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. *Genes Dev* 16: 2465–2478, 2002.
17. Daido S, Kanzawa T, Yamamoto A, Takeuchi H, Kondo Y, and Kondo S. Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. *Cancer Res* 64: 4286–4293, 2004.
18. Dayan F, Roux D, Brahimi-Horn MC, Pouyssegur J, and Mazure NM. The oxygen sensor factor-inhibiting hypoxia-inducible factor-1 controls expression of distinct genes through the bifunctional transcriptional character of hypoxia-inducible factor-1alpha. *Cancer Res* 66: 3688–3698, 2006.
19. Deas E, Plun-Favreau H, and Wood NW. PINK1 function in health and disease. *EMBO Mol Med* 1: 152–165, 2009.
20. Ding WX, Ni HM, Li M, Liao Y, Chen X, Stolz DB, Dorn GW, and Yin XM. Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming. *J Biol Chem* 285: 27879–27890, 2010.
21. Diwan A, Koesters AG, Odley AM, Pushkaran S, Baines CP, Spike BT, Daria D, Jegga AG, Geiger H, Aronow BJ, Molkentin JD, Macleod KF, Kalfa TA, and Dorn GW. Unrestrained erythroblast development in Nix-/- mice reveals a mechanism for apoptotic modulation of erythropoiesis. *Proc Natl Acad Sci U S A* 104: 6794–6799, 2007.
22. Diwan A, Krenz M, Syed FM, Wansapura J, Ren X, Koesters AG, Li H, Kirshenbaum LA, Hahn HS, Robbins J, Jones WK, and Dorn GW. Inhibition of ischemic cardiomyocyte apoptosis through targeted ablation of Bnip3 restrains postinfarction remodeling in mice. *J Clin Invest* 117: 2825–2833, 2007.
23. Diwan A, Matkovich SJ, Yuan Q, Zhao W, Yatani A, Brown JH, Molkentin JD, Kranias EG, and Dorn GW. Endoplasmic reticulum-mitochondria crosstalk in NIX-mediated murine cell death. *J Clin Invest* 119: 203–212, 2009.
24. Diwan A, Wansapura J, Syed FM, Matkovich SJ, Lorenz JN, and Dorn GW. Nix-mediated apoptosis links myocardial fibrosis, cardiac remodeling, and hypertrophy decompensation. *Circulation* 117: 396–404, 2008.
25. Dorn GW. Mitochondrial pruning by Nix and BNip3: an essential function for cardiac-expressed death factors. *J Cardiovasc Transl Res* 3: 374–383, 2010.
26. Elmore SP, Qian T, Grissom SF, and Lemasters JJ. The mitochondrial permeability transition initiates autophagy in rat hepatocytes. *FASEB J* 15: 2286–2287, 2001.
27. Erkan M, Kleeff J, Esposito I, Giese T, Ketterer K, Buchler MW, Giese NA, and Friess H. Loss of BNIP3 expression is a late event in pancreatic cancer contributing to chemoresistance and worsened prognosis. *Oncogene* 24: 4421–4432, 2005.
28. Fei P, Wang W, Kim SH, Wang S, Burns TF, Sax JK, Buzzai M, Dicker DT, McKenna WG, Bernhard EJ, and El-Deiry WS. Bnip3L is induced by p53 under hypoxia, and its knockdown promotes tumor growth. *Cancer Cell* 6: 597–609, 2004.
29. Frazier DP, Wilson A, Graham RM, Thompson JW, Bishopric NH, and Webster KA. Acidosis regulates the stability, hydrophobicity, and activity of the BH3-only protein Bnip3. *Antioxid Redox Signal* 8: 1625–1634, 2006.
30. Frezza C, Cipolat S, Martins de BO, Micaroni M, Beznoussenko GV, Rudka T, Bartoli D, Polishuck RS, Danial NN, De SB, and Scorrano L. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell* 126: 177–189, 2006.
31. Galvez AS, Brunskill EW, Marreez Y, Benner BJ, Regula KM, Kirschenbaum LA, and Dorn GW. Distinct pathways regulate proapoptotic Nix and BNip3 in cardiac stress. *J Biol Chem* 281: 1442–1448, 2006.
32. Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, and Springer W. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* 12: 119–131, 2010.
33. Giatromanolaki A, Koukourakis MI, Sowter HM, Sivridis E, Gibson S, Gatter KC and Harris AL. BNIP3 expression is linked with hypoxia-regulated protein expression and with poor prognosis in non-small cell lung cancer. *Clin Cancer Res* 10: 5566–5571, 2004.
34. Gronowicz G, Swift H, and Steck TL. Maturation of the reticulocyte *in vitro*. *J Cell Sci* 71: 177–197, 1984.
35. Guo K, Searfoss G, Krolikowski D, Pagnoni M, Franks C, Clark K, Yu KT, Jaye M, and Ivashchenko Y. Hypoxia induces the expression of the pro-apoptotic gene BNIP3. *Cell Death Differ* 8: 367–376, 2001.
36. Hamacher-Brady A, Brady NR, Logue SE, Sayen MR, Jinno M, Kirshenbaum LA, Gottlieb RA, and Gustafsson AB. Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. *Cell Death Differ* 14: 146–157, 2007.
37. Haworth RA and Hunter DR. The Ca2+-induced membrane transition in mitochondria. II. Nature of the Ca2+ trigger site. *Arch Biochem Biophys* 195: 460–467, 1979.
38. Heynen MJ, Tricot G, and Verwilghen RL. Autophagy of mitochondria in rat bone marrow erythroid cells. Relation to nuclear extrusion. *Cell Tissue Res* 239: 235–239, 1985.
39. Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi M, Noda T, and Ohsumi Y. A ubiquitin-like system mediates protein lipidation. *Nature* 408: 488–492, 2000.
40. Imazu T, Shimizu S, Tagami S, Matsushima M, Nakamura Y, Miki T, Okuyama A, and Tsujimoto Y. Bcl-2/E1B 19 kDa-interacting protein 3-like protein (Bnip3L) interacts with bcl-2/Bcl-xL and induces apoptosis by altering mitochondrial membrane permeability. *Oncogene* 18: 4523–4529, 1999.
41. Kanki T, Wang K, Cao Y, Baba M, and Klionsky DJ. Atg32 is a mitochondrial protein that confers selectivity during mitophagy. *Dev Cell* 17: 98–109, 2009.
42. Kanzawa T, Zhang L, Xiao L, Germano IM, Kondo Y, and Kondo S. Arsenic trioxide induces autophagic cell death in malignant glioma cells by upregulation of mitochondrial cell death protein BNIP3. *Oncogene* 24: 980–991, 2005.

43. Kim H, Rafiuddin-Shah M, Tu HC, Jeffers JR, Zambetti GP, Hsieh JJ, and Cheng EH. Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nat Cell Biol* 8: 1348–1358, 2006.
44. Kim I, Rodriguez-Enriquez S, and Lemasters JJ. Selective degradation of mitochondria by mitophagy. *Arch Biochem Biophys* 462: 245–253, 2007.
45. Kim JY, Cho JJ, Ha J, and Park JH. The carboxy terminal C-tail of BNip3 is crucial in induction of mitochondrial permeability transition in isolated mitochondria. *Arch Biochem Biophys* 398: 147–152, 2002.
46. Kirkin V, McEwan DG, Novak I, and Dikic I. A role for ubiquitin in selective autophagy. *Mol Cell* 34: 259–269, 2009.
47. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, and Shimizu N. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392: 605–608, 1998.
48. Kong DK, Georgescu SP, Cano C, Aronovitz MJ, Iovanna JL, Patten RD, Kyriakis JM, and Goruppi S. Deficiency of the transcriptional regulator p8 results in increased autophagy and apoptosis, and causes impaired heart function. *Mol Biol Cell* 21: 1335–1349, 2010.
49. Koop EA, van LT, van Wichen DF, de Weger RA, Wall E, and van Diest PJ. Expression of BNIP3 in invasive breast cancer: correlations with the hypoxic response and clinicopathological features. *BMC Cancer* 9: 175, 2009.
50. Koury MJ, Koury ST, Kopsombut P, and Bondurant MC. *In vitro* maturation of nascent reticulocytes to erythrocytes. *Blood* 105: 2168–2174, 2005.
51. Kubasiak LA, Hernandez OM, Bishopric NH, and Webster KA. Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3. *Proc Natl Acad Sci U S A* 99: 12825–12830, 2002.
52. Kubli DA, Quinsay MN, Huang C, Lee Y, and Gustafsson AB. Bnip3 functions as a mitochondrial sensor of oxidative stress during myocardial ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 295: H2025–H2031, 2008.
53. Kundu M, Lindsten T, Yang CY, Wu J, Zhao F, Zhang J, Selak MA, Ney PA, and Thompson CB. Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. *Blood* 112: 1493–1502, 2008.
54. Lai J, Flanagan J, Phillips WA, Chenevix-Trench G, and Arnold J. Analysis of the candidate 8p21 tumour suppressor, BNIP3L, in breast and ovarian cancer. *Br J Cancer* 88: 270–276, 2003.
55. Lam M, Oleinick NL, and Nieminen AL. Photodynamic therapy-induced apoptosis in epidermoid carcinoma cells. Reactive oxygen species and mitochondrial inner membrane permeabilization. *J Biol Chem* 276: 47379–47386, 2001.
56. Landes T, Emorine LJ, Courilleau D, Rojo M, Belenguer P and Arnaune-Pelloquin L. The BH3-only Bnip3 binds to the dynamin Opa1 to promote mitochondrial fragmentation and apoptosis by distinct mechanisms. *EMBO Rep* 11: 459–465, 2010.
57. Lee JY, Nagano Y, Taylor JP, Lim KL, and Yao TP. Disease-causing mutations in parkin impair mitochondrial ubiquitination, aggregation, and HDAC6-dependent mitophagy. *J Cell Biol* 189: 671–679, 2010.
58. Li Y, Wang Y, Kim E, Beemiller P, Wang CY, Swanson J, You M, and Guan KL. Bnip3 mediates the hypoxia-induced inhibition on mammalian target of rapamycin by interacting with Rheb. *J Biol Chem* 282: 35803–35813, 2007.
59. Liu W, Xie CC, Zhu Y, Li T, Sun J, Cheng Y, Ewing CM, Dalrymple S, Turner AR, Sun J, Isaacs JT, Chang BL, Zheng SL, Isaacs WB, and Xu J. Homozygous deletions and recurrent amplifications implicate new genes involved in prostate cancer. *Neoplasia* 10: 897–907, 2008.
60. Low BC, Seow KT, and Guy GR. Evidence for a novel Cdc42GAP domain at the carboxyl terminus of BNIP-2. *J Biol Chem* 275: 14415–14422, 2000.
61. Maiuri MC, Le TG, Criollo A, Rain JC, Gautier F, Juin P, Tasdemir E, Pierron G, Troulinaki K, Tavernarakis N, Hickman JA, Geneste O, and Kroemer G. Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. *EMBO J* 26: 2527–2539, 2007.
62. Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del PP, Burden SJ, Di LR, Sandri C, Zhao J, Goldberg AL, Schiaffino S, and Sandri M. FoxO3 controls autophagy in skeletal muscle *in vivo*. *Cell Metab* 6: 458–471, 2007.
63. Manka D, Spicer Z, and Millhorn DE. Bcl-2/adenovirus E1B 19 kDa interacting protein-3 knockdown enables growth of breast cancer metastases in the lung, liver, and bone. *Cancer Res* 65: 11689–11693, 2005.
64. Matsui M, Yamamoto A, Kuma A, Ohsumi Y, and Mizushima N. Organelle degradation during the lens and erythroid differentiation is independent of autophagy. *Biochem Biophys Res Commun* 339: 485–489, 2006.
65. Matsushima M, Fujiwara T, Takahashi E, Minaguchi T, Eguchi Y, Tsujimoto Y, Suzumori K, and Nakamura Y. Isolation, mapping, and functional analysis of a novel human cDNA (BNIP3L) encoding a protein homologous to human NIP3. *Genes Chromosomes Cancer* 21: 230–235, 1998.
66. Metukuri MR, Beer-Stolz D, Namas RA, Dhupar R, Torres A, Loughran PA, Jefferson BS, Tsung A, Billiar TR, Vodovotz Y, and Zamora R. Expression and subcellular localization of BNIP3 in hypoxic hepatocytes and liver stress. *Am J Physiol Gastrointest Liver Physiol* 296: G499–G509, 2009.
67. Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, Klionsky DJ, Ohsumi M, and Ohsumi Y. A protein conjugation system essential for autophagy. *Nature* 395: 395–398, 1998.
68. Mortensen M, Ferguson DJ, Edelmann M, Kessler B, Morten KJ, Komatsu M, and Simon AK. Loss of autophagy in erythroid cells leads to defective removal of mitochondria and severe anemia *in vivo*. *Proc Natl Acad Sci U S A* 107: 832–837, 2010.
69. Murai M, Toyota M, Satoh A, Suzuki H, Akino K, Mita H, Sasaki Y, Ishida T, Shen L, Garcia-Manero G, Issa JP, Hinoda Y, Tokino T, and Imai K. Aberrant DNA methylation associated with silencing BNIP3 gene expression in haematopoietic tumours. *Br J Cancer* 92: 1165–1172, 2005.
70. Murai M, Toyota M, Suzuki H, Satoh A, Sasaki Y, Akino K, Ueno M, Takahashi F, Kusano M, Mita H, Yanagihara K, Endo T, Hinoda Y, Tokino T, and Imai K. Aberrant methylation and silencing of the BNIP3 gene in colorectal and gastric cancer. *Clin Cancer Res* 11: 1021–1027, 2005.
71. Nakajima K, Hirose H, Taniguchi M, Kurashina H, Arasaki K, Nagahama M, Tani K, Yamamoto A, and Tagaya M. Involvement of BNIP1 in apoptosis and endoplasmic reticulum membrane fusion. *EMBO J* 23: 3216–3226, 2004.
72. Narendra D, Tanaka A, Suen DF, and Youle RJ. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol* 183: 795–803, 2008.
73. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, Cookson MR, and Youle RJ. PINK1 is selectively

stabilized on impaired mitochondria to activate Parkin. *PLoS Biol* 8: e1000298, 2010.
74. Narendra DP, Kane LA, Hauser DN, Fearnley IM, and Youle RJ. p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. *Autophagy* 6: 1090–1106, 2010.
75. Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T, Komatsu M, Otsu K, Tsujimoto Y, and Shimizu S. Discovery of Atg5/Atg7-independent alternative macroautophagy. *Nature* 461: 654–658, 2009.
76. Novak I, Kirkin V, McEwan DG, Zhang J, Wild P, Rozenknop A, Rogov V, Lohr F, Popovic D, Occhipinti A, Reichert AS, Terzic J, Dotsch V, Ney PA, and Dikic I. Nix is a selective autophagy receptor for mitochondrial clearance. *EMBO Rep* 11: 45–51, 2010.
77. Nutt LK, Pataer A, Pahler J, Fang B, Roth J, McConkey DJ, and Swisher SG. Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca2+ stores. *J Biol Chem* 277: 9219–9225, 2002.
78. Ohi N, Tokunaga A, Tsunoda H, Nakano K, Haraguchi K, Oda K, Motoyama N, and Nakajima T. A novel adenovirus E1B19K-binding protein B5 inhibits apoptosis induced by Nip3 by forming a heterodimer through the C-terminal hydrophobic region. *Cell Death Differ* 6: 314–325, 1999.
79. Okami J, Simeone DM, and Logsdon CD. Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. *Cancer Res* 64: 5338–5346, 2004.
80. Okamoto K, Kondo-Okamoto N, and Ohsumi Y. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. *Dev Cell* 17: 87–97, 2009.
81. Papandreou I, Krishna C, Kaper F, Cai D, Giaccia AJ, and Denko NC. Anoxia is necessary for tumor cell toxicity caused by a low-oxygen environment. *Cancer Res* 65: 3171–3178, 2005.
82. Papandreou I, Lim AL, Laderoute K, and Denko NC. Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. *Cell Death Differ* 15: 1572–1581, 2008.
83. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, and Levine B. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122: 927–939, 2005.
84. Quinsay MN, Lee Y, Rikka S, Sayen MR, Molkentin JD, Gottlieb RA, and Gustafsson AB. Bnip3 mediates permeabilization of mitochondria and release of cytochrome c via a novel mechanism. *J Mol Cell Cardiol* 48: 1146–1156, 2010.
85. Quinsay MN, Thomas RL, Lee Y, and Gustafsson AB. Bnip3-mediated mitochondrial autophagy is independent of the mitochondrial permeability transition pore. *Autophagy* 6: 17–24, 2010.
86. Ray R, Chen G, Vande VC, Cizeau J, Park JH, Reed JC, Gietz RD, and Greenberg AH. BNIP3 heterodimerizes with Bcl-2/Bcl-X(L) and induces cell death independent of a Bcl-2 homology 3 (BH3) domain at both mitochondrial and nonmitochondrial sites. *J Biol Chem* 275: 1439–1448, 2000.
87. Regula KM, Ens K, and Kirshenbaum LA. Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. *Circ Res* 91: 226–231, 2002.
88. Sandoval H, Thiagarajan P, Dasgupta SK, Schumacher A, Prchal JT, Chen M, and Wang J. Essential role for Nix in autophagic maturation of erythroid cells. *Nature* 2008.
89. Scherz-Shouval R and Elazar Z. Regulation of autophagy by ROS: physiology and pathology. *Trends Biochem Sci* 36: 30–38, 2010.
90. Schwarten M, Mohrluder J, Ma P, Stoldt M, Thielmann Y, Stangler T, Hersch N, Hoffmann B, Merkel R, and Willbold D. Nix directly binds to GABARAP: a possible crosstalk between apoptosis and autophagy. *Autophagy* 5: 690–698, 2009.
91. Schweers RL, Zhang J, Randall MS, Loyd MR, Li W, Dorsey FC, Kundu M, Opferman JT, Cleveland JL, Miller JL, and Ney PA. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. *Proc Natl Acad Sci U S A* 104: 19500–19505, 2007.
92. Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, and Korsmeyer SJ. BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. *Science* 300: 135–139, 2003.
93. Shaida N, Launchbury R, Boddy JL, Jones C, Campo L, Turley H, Kanga S, Banham AH, Malone PR, Harris AL, and Fox SB. Expression of BNIP3 correlates with hypoxia-inducible factor (HIF)-1alpha, HIF-2alpha and the androgen receptor in prostate cancer and is regulated directly by hypoxia but not androgens in cell lines. *Prostate* 68: 336–343, 2008.
94. Sowter HM, Ferguson M, Pym C, Watson P, Fox SB, Han C, and Harris AL. Expression of the cell death genes BNip3 and NIX in ductal carcinoma *in situ* of the breast; correlation of BNip3 levels with necrosis and grade. *J Pathol* 201: 573–580, 2003.
95. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, and Harris AL. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. *Cancer Res* 61: 6669–6673, 2001.
96. Sulistijo ES, Jaszewski TM, and MacKenzie KR. Sequence-specific dimerization of the transmembrane domain of the "BH3-only" protein BNIP3 in membranes and detergent. *J Biol Chem* 278: 51950–51956, 2003.
97. Sulistijo ES and MacKenzie KR. Sequence dependence of BNIP3 transmembrane domain dimerization implicates side-chain hydrogen bonding and a tandem GxxxG motif in specific helix-helix interactions. *J Mol Biol* 364: 974–990, 2006.
98. Tracy K, Dibling BC, Spike BT, Knabb JR, Schumacker P, and Macleod KF. BNIP3 is an RB/E2F target gene required for hypoxia-induced autophagy. *Mol Cell Biol* 27: 6229–6242, 2007.
99. Twig G, Elorza A, Molina AJ, Mohamed H, Wikstrom JD, Walzer G, Stiles L, Haigh SE, Katz S, Las G, Alroy J, Wu M, Py BF, Yuan J, Deeney JT, Corkey BE, and Shirihai OS. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *EMBO J* 27: 433–446, 2008.
100. Vande Velde C, Cizeau J, Dubik D, Alimonti J, Brown T, Israels S, Hakem R, and Greenberg AH. BNIP3 and genetic control of necrosis-like cell death through the mitochondrial permeability transition pore. *Mol Cell Biol* 20: 5454–5468, 2000.
101. Wang J, Ni H, Chen L, Liu YX, Chen CB, and Song WQ. Preparation and analysis of cSNP chip on hepatocellular carcinoma-related genes. *Hepatobiliary Pancreat Dis Int* 4: 398–402, 2005.
102. Xie Z and Klionsky DJ. Autophagosome formation: core machinery and adaptations. *Nat Cell Biol* 9: 1102–1109, 2007.
103. Yang Z and Klionsky DJ. Eaten alive: a history of macroautophagy. *Nat Cell Biol* 12: 814–822, 2010.
104. Yasuda M, Han JW, Dionne CA, Boyd JM, and Chinnadurai G. BNIP3alpha: a human homolog of mitochon-

drial proapoptotic protein BNIP3. *Cancer Res* 59: 533–537, 1999.
105. Yasuda M, Sa-Eipper C, Gong XL, and Chinnadurai G. Regulation of apoptosis by a *Caenorhabditis elegans* BNIP3 homolog. *Oncogene* 17: 2525–2530, 1998.
106. Yasuda M, Theodorakis P, Subramanian T, and Chinnadurai G. Adenovirus E1B-19K/BCL-2 interacting protein BNIP3 contains a BH3 domain and a mitochondrial targeting sequence. *J Biol Chem* 273: 12415–12421, 1998.
107. Yussman MG, Toyokawa T, Odley A, Lynch RA, Wu G, Colbert MC, Aronow BJ, Lorenz JN, and Dorn GW. Mitochondrial death protein Nix is induced in cardiac hypertrophy and triggers apoptotic cardiomyopathy. *Nat Med* 8: 725–730, 2002.
108. Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, Gonzalez FJ, and Semenza GL. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem* 283: 10892–10903, 2008.
109. Zhang J and Ney PA. NIX induces mitochondrial autophagy in reticulocytes. *Autophagy* 4: 354–356, 2008.
110. Zhang J, Randall MS, Loyd MR, Dorsey FC, Kundu M, Cleveland JL, and Ney PA. Mitochondrial clearance is regulated by Atg7-dependent and -independent mechanisms during reticulocyte maturation. *Blood* 114: 157–164, 2009.
111. Zhang Z, Yang X, Zhang S, Ma X, and Kong J. BNIP3 upregulation and EndoG translocation in delayed neuronal death in stroke and in hypoxia. *Stroke* 38: 1606–1613, 2007.
112. Zhu X, Zhou W, Cui Y, Zhu L, Li J, Feng X, Shao B, Qi H, Zheng J, Wang H, and Chen H. Pilocarpine protects cobalt chloride-induced apoptosis of RGC-5 cells: involvement of muscarinic receptors and HIF-1 alpha pathway. *Cell Mol Neurobiol* 30: 427–435, 2010.

Address correspondence to:
Prof. Paul A. Ney
Department of Biochemistry
St. Jude Children's Research Hospital
262 Danny Thomas Place
Memphis, TN 38105

E-mail: paul.ney@stjude.org

Date of first submission to ARS Central, November 9, 2010; date of acceptance, December 2, 2010.

Abbreviations Used

BAK = BCL-2-antagonist/killer
BAX = BCL-2-associated X protein
BCL-2 = B-cell leukemia/lymphoma 2
BCL-X_L = BCL-2-like 1
BH3 = BCL-2 homology domain 3
BNIP3 = BCL-2/adenovirus E1B interacting protein 3
E1B-19K = E1B 19kDa protein
ER = endoplasmic reticulum
LIR = LC3-interaction region
MOMP = mitochondrial outer membrane permeabilization
MPT = mitochondrial permeability transition
NIX = Nip-like protein X
PE = phosphatidylethanolamine
ROS = reactive oxygen species
SR = sarcoplasmic reticulum
ULK1 = Unc-51 like kinase 1

This article has been cited by:

1. Koji Okamoto, Noriko Kondo-Okamoto. 2012. Mitochondria and autophagy: Critical interplay between the two homeostats. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1820**:5, 595-600. [CrossRef]