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Enhancing lysosome biogenesis attenuates BNIP3-induced cardiomyocyte death

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Keywords: BNIP3, autophagy, cardiomyocyte death, lysosomes, TFEB

Abbreviations: BNIP3, BCL2/adenovirus E1B 19 kd-interacting protein; FRET, Forster resonance energy transfer; NRCM, neonatal rat cardiac myocyte; CQ, chloroquine; SMA, 3-methyladenine

Hypoxia-inducible pro-death protein BNIP3 (BCL-2/adenovirus E1B 19-kDa interacting protein), provokes mitochondrial permeabilization causing cardiomyocyte death in ischemia-reperfusion injury. Inhibition of autophagy accelerates BNIP3-induced cell death, by preventing removal of damaged mitochondria. We tested the hypothesis that stimulating autophagy will attenuate BNIP3-induced cardiomyocyte death. Neonatal rat cardiac myocytes (NRCMs) were adenovirally transduced with BNIP3 (or LacZ as control; at multiplicity of infection = 100); and autophagy was stimulated with rapamycin (100 nM). Cell death was assessed at 48 h. BNIP3 expression increased autophagosome abundance 8-fold and caused a 3.6-fold increase in cardiomyocyte death as compared with control. Rapamycin treatment of BNIP3-expressing cells led to further increase in autophagosome number without affecting cell death. BNIP3 expression led to accumulation of autophagosome-bound LC3-II and p62, and an increase in autophagosomes, but not autolysosomes (assessed with dual fluorescent mCherry-GFP-LC3 expression). BNIP3, but not the transmembrane deletion variant, interacted with LC3 and colocalized with mitochondria and lysosomes. However, BNIP3 did not target to lysosomes by subcellular fractionation, provoke lysosome permeabilization or alter lysosome pH. Rather, BNIP3-induced autophagy caused a decline in lysosome numbers with decreased expression of the lysosomal protein LAMP-1, indicating lysosome consumption and consequent autophagosome accumulation. Forced expression of transcription factor EB (TFEB) in BNIP3-expressing cells increased lysosome numbers, decreased autophagosomes and increased autolysosomes, prevented p62 accumulation, removed depolarized mitochondria and attenuated BNIP3-induced death. We conclude that BNIP3 expression induced autophagosome accumulation with lysosome consumption in cardiomyocytes. Forced expression of TFEB, a lysosomal biogenesis factor, restored autophagosome processing and attenuated BNIP3-induced cell death.

Introduction

In myocardial ischemia-reperfusion injury, programmed cell death causes substantial cardiac myocyte loss in addition to accidental necrosis triggered by lack of oxygen and nutrients in the ischemic core. 1 The BCL-2 family of proteins is a key regulator of the initiation and execution of programmed cell death pathways. 2 BNIP3 3, 4 a pro-death member of this family, 5-7 is transcriptionally upregulated in hypoxic cardiac myocytes, 6, 8 and causes mitochondrial permeabilization 6 and dysfunction 7 leading to cell death, which is an important determinant of cardiac dysfunction 9 and post-infarction remodeling following ischemia-reperfusion injury. 10 In the setting of increased BNIP3 expression, as happens with cardiac ischemia-reperfusion injury, 2 cardiomyocyte autophagy is upregulated. 9, 26-28 Autophagy is an evolutionarily conserved lysosomal degradative pathway to remove damaged intracellular constituents that facilitates cellular homeostasis, and promotes cell survival under stress such as nutrient deprivation and hypoxia. 29 Induction of autophagy is protective in the ischemic heart, 29-31, 36 but has been implicated in causing cardiomyocyte death in myocardial reperfusion injury. 11 Forced expression of BNIP3 stimulates autophagy in cardiac myocytes, 33, 35, 36 with a dose-dependent increase in autophagosome abundance. 13 While BNIP3-induced autophagy has been implicated in causing cell death in cancerous cells, 37-39 induction of autophagy in the setting of BNIP3 expression is protective in cardiac myocytes, as inhibition of autophagosome formation either pharmacologically [with 3-methyladenine (3MA)] 47 or with co-expression of dominant negative autophagy-related (Atg) protein 5 (Arg5) 48 increases BNIP3-induced cardiomyocyte death. Conversely, enhancing autophagosome formation with forced expression of Arg5 and BECN1 appears to attenuate BNIP3-induced cell death in HL-1 cardiac myocytes. 34, 35 It is not known whether further induction of protective autophagy in BNIP3-expressing cardiac myocytes is
limited by the availability of constituents of the autophagic machinery, or is actively suppressed, whereby it is unable to fully protect cells from BNIP3-induced cell death.

BNIP3 permeabilizes cardiac mitochondria, promotes mitochondrial fission, and renders them dysfunctional, and the damaged mitochondria are removed via macroautophagy, to ensure cellular viability. Indeed, BNIP3 has been proposed as a key mediator for autophagic removal of damaged mitochondria under hypoxic stress and in unstressed cardiac myocytes. This process is mediated by the transcription factor EB (TFEB), in upregulating synthesis of autophagy proteins and stimulating lysosomal biogenesis in a coordinated fashion to facilitate starvation-induced autophagy. We propose a model whereby TFEB initiates a strategy for enhancing flux through the macroautophagy pathway, rather than stimulating autophagosome formation alone, which may accelerate removal of BNIP3-damaged mitochondria.

Recent studies have identified a critical role for transcription factor EB (TFEB), in upregulating synthesis of autophagy proteins and stimulating lysosomal biogenesis in a coordinated fashion to facilitate starvation-induced autophagy. We therefore evaluated whether further induction of autophagosome formation with rapamycin treatment enhances BNIP3-induced cell death. Our results implicate lysosomal consumption as a rate-limiting factor in BNIP3-induced autophagy, whereby a strategy for enhancing flux through the macroautophagy pathway, rather than stimulating autophagosome formation alone, may accelerate removal of BNIP3-damaged mitochondria.

BNIP3 expression induced autophagy with a preponderance of autophagosome-bound LC3-II, without a change in LC3 transcription, as compared with control. Interestingly, while rapamycin increased autophagosome numbers (Fig. 1A and B), LC3-II abundance in the presence of CQ to autophagosome numbers, as previously described. In contrast, nutrient deprivation and rapamycin increased LC3-II abundance (Fig. 1C) and increased LC3-II expression with a trend toward reduction in p62 accumulation (Fig. S1A), suggesting BNIP3-induced impaired clearance of p62. Rapamycin increased LC3-II expression (Fig. 1C) as compared with control. Importantly, while rapamycin increased LC3-II expression in BNIP3-expressing cells, p62 levels did not decline (Fig. 1C), suggesting lack of p62 clearance despite further induction of autophagosome formation by rapamycin. BNIP3 expression provoked a ~3.6-fold increase in cell death as compared with controls (Fig. 1D). Rapamycin, a potent inducer of autophagy in cardiomyocytes, 21,22 and in unstressed cardiac myocytes, 23 further induction of autophagosome formation by rapamycin. We therefore evaluated whether further induction of autophagosome formation with rapamycin treatment either simultaneously or 24 h after induction of BNIP3 expression (Fig. 1C), did not affect BNIP3-induced cell death (Fig. 1D). Inhibition of autophagosome formation with 3MA increased cell death in both control and BNIP3-expressing cells, confirming a beneficial role for autophagy in cardiomyocyte homeostasis and protection from BNIP3-induced cell death, as previously described. 7

BNIP3 induces autophagosome accumulation in cardiac myocytes. Accumulation of p62 with increased autophagosome abundance in BNIP3-expressing cells (Fig. 1C), suggests impairment of autophagosome processing. Accordingly, we examined autophagosome abundance in the absence of chloroquine (CQ), a lysosomal acidification inhibitor, which leads to accumulation of autophagosomes due to impairment in autophagosome-lysosome fusion and autophagosome removal. 82 Cumulative flux, expressed as a ratio of autophagosome abundance in the presence of CQ to autophagosome numbers in its absence, was partially impaired with BNIP3 expression as compared with controls. Cells demonstrated basal autophagy with a preponderance of autophagosome-bound LC3-II as compared with control (Fig. 1C). In contrast, nutrient deprivation and rapamycin led to autophagy induction with better preserved flux as compared with BNIP3-expressing cells (Fig. 1C). Rapamycin increased autophagosome numbers (Fig. 1C) as compared with control. Interestingly, while rapamycin increased LC3-II expression in BNIP3-expressing cells, p62 levels did not decline (Fig. 1C), suggesting lack of p62 clearance despite further induction of autophagosome formation by rapamycin. BNIP3 expression provoked a ~3.6-fold increase in cell death as compared with controls (Fig. 1D). Rapamycin, a potent inducer of autophagy in cardiomyocytes, 21,22 and in unstressed cardiac myocytes, 23 further induction of autophagosome formation by rapamycin. We therefore evaluated whether further induction of autophagosome formation with rapamycin treatment either simultaneously or 24 h after induction of BNIP3 expression (Fig. 1C), did not affect BNIP3-induced cell death (Fig. 1D). Inhibition of autophagosome formation with 3MA increased cell death in both control and BNIP3-expressing cells, confirming a beneficial role for autophagy in cardiomyocyte homeostasis and protection from BNIP3-induced cell death, as previously described. 7

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treatment of cells transduced with control (LacZ) adenovirus increased autophagosome-bound LC3-II abundance and led to p62 accumulation (Fig. 2E), which was associated with a 1.8-fold increase in cell death (Fig. 2F), likely secondary to autophagosome accumulation (Fig. 2A and B) and lack of homeostatic clearance of autophagic cargo such as damaged mitochondria. In contrast, while CQ treatment of BNIP3-expressing cells caused a further increase in LC3-II abundance (as compared with BNIP3-expressing cells treated with diluent; Fig. 2E), there was no further p62 accumulation (Fig. 2E), suggesting impaired autophagosome clearance in BNIP3-expressing cells. In contrast to the effect of CQ on cell death in control cells, the effect of chloroquine treatment on cell death in BNIP3-expressing cells was marginal (1.2 fold; Fig. 2F), correlating with an underlying impairment in clearance of autophagosomes and possibly damaged mitochondria in BNIP3-expressing cells in the absence of CQ treatment. Interestingly, CQ treatment provoked an accumulation of the monomeric, but not the dimeric forms of BNIP3 (Fig. 2E) and rapamycin treatment preferentially reduced monomeric BNIP3 protein levels (Fig. 2C), indicating an underlying impairment in
Figure 2. BNIP3 induces autophagosome accumulation in NRCMs. (A) Representative epifluorescence images (630x/C190 magnification) demonstrating cellular localization of GFP-LC3 in NRCMs adenovirally transduced with BNIP3 (Red; for FLAG) or LacZ (as control) for 48 h, and treated with chloroquine (10 μM, black bars) or diluent (white bars) for 24 h prior to fixation. Nuclei are blue (DAPI). (B) Quantification of punctate GFP-LC3 dots in cells treated as in (A). (C) Quantification of punctate GFP-LC3 dots in cells subjected to nutrient deprivation or treated with rapamycin (100 nM) for 4 h in the presence of chloroquine (10 μM, black bars) or diluent (white bars); p values are by post-hoc test. *p < 0.05 vs. diluent-treated control group. # p < 0.05 vs. CQ-treated control group (n = 15–25 nuclei/group). (D) Representative epifluorescence images (630x/C190 magnification) demonstrating cellular localization of mCherry-GFP-LC3 in NRCMs adenovirally transduced with Bnip3 or LacZ (as control) for 48 h; subjected to nutrient deprivation or treated with rapamycin (100 nM) for 4 h. (E) Quantitation of autophagosomes (green+red; white bars), autolysosomes (red, black bars) and both (gray bars) in NRCMs treated as in (C) (n = 20–40 nuclei/group); p values are by post-hoc test. *p < 0.05 for autophagosomes vs. control; # p < 0.05 for autolysosomes vs. control and $ p < 0.05 for both vs. control (n = 15–25 nuclei/group). (F) Immunoblot demonstrating LC3, p62 and BNIP3 (FLAG) expression in NRCMs adenovirally transduced with BNIP3 or LacZ (Con) for 48 h, and treated with chloroquine (10 μM/L) or diluent for 24 h. Expression of α-sarcomeric actin (αSA) was assessed as loading control. (F) Cell death in NRCMs treated as in E (n = 8–24/group).
clearance of BNIP3-dimer and likely mitochondria that are permeabilized only by the dimeric forms of BNIP3, during BNIP3-induced autophagy.\(^5,\)\(^6,\)\(^7,\)\(^8,\)\(^9\) The transmembrane domain is essential for the interaction of BNIP3 with LC3. The C-terminal transmembrane domain of BNIP3 is essential for targeting mitochondria\(^4,\)\(^5\) and the endoplasmic reticulum,\(^2,\)\(^6\) inducing mitochondrial permeabilization and causing cell death.\(^4,\)\(^5,\)\(^9,\)\(^10,\)\(^11\) Recent studies have identified an interaction between BNIP3 and LC3,\(^4,\) likely via amino acid sequences upstream of the C-terminal transmembrane domain, based on similarities with a related protein NIX/BNIP3L.\(^37,\)\(^38\) suggesting that BNIP3 acts as a 'receptor' for targeting mitochondria to autophagosomes.\(^3,\)\(^4,\)\(^5,\)\(^9,\)\(^10,\)\(^11\) Interestingly, an endogenous hypoxia-inducible splice variant of BNIP3 lacking the transmembrane domain (BNIP3Δex3), but retaining the higher affinity LC3 interaction region (LR; homologous to LR labeled W35 in BNIP3L\(^37\)) was recently identified.\(^39\) It is conceivable that at high levels of BNIP3 expression, such as may occur during hypoxia,\(^3,\)\(^4,\)\(^9\) the interaction between concomitantly hypoxia-upregulated BNIP3Δex3\(^37\) and LC3 protein involving the non-transmembrane segment of the BNIP3 protein, such as observed in silico with BNIP3L/NIX,\(^37,\)\(^38\) leads to sequestration of the available LC3, preventing its role in autophagic removal of damaged mitochondria. To examine this premise, we assessed the interaction of a transmembrane deletion mutant of BNIP3 (BNIP3\(^\Delta\)TM; with all putative LIR regions\(^37\) intact) and LC3, each tagged with a FRET compatible fluorophore partner in HEK 293 cells. As previously demonstrated,\(^4\) BNIP3\(^\Delta\)TM demonstrated diffuse cellular localization (Fig. 3A; Fig. S2), and did not induce autophagy,\(^10\) assessed as increased punctate LC3 localization, as compared with controls (Fig. 3A; Fig. S2). Interestingly, induction of autophagy by rapamycin treatment led to markedly increased punctate LC3 localization, but did not alter the subcellular localization of BNIP3\(^\Delta\)TM (Fig. S2), suggesting that the interaction between BNIP3 and LC3 requires activation of autophagy with an intact transmembrane domain. Indeed, assessment between full-length BNIP3, and BNIP3\(^\Delta\)TM with LC3 by FRET (Fig. 3A and B) and co-immunoprecipitation as previously described for BNIP3 and LC3\(^9\) (Fig. 3C), confirmed an obligate role for the transmembrane domain in the interaction between BNIP3 and LC3, potentially involving additional proteins recruited upon BNIP3-induced mitochondrial permeabilization and dysfunction.\(^3,\)\(^4,\)\(^5,\)\(^9,\)\(^10,\)\(^11\) Also, while full-length BNIP3 colocalized both with LC3 and mitochondria in NRCMs, BNIP3\(^\Delta\)TM did not (Fig. S3), indicating that only the full-length BNIP3 protein acts as a receptor to target damaged mitochondria into autophagosomes, as observed with the closely related protein, BNIP3L/NIX.\(^37,\)\(^41\) This suggests that increased BNIP3 expression results in accumulation of autophagosomes containing full-length BNIP3-targeted mitochondria,\(^7,\)\(^9,\)\(^10,\)\(^13,\)\(^16\) with potential deleterious consequences secondary to impaired removal of these damaged organelles.

![Figure 3](image-url)
BNIP3 does not target lysosomes or alter lysosome pH. Accumulation of autophagosomes in the setting of increased BNIP3 expression may occur due to inhibition of subsequent autophagosome processing. Additionally, previous studies have suggested direct targeting of BCL-2 family proteins to lysosomes as a potential mechanism for activation of cell death.44,45 Accordingly, we examined whether BNIP3 targets to lysosomes or alters lysosomal integrity. BNIP3 colocalizes together with both lysosomes and mitochondria in NRCMs (Fig. 4A, see arrows, bottom panel). This may indicate that BNIP3 targets to the lysosomes independently or via localization on mitochondria engulfed within autophagosomes that have subsequently fused with lysosomes. Accordingly, to examine the organelle-specific localization of BNIP3, we performed subcellular fractionation to isolate fractions enriched in lysosomes, mitochondria and the endoplasmic/sarcoplasmic reticulum in HL-1 cardiac myocytes, a cell line that displays mammalian cardiomyocyte physiology and can be easily expanded as necessary.46 As previously demonstrated, BNIP3 protein segregated to subfractions enriched in the mitochondrial marker COX IV, and the endoplasmic reticulum marker CA10N, but not a lysosomal marker, LAMP-1 (Fig. 4B). Taken together, these data indicate that BNIP3 protein does not target lysosomes independently of being localized to mitochondria, and suggest that autophagosome-lysosome fusion in the setting of BNIP3-induced autophagy is not disrupted. Additionally, BNIP3 did not cause lysosome permeabilization (Fig. 5D) or alter lysosomal pH in NRCMs (Fig. 4G).

BNIP3-induced autophagosome formation leads to lysosome consumption. We next examined whether BNIP3 affects lysosome abundance. Expression of full-length BNIP3, but not BNIP1ATM, provoked a ~20% reduction in lysosome numbers in NRCMs as assessed by uptake of two pH-dependent lysosome probes, LysoTracker red (BNIP3 provoked an increase in green fluorescent JC-1 monomers (Fig. 6A, and B), bottom) and markedly increased green to red fluorescence ratio (vs controls, Fig. 7C). This was likely secondary to enhanced removal of damaged mitochondria by a global induction of the autophagic machinery driven by TFEB in the setting of BNIP3 expression, as BNIP3 provoked an increase in green fluorescent JC-1 monomers (Fig. 7A and B, top) with a reduction in red fluorescent JC-1 J-aggregates (Figs. 7A and B, bottom) and markedly increased green to red fluorescence ratio (vs controls, Fig. 7C), indicating increased depolarized mitochondria and reduced numbers of normally polarized mitochondria, and suggesting accumulation of damaged mitochondria (Fig. 7A-C), which was reversed by co-expression of TFEB (Figs. 7A-C).

**Discussion**

In this study, we demonstrated that BNIP3-induced autophagy in cardiac myocytes was rate limited by lysosome consumption, which led to upstream autophagosome accumulation. Expression of transcription factor EB (TFEB) stimulated lysosome biogenesis and restored processing of autophagosomes. The resultant enhanced flux through the macroautophagy pathway attenuated BNIP3-induced cell death. The primary stimulus for autophagy induction with increased BNIP3 expression, as occurs with hypoxic insult, appears to be BNIP3 targeting to the organelle, in particular the mitochondria. Induced, our data confirm an obligate role for the transmembrane domain of BNIP3 protein, which is essential for organelle targeting, in inducing autophagy. BNIP3 induces mitochondrial damage by multiple mechanisms, such as mitochondrial outer membrane permeabilization in concert with Bax...
and Bak, mitochondrial permeability transition by a novel cyclophilin D-independent mechanism, mitochondrial fragmentation in concert with Opa1 and Drp1, and mitochondrial energetic dysfunction via protease-mediated cleavage of oxidative phosphorylation/electron transport chain proteins. The obligate role of the transmembrane domain in the interaction of BNIP3 with LC3 proteins (see Fig. 3) suggests that mitochondrially-localized BNIP3 interacts with autophagosome-bound LC3-II as a
Recent studies have employed bafilomycin A1 to inhibit lysosome acidification and assess cumulative flux through the macroautophagy pathway in BNIP3-expressing cells, and suggest that autophagic flux is intact in this setting. While a BNIP3-induced increase in autophagosomes is incontrovertible, the ratio of autophagosome abundance with bafilomycin A1 treatment as

Receptor to facilitate autophagic removal of these damaged mitochondria.  

Figure 5. BNIP3-induced autophagy is associated with reduced lysosome abundance, which is restored by co-expression of TFEB. (A) Representative epifluorescence images (630X) demonstrating lysosome distribution (by LysoTracker red staining) in cells adenovirally transduced with Bnip3, TFEB (green, at 100 MOI), BNIP3+TFEB (at 100 MOI each) for 48 h. Nuclei are blue (Hoechst dye). Adenovirus coding for LacZ expression was added as necessary to result in equivalent MOIs (at total 200 MOI per treatment); (B) Flow cytometric analysis of LysoTracker red staining in cells treated as in (A). Control is depicted in black, BNIP3 in green, TFEB in red and BNIP3+TFEB in blue. (C) Assessment of LysoTracker red expression by flow cytometry in NRCMs expressing BNIP3, BNIP3\(\Delta\)TM, TFEB, BNIP3+TFEB for 48 h; and in BNIP3 expressing cells treated for 24 h with 3MA (7 mmol/L). (D) Representative immunoblots demonstrating LC3, p62, BNIP3 (FLAG), BNIP3\(\Delta\)TM (HA), TFEB (both rat TFEB and HA tagged human TFEB) and LAMP1 expression, with α-sarcomeric actin (αSA) in NRCMs adenovirally transduced with LacZ (control), BNIP3, BNIP3\(\Delta\)TM, TFEB and BNIP3+TFEB as in (A) (for 48 h). (E-H) Quantitative assessment of LC3-II/α-sarcomeric actin ratio (E), total LC3 (F), p62 (G) and LAMP1 (H) abundance in NRCMs treated as in D (n = 3–7/group). P values are by post-hoc test.
compared with no treatment was low in BNIP3-expressing cells when compared with a similar ratio in controls (1.6 in BNIP3-expressing cells vs. 5 in controls). These data are comparable to our results with CQ (which also inhibits lysosomal acidification; see Fig. 2A and B), and taken together, suggest a partial impairment in autophagosome processing in BNIP3-expressing cells.

Our data also suggest that under conditions of high levels of BNIP3 expression as may occur with myocardial ischemia-reperfusion injury, autophagosome processing is impaired, as indicated by accumulation of p62, a protein that gets consumed in the autophagic process, and accumulation of autophagosomes without a commensurate increase in autolysosomes. Impaired autophagosome clearance prevents removal of BNIP3-damaged mitochondria, similar to that observed with preventing autophagosome formation (e.g., with 3MA, or dominant negative Atg5 or autophagosome processing (with CQ, see Fig. 2, or bafilomycin A1 treatment), and impairs the role for autophagy in preventing BNIP3-mediated programmed cell death.

Previous studies have demonstrated that inducing autophagosome formation with transfection of Atg5 or Beclin1 attenuates BNIP3-induced cell death. We observed that stimulating autophagosome formation with rapamycin does not attenuate BNIP3-induced cell death (Fig. 1). The observed differences may be attributable to level of BNIP3 expression, whereby autophagosome accumulation is only observed at high levels (as obtained with adenoviral transduction of Bnip3), but not at lower levels typically achieved with transfection-based-methods in cardiac myocytes. Alternatively, the previous studies may indicate a specific impairment in ATG5 and/or BECN1 levels or function in the context of BNIP3-induced autophagy, whereby restoring
could provoke a lysosomal pathway for cell death.51 Indeed, BNIP3 did not target to or permeabilize lysosomes whereby it did not occur with the transmembrane deletion variant prevented by inhibition of autophagosome formation with 3MA, lysosome consumption in the autophagy process, as it was some abundance in cardiac myocytes. This appears to be due to normalization or acceleration of this process. These hypotheses require further investigation.

We found that BNIP3 expression triggers a decline in lysosome abundance in cardiac myocytes. This appears to be due to lysosome consumption in the autophagy process, as it was prevented by inhibition of autophagosome formation with SMA, and did not occur with the transmembrane deletion variant BNIP3TM which does not induce autophagy. Importantly, BNIP3 did not target to or permeabilize lysosomes whereby it could provoke a lysosomal pathway for cell death.33 Indeed, enhancing lysosomal biogenesis in that setting would have increased BNIP3-induced cell death, akin to adding fuel to the fire. The decline in lysosome numbers with BNIP3-induced autophagy indicates a lack of recruitment of, or an active suppression of mechanisms to enhance lysosomal biogenesis endogenously as occurs in starvation-induced autophagy.28 Notably, rapamycin treatment, which results in an initial depletion of lysosomes followed by a rapid restoration of lysosome abundance,33 did not attenuate BNIP3-induced cell death, suggesting that the observed beneficial effects of TFEB relate to its ability to coordinately upregulate the entire autophagic machinery.28 It is interesting to speculate that active suppression of autophagy by limiting the process at various steps is a mechanism inherent to the programmed cell death process, which prevents the pro-survival function of lysosome-mediated autophagy, and requires further study.

Materials and Methods

Cardiac myocyte culture. Neonatal rat cardiac myocyte (NRCM) cultures were prepared as described.14 Briefly, hearts were removed from 1-d-old Sprague-Dawley rats, the atria and great vessels were trimmed off, and tissue was finely minced followed by sequential digestion with 0.5 mg/ml collagenase (WAKO, LK03303). Ventricular cardiomyocytes were separated from fibroblasts by differential plating, and were cultured in gelatin-coated 12-well tissue culture plates (0.4 hearts/well) in media containing Dulbecco's modified Eagle's medium, 10% horse serum, 5% fetal calf serum, 100 U/ml penicillin, streptomycin and L-glutamine. Nutrient deprivation was induced as previously described.33 HL-1 cardiac myocytes were a kind gift from Dr. William Claycomb, Louisiana State University, New Orleans, and were cultured as described.14 Generation of viral constructs. The coding sequence for rat Maplc3β (microtubule-associated protein 1 light chain 3β) was cloned in frame with GFP in pAcGFP-C vector (Clontech, 632470). The GFP-LC3 coding sequence was then cloned downstream of mCherry in pLVx-mCherry-C vector (Clontech, 632561) and lentiviral particles coding for expression of dual fluorescent tandem tagged mCherry-GFP-LC3 were generated per the manufacturer's instructions at the Hope Center Viral Vector Core at Washington University School of Medicine. Transduction of lentiviruses was facilitated with polybrene, at 8 μg/ml (Sigma, H9268). Codon-optimizing treatment for human BNIP3, BNIP3ATM (see Supplementary methods for details) and human TFEB, with N-terminal FLAG (DYKDDDDK) on BNIP3) and HA (YPYDVPDYA; on BNIP3ATM and TFEB) tags were cloned into pENTR-TOPO vector (Invitrogen, 45.0218) and recombinant adenoaviral constructs generated with Clonase
mediated recombination (Virapower, Invitrogen, K493000). Adenoviruses were generated in HEK293A cells and titered per the manufacturer’s instructions.

Assessment of cell death. Cell death assays were performed in 96-well plates and chamber slides (Nunc, Fisher, 177429) formatted with the Live-Dead Cytotoxicity Viability kit for Mammalian cells (Invitrogen, L3224) per manufacturer’s instructions as described. Quantitative assessment of fluorescence was performed with BioTek Synergy-2 microplate reader equipped with the appropriate filter sets (Green: Excitation 485 ± 10 nm, Emission 528 ± 10 nm; Red: Excitation 540 ± 7.5 nm, Emission 620 ± 20 nm) at Chemical Genetics Screening Core at Washington University School of Medicine. TUNEL staining was performed as described.

Immunofluorescence imaging. Imaging for GFP-LC3 and mCherry-GFP-LC3 localization, and immunofluorescence for FLAG and HA epitopes (with Alexa Fluor 488 and 594 tagged secondary antibodies from Invitrogen, A21202 and A21207) were performed on 4% paraformaldehyde fixed cells using Axioskop upright microscope, AxioCam HRC camera and Plan Neofluar objective (0.8X, NA 1.25; Zeiss) fitted with appropriate filter cubes. Images were acquired and analyzed using Zeiss Axiosvision software. Confocal imaging was performed on a Zeiss LSM 510 NLO Meta using an Achroplan 63X (NA 0.95) water objective and Zeiss LSM software. Punctate fluorescent tagged LC3 dots were counted and expressed as number per nucleus using Image J software (NIH) as described. Organotypic imaging was performed using Lysotracker red (L7545) and green (L7526), Mitotracker deep red FM (M22426), Hoeplast dye (H3570), JC-1 (T3168; all from Invitrogen) and DAPI (Vector Labs; H-1200) per manufacturer’s protocols. Acridine orange (Biotium, 40039) staining was performed as described. Assessment of lysosomal pH was performed with pH-sensitive dye LysoSensor yellow/ blue DND-160 (Invitrogen, L7545) following manufacturer’s instructions as described. Organelle imaging was performed on 4% paraformaldehyde fixed cells using Axioskop upright microscope, AxioCam HRC camera and Plan Neofluar objective (0.8X, NA 1.25; Zeiss) fitted with appropriate filter cubes. Images were acquired and analyzed using Zeiss Axiosvision software. Confocal imaging was performed on a Zeiss LSM 510 NLO Meta using an Achroplan 63X (NA 0.95) water objective and Zeiss LSM software. Punctate fluorescent tagged LC3 dots were counted and expressed as number per nucleus using Image J software (NIH) as described. Organotypic imaging was performed using Lysotracker red (L7545) and green (L7526), Mitotracker deep red FM (M22426), Hoeplast dye (H3570), JC-1 (T3168; all from Invitrogen) and DAPI (Vector Labs; H-1200) per manufacturer’s protocols. Acridine orange (Biotium, 40039) staining was performed as described. Assessment of lysosomal pH was performed with pH-sensitive dye LysoSensor yellow/ blue DND-160 (Invitrogen, L7545) following manufacturer’s instructions as described.

Subcellular fractionation. HL-1 cells (7 × 10^6 cells per group) were adenoenovirally transduced with LacZ (control) and Bnip3 [at MOI (multiplicity of infection) = 100] for 48 h and subjected to subcellular fractionation on a discontinuous nycodenz gradient using procedures modified from protocols previously described. Briefly, cells were homogenized in a medium containing 0.25 M sucrose, 1 mM Na₂EDTA, 10 mM HEPES adjusted to pH 7.4 with NaOH. Homogenates were cleared off unbroken cells with a brief 120 g spin and a mitochondria lysosome rich fraction was sedimented at 20,000 g for 20 min. This fraction was layered on a discontinuous nycodenz (Optiprep, Sigma, D1556) gradient (19%, 27% and 30%) and subjected to ultracentrifugation at 110,000 g for 2 h in a swinging bucket rotor. Three-milliliter fractions were collected from the gradient, and lysosomes, and mitochondria with mitochondria-associated membranes were recovered from the top and 3rd (from top) fractions, respectively. The supernatant from the 20,000 g spin was subjected to ultracentrifugation at 100,000 g for 1 h to recover endoplasmic reticulum rich fraction; with resultant supernatant concentrated as cytosol using Ultracel-10K protein filters (Millipore, UFC801024).

Assessment of FRET interaction. Constructs coding for DsRed-BNIP3 or DsRed-BNIP3ATM (see supplementary methods for details) were co-transfected with GFP-LC3 in HEK293 cells. Construct encoding for DsRed-monomer-GFP fusion protein was employed as positive control. Normalized FRET was assessed by confocal microscopy and BioTek Synergy-2 microplate reader equipped with the appropriate filter sets as described.

Flow cytometry. NRCMs were incubated with Lysotracker Red (1 μmol/L for 15 min at 37°C in 5% CO₂) or JC-1 (10 μg/ml for 10 min at 37°C in 5% CO₂) and subjected to flow cytometry on FACScan instrument (Becton-Dickinson) as described. Cytofluor software (CyFlo) was employed to analyze 20,000 events per run.

Co-immunoprecipitation studies. NIH 3T3 fibroblasts were transduced with adenoviruses to co-express BNIP3 or BNIP5ATM and GFP-LC3 (all at 100 MOI); and crude extracts prepared as described. One and a half mg of total protein was incubated with anti-GFP (Abcam, ab02960) and normal rabbit IgG; and immunoprecipitation performed using Dynabeads® Protein G (Invitrogen, 1000-02D).

Immunoblotting. Immunoblotting was performed on cardiac and cellular extracts using previously described techniques. Antibodies employed were as follows: FLAG (Sigma, F31655); HA (Sigma, H69088); Bnip3 (Abcam, Ab10433); GFP (Clontech, 632375); LAMP1 (Abcam, AB24170); LC3 (Novus Biologicals, NB100-2220); p62 (Abcam, ab54160); and -actinomycin actin (Abcam, ab2249); [Image J] software was employed for quantitative analysis. Protein abundance was normalized to β-tubulin-acting protein expression and reported as fold change vs. control. Chemicals employed were obtained as follows: rapamycin (EMD4Biosciences, 555212); chloroquine (Sigma, CC6828); 3-methyladenine (EMD4Biosciences, 189490); and staurosporine (Sigma, S6042). Statistical analysis. Results are expressed as mean ± SEM. Statistical differences were assessed with the unpaired 2-tailed Student’s-t test for two experimental groups and one-way ANOVA for multiple groups with SPSS software. Bonferroni’s post-hoc testing was employed after ANOVA for testing for significant differences between groups. A two-tailed p value of less than 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest. No potential conflicts of interest were disclosed.

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