UNIVERSITY OF CALIFORNIA, SAN DIEGO

Bnip3 Interacts with LC3 to Induce Selective Removal of Endoplasmic Reticulum and Mitochondria via Autophagy

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

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Professor Aaron Coleman

2011

The Thesis of Rita Akram Hanna is approved, and it is acceptable

in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2011

DEDICATION

Dedicated to all the great people in my life who provided me with support, especially my family and my PI whom without their help and encouragement I could have not achieved my dreams

EPIGRAPH

Every day you may make progress. Every step may be fruitful. Yet there will stretch out before you an ever-lengthening, ever-ascending, ever-improving path. You know you will never get to the end of the journey. But this, so far from discouraging, only adds to the joy and glory of the climb.

- Winston Churchill

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LIST OF ABBREVIATIONS

Baf. A1	Bafilomycin
Bcl-2	B-cell CLL/lymphoma 2
β-gal	β-galactosidase
Bnip3	Bcl-2/adenovirus E1B 19kDa interacting protein 3
BZ	Border zone
CsA	Cyclosporin A
DMEM	Dulbecco's Modified Eagle Medium
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
GABARAP	γ-aminobutyric acid type A receptor-associated protein
GFP	Green fluorescent protein
IP	Immunoprecipitation
LAD	Left Anterior Descending Artery
LC3	Light Chain 3 of Microtubule Associated

	Protein
MI	Myocardial Infarction
mPTP	Mitochondrial permeability transition pore
p62	Polyubiquitin-binding protein
RZ	Remote zone
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TM	Transmembrane Domain
TOM20	Translocase of Outer Mitochondrial Membrane 20

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ACKNOWLEDGMENTS

First and foremost, I would like to acknowledge my P.I., Dr. Åsa Gustafsson, who has supported me throughout my thesis with her patience and vast knowledge. I attribute the level of my master's degree to her continued support and effort. This thesis would not have been possible without her encouragement and guidance; she was the main proponent to my success.

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ABSTRACT OF THE THESIS

Bnip3 Interacts with LC3 to Induce Selective Removal of Endoplasmic Reticulum and Mitochondria via Autophagy

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Bcl-2 family proteins are known to regulate mitochondrial integrity and apoptosis. More recently, they have been found to play a role in regulating autophagy. Autophagy is a process involved in removing excess or damaged organelles. Bnip3 is a pro-apoptotic BH3-only protein which is known to cause mitochondrial dysfunction and cell death. Bnip3 is also a potent inducer of mitochondrial autophagy. In this study I have investigated the mechanism by which Bnip3 promotes removal of mitochondria via autophagy. Bnip3 contains a

C-terminal transmembrane (TM) domain that is essential for homodimerization and pro-apoptotic function. Here, I show that Bnip3 homodimerization is also a requirement for induction of autophagy. Mutations in Bnip3 that disrupt homodimerization, but do not interfere with mitochondrial localization, failed to induce autophagy. In addition, I found that endogenous Bnip3 was localized to both the mitochondria and the endoplasmic reticulum (ER) in HeLa cells. To investigate the effects of Bnip3 at ER on autophagy, Bnip3 was targeted specifically to mitochondria or ER by substituting the Bnip3 TM domain with that of Acta or cytochromeb_5, respectively. Interestingly, Bnip3 induced significant autophagy in cells from both sites. Moreover, Bnip3 induced removal of mitochondria and ER by autophagy via binding to LC3 on the autophagosome. Ablation of the Bnip3-LC3 interaction had no effect on the induction of general autophagy but significantly reduced autophagy of mitochondria and ER. Thus, our data suggest that the Bnip3 homodimer functions as an autophagy receptor to ensure removal of mitochondria and ER.

INTRODUCTION

Cardiovascular Disease

Cardiovascular disease is a major cause of morbidity and mortality in the United States and is expected to increase as our population ages (LaRosa 2001). Cardiovascular diseases, such as ischemic heart disease, lead to loss of cardiac myocytes which are responsible for contractility of the heart. When these cells are exposed to stressors such as deprivation of oxygen or nutrients, they activate cell death pathways which lead to loss of the cardiac myocytes. Cardiac myocytes are terminally differentiated cells and cannot be replaced. With fewer cardiac myocytes, the heart is unable to sustain contractility and ultimately lead to development of heart failure (Searle et al., 1982).

Autophagy

Autophagy is a process involved in degradation of long-lived proteins and organelles. Autophagy occurs at a low level under normal conditions in cells (Levine et al., 2004). However, autophagy is also rapidly upregulated in response to nutrient shortage to regenerate fatty acid and amino acids which serve as building blocks to make ATP and proteins (Lum et al., 2005). Autophagy is also important in removing protein aggregates, and damaged or excess organelles in the cell. This allows the cell to maintain a healthy and balanced intracellular environment (Klionsky et al., 2000, Tanaka et al., 2000, Nishino et al., 2000).

Upon initiation of autophagy, a vesicle membrane starts forming, a process known as vesicle nucleation (Fig. 1). Next, the membrane expands and elongates, leading to the formation of an isolation membrane or phagophore. The phagophore will engulf material in the cytosol and then close to form the double-membrane vesicle called the autophagosome. The autophagosome will subsequently fuse with a lysosome for further digestion to form the autophagolysosome. The lysosome contains enzyme, such as cathepsins B, D, L, and lysosomal cysteine proteases that are involved in degradation of the autophagosome and its content (Levine et al., 2008).

Autophagy can be divided into three main types: chaperone-mediated autophagy, microautophagy, and macroautophagy, which is the most common form of autophagy and is generally referred to as autophagy (Levine et al., 2004).

Autophagy in the Heart

Autophagy is also an important process in the heart and a defect in this process has severe consequences for the heart. For instance, Nakai et al. reported that cardiac specific deletion of Atg5, a protein that is required for formation of autophagosomes, resulted in development of cardiac dysfunction. Interestingly, loss of Atg5 led to rapid accumulation of mitochondria in the heart, suggesting that autophagy plays an important role in the normal turnover of mitochondria (Nakai et al., 2007). Many studies have reported that autophagy is altered in cardiovascular diseases. For instance, Dammrich et al. reported that autophagy is inhibited during cardiac hypertrophy to help maintain a balance

between the processes of growth and degradation under stressful conditions (Dammrich et al., 1983). Autophagy is also enhanced in the failing heart (Shimomura et al., 2001, Nishino et al., 2000). Moreover, a defect in the autophagic-lysosomal pathway has been implicated in Danon's disease which leads to dilated cardiomyopathy (Tanaka et al., 2000, Nishino et al., 2000). Other studies have found that autophagy is enhanced in response to stress as ATP depletion (Lum et al., 2005), opening of the mitochondrial permeability transition pore (mPTP) (Elmore et al., 2001, Arrington et al., 2006, Teckman et al., 2004), hypoxia (Zhang et al., 2008), or production of oxygen species (Khadour et al., 2002, Hickson-Bick et al., 2008).

The functional role of autophagy in the heart in response to stress is still unclear. For instance, several studies have shown that increased autophagy promotes survival by removing protein aggregates and damaged organelles that can be detrimental to the cells or recycling building blocks that can be used for survival (Kim et al., 2007, Levine et al., 2004). Moreover, enhancing autophagy has been reported to protect against IR injury in a cardiac cell line (Hamacher-Brady et al., 2006). In contrast, Matsui et al. reported that enhanced autophagy was detrimental to cells during reperfusion after ischemic injury (Matsui et al., 2007). Clearly, further studies are needed to fully understand whether autophagy plays a protective or detrimental function in the heart.

Bnip3 and Autophagy

Bcl-2 family proteins are known to regulate mitochondrial integrity and apoptosis. More recently, they have been found to play a role in regulating autophagy. The Bcl-2 family is composed of pro- and anti-apoptotic proteins. Anti-apoptotic proteins such as Bcl-2 and Bcl-X_L promote survival of cells by preventing activation of the pro-apoptotic members (Adams et al., 1998). The anti-apoptotic proteins contain four so-called Bcl-2 homology domains or BH domains. The pro-apoptotic protein consists of two subfamilies. Proteins in one subfamily contain three BH domains (BH1-BH3) and include Bax and Bak. The other subfamily is the "BH3-only" proteins which share only the BH3 domain (Huang et al., 2000). Bnip3 is a BH3-only protein which is localized primarily to the mitochondria (Hamacher-Brady et al., 2007). Bnip3 is known to contribute to myocardial I/R injury by perturbing mitochondrial function (Hamacher Brady et al., 2007). Bnip3 is also upregulated in response to hypoxia (Regula et al., 2002, Kubasiak et al., 2002). For instance, Kubasiak et al. reported that Bnip3 was barely detectable under normal conditions in neonatal myocytes but that Bnip3 was significantly upregulated under anaerobic conditions (Kubasiak et al., 2002). Bnip3 is known to cause mitochondrial dysfunction and cell death via activation of Bax/Bak (Kubli et al., 2007). Bnip3 has also been shown to induce opening of the mitochondrial permeability transition pore (mPTP) (Regula et al., 2002). Interestingly, Bnip3 induces autophagy independent of Bax/Bak and opening of the mPTP (Rikka et al., 2011, Quinsay et al., 2010), suggesting that autophagy is induced independent of the cell death pathways.

Bnip3 contains a transmembrane (TM) domain at the COOH-terminus which is important in anchoring Bnip3 in the outer membrane of the mitochondria (Yasuda et al., 1998). The TM domain is also important for homodimerization and for its pro-apoptotic function (Regula et al., 2002, Sulistijo et al., 2003, Sulitijo et al., 2006, Metcalf et al., 2007). Our laboratory has previously reported that Bnip3 forms a homodimer upon activation in cells (Kubli et al., 2008). We also discovered that Bnip3 contains a conserved cysteine residue that is important in stabilizing the active homodimer (Kubli et al., 2008).

Although Bnip3 is primarily localized to the mitochondria, studies have indicated that some of Bnip3 also found in the endoplasmic reticulum (ER) (Ray et al., 2000). Interestingly, Zhang et al. reported that targeting of Bnip3 to the ER promoted cell death by increasing the release of Ca^{2+} with subsequent uptake of the Ca^{2+} by the adjacent mitochondria. Mitochondrial Ca^{2+} overload is known to cause opening of the mPTP and loss of mitochondrial membrane potential (Zhang et al., 2009, Zhu et al., 1996). The same study found that mitochondria-targeted Bnip3 also induced cell death but via activation of Bax/Bak (Zhang et al., 2009). However, it is still unknown whether Bnip3 induces autophagy from ER.

RATIONALE

Our laboratory has discovered that Bnip3 is a potent inducer of autophagy in cells and that Bnip3 specifically promotes removal of mitochondria via autophagy. However, exactly how Bnip3 promotes mitochondrial autophagy is currently unknown. In my research, I have investigated the mechanisms by which

Bnip3 promotes autophagy in cells and whether Bnip3 can directly act as an autophagy receptor to promote clearance of mitochondria via autophagy. I have examined the hypothesis that the Bnip3 homodimer functions as an autophagy receptor to ensure removal of organelles such as mitochondria and ER.

The following questions have been investigated to answer the hypothesis:

1) Is homodimerization of Bnip3 required for induction of autophagy?
2) Can Bnip3 directly interact with LC3 and/or GABARAP on the autophagosome to promote removal of mitochondria via autophagy?
3) Can Bnip3 promote autophagy of ER?

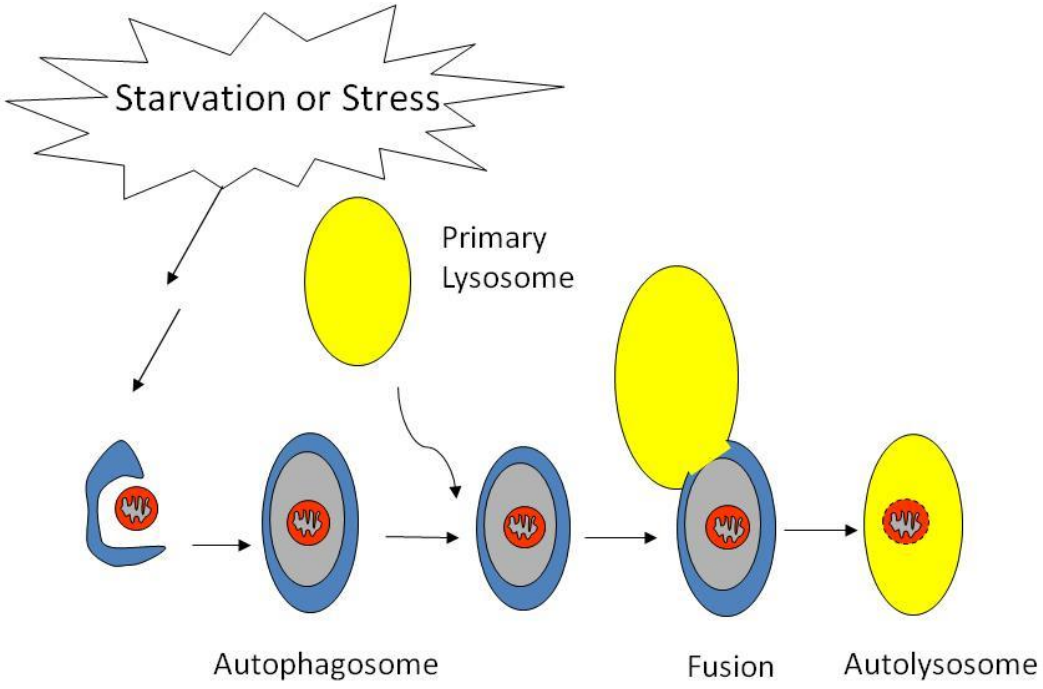

Figure 1. The autophagic-lysosomal pathway.

MATERIALS AND METHODS

cDNA Constructs

The Bnip3Acta and Bnip3Cb5 constructs were generously provided by Dr. Gary Isom, Purdue University (Zhang et al., 2009). The Bnip3W18A and Cb5W18A were prepared using site directed mutagenesis by PCR with Bnip3 wild type and Cb5 constructs as templates, respectively.

Cell Culture and Transient Transfections

HeLa cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium) plus 10% (v/v) FBS (fetal bovine serum), 100 units/ml penicillin and 100 µg/ml streptomycin. HeLa cells were transiently transfected using FuGeneR 6 (Roche) according to the manufacturer's instructions. For immunoprecipitation experiments, the cells were transfected for 24 h. For fluorescence microscopy experiments, cells were transfected for 48 h.

Adenoviral Infection

HeLa cells were infected with adenoviruses encoding GFP-LC3, β-galactosidase (β-gal), Bnip3, or Bnip3ΔTM in DMEM + 2% heat-inactivated serum. After 3 h, the cells were rinsed in PBS and incubated in growth medium (DMEM + 10% FBS). All the experiments are performed at 24 h or 48 h post-infection.

Western Blotting and Co-Immunoprecipiation Experiments

HeLa cells were lysed in ice cold buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100 and Complete™ protease inhibitor cocktail (Roche)) and then cleared by centrifugation at 20,000 xg for 20 min at 4°C. Proteins in the supernatants were separated by SDS/PAGE, transferred to a nitrocellulose membrane, and immunoblotted with antibodies. The protein concentrations were determined by the Coomassie Blue binding assay (Pierce) with BSA standards. Blots were quantified and analyzed using Quantity One software (BioRad).

For the normoxic and hypoxic experiments, HeLa cells were overexpressed for 24 h. Cells for hypoxia were transferred to hypoxic culture chamber for 24 h. To prepare lysates, cells were rinsed in PBS and then resuspended in 1 ml of ice-cold isolation buffer (200 mM sucrose, 1 mM EGTA-Tris, and 10 mM Tris-MOPS, pH 7.4). The cells were lysed with dounce homogenizer (40 strokes with a tight pestle). The cellular fractions were separated by centrifugation at different speeds: Mitochondria- 8,000 xg for 10 min, ER- 100,000 xg for 90 min.

In vivo Tissues Isolation

Adult C57BL (8-10 weeks old) male mice were subjected to myocardial infarction by permanent ligation of the left descending coronary artery (LAD) (Huang et al., 2010). Tissue was isolated from the border and remote zones at 0, 4, 8, 24, 48 h post MI (Huang et al., 2010).The tissues were homogenized by

polytron in ice-cold lysis buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100 and Complete™ protease inhibitor cocktail (Roche)), incubated on ice for 30 min, and then cleared by centrifugation at 20,000 xg for 20 min at 4°C. Proteins in the supernatants were separated by SDS/PAGE, transferred to a nitrocellulose membrane, and immunoblotted with antibodies. The protein concentrations were determined by the Coomassie Blue binding assay (Pierce) with BSA standards. Blots were quantified and analyzed using Quantity One software (BioRad).

Immunofluorescence Analysis

Cells overexpressing vector or Bnip3 constructs plus GFP-LC3 or GABARAP-GFP for 48 h were fixed with 4% (w/v) paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 in PBS and then blocked in 5% goat serum. The cells were incubated with anti-TOM20 or Calnexin and then with goat anti-rabbit Alexa-594 secondary antibody. Cells were examined using a Carl Zeiss AxioObserver Z1 equipped with a motorized z-stage and ApoTome for optical sectioning. To assess autophagy cells overexpressing GFP-LC3 were examined at 60x magnification and classified as: (a) cells with diffuse GFP-LC3 fluorescence, or as (b) cells with numerous GFP-LC3 puncta (>30 dots/cell), representing autophagosomes.

Immunoprecipitation

HeLa cells were lysed in ice cold buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100 and Complete™

protease inhibitor cocktail (Roche) and then cleared by centrifugation at 20,000 xg for 20 min at 4°C. The protein concentrations were determined by the Coomassie Blue binding assay (Pierce) with BSA standards. Cell lysates were precleared with protein G PLUS agarose (Santa Cruz Biotechnology) for 1 h, and then incubated with anti-GFP or anti-GABARAP o/n to immunoprecipitate GFP-LC3 or GABARAP-GFP, respectively. Immune complexes were captured with protein G PLUS agarose beads, centrifuged, washed four times in PBS, and solubilized in 2X SDS sample buffer. Proteins were analyzed by western blotting with anti-Bnip3 to determine how much of Bnip3 co-immunoprecipitated with LC3 or GABARAP, or anti-GFP or GBARAP to verify that equal amounts of GFP-LC3 or GABARAP-GFP were immunoprecipitated.

Statistical Analysis

All values are expressed as means ± Standard Error of Mean (S.E.M). Statistical analyses were performed using Student's t-test to identify statistical significance between groups. $P<0.05$ was considered significant.

Chapter 1: Homodimerizationof Bnip3 is Required for Induction of Autophagy

Introduction

We have previously demonstrated that Bnip3 must form a homodimer before it can induce cell death (Kubli et al., 2008). Deletion of the transmembrane (TM) domain disrupts the Bnip3 ability to form a homodimer and induce cell death (Kubli et al., 2008, Sulistijo et al., 2003, Sulistijo et al., 2006, Metcalf et al., 2007, Regula et al., 2002). A histidine residue at position 173 and a glycine residue at position 180 in the TM have been demonstrated to be important for dimerization and cell death (Sulistijo et al., 2003, Sulistijo et al., 2006, Kubli et al., 2008, Kim et al., 2005). Moreover, Bnip3 contains a conserved cysteine in the cytosolic N-terminal region which is important in stabilizing the Bnip3 homodimer (Kubli et al., 2008). Since it has been shown that the activation of cell death is an independent process from the induction of autophagy (Rikka et al., 2011), I investigated whether Bnip3 homodimerization is also required for induction of autophagy.

Bnip3-Mediated Autophagy is Dependent on Homodimerization of Bnip3

To investigate if an intact TM domain is required for activation of autophagy by Bnip3, I assessed induction of autophagy in HeLa cell overexpressing full length Bnip3 or a TM deletion mutant of Bnip3, Bnip3ΔTM. HeLa cells were infected with vector, Bnip3, or Bnip3ΔTM plus GFP-LC3 to monitor formation of autophagosomes. I found that deletion of the TM domain completely abrogated induction of autophagy (Fig. 2A). Since the TM domain is

also important in targeting Bnip3 to membranes (Yasuda et al., 1998, Sulistijo et al., 2003, Sulistijo et al., 2006, Metcalf et al., 2007), Bnip3ΔTM is localized to the cytosol. Therefore, it was still unclear whether the failure to induce autophagy was due to lack of homodimerization or failure to localize to the mitochondria. Therefore, I investigated whether Bnip3G180F or Bnip3H173A were able to induce autophagy. These mutants are unable to form homodimers but are still localized to the mitochondria (Sulistijo et al., 2003, Sulistijo et al., 2006, Frazier et al, 2006, Kubli et al., 2008, Kim et al., 2005). I found that these mutants were unable to induce autophagy in HeLa cells (Fig. 2B). Bnip3 contains a conserved cysteine residue that is important in stabilizing the homodimer (Kubli et al., 2008) and I found that Bnip3C64A was a significantly weaker inducer of autophagy compared to wild type Bnip3 (Fig. 2B). Moreover, the BH3 domain contains two conserved residues, leucine and aspartic acid (LXXXXD, in human Bnip3: LKKNSD) which are important for pro-apoptotic function of the BH3 proteins (Kelekar et al., 1998).The conserved aspartic acid in human Bnip3 is at position 115 in the BH3 domain of Bnip3 (Sattler et al., 1997). Interestingly, mutation of this residue to a glycine decreased Bnip3 ability to induce autophagy (Fig. 2B). These findings suggest that homodimerization and the BH3-domain of Bnip3 are important for induction of autophagy.

B. Bnip3 and GABARAP

Similar to LC3, the γ-aminobutyric-acid-type-A-receptor-associated protein (GABARAP) is a mammalian homologue of yeast Atg8 and has been reported to associate with the autophagosomal membrane (Mohrluder et al., 2009, Longatti

et al., 2009). Therefore, I investigated if GABARAP translocated to autophagosomes in response to Bnip3 overexpression. To verify that GABARAP co-localized with autophagosomes, HeLa cells were co-transfected with vector or Bnip3 plus GABARAP-GFP and mCherry-LC3. I found that in control transfected cells, GABARAP-GFP and mCherry-LC3 were both localized primarily in the cytosol but in Bnip3 overexpressing cells, GABARAP-GFP positive punctate co-localized with mCherry-LC3 positive punctate (Fig. 3A), confirming that GABARAP is recruited to autophagosomes in response to Bnip3. Moreover, only wild type Bnip3, but not the mutants, caused a significant increase in cells with punctate patterns of GABARAP-GFP indicating activation of autophagy (Fig. 3B). This suggests that GABARAP is also involved in Bnip3-mediated autophagy. Western blot analysis of lysates confirmed that all the Bnip3 constructs were expressed in the HeLa cells (Fig. 3C).

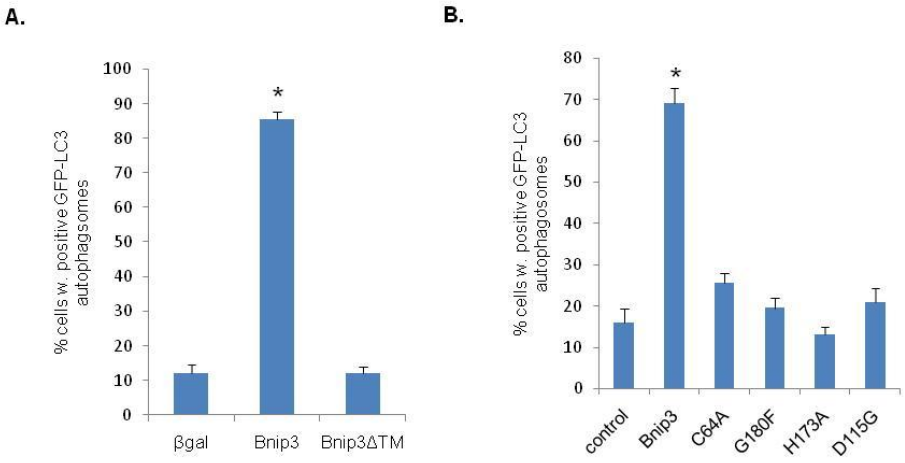

Figure 2. Bnip3-mediated autophagy is dependent on homodimerization of Bnip3. HeLa cells overexpressing Bnip3 or Bnip3 mutants were scored for the presence of GFP-LC3 positive autophagosomes. **A.** Bnip3 induced significant autophagy compared to β-gal (*$p<0.05$, n=3). **B.** Bnip3 but not the mutants induced significant autophagy compared to control (*$p<0.05$, n=4).

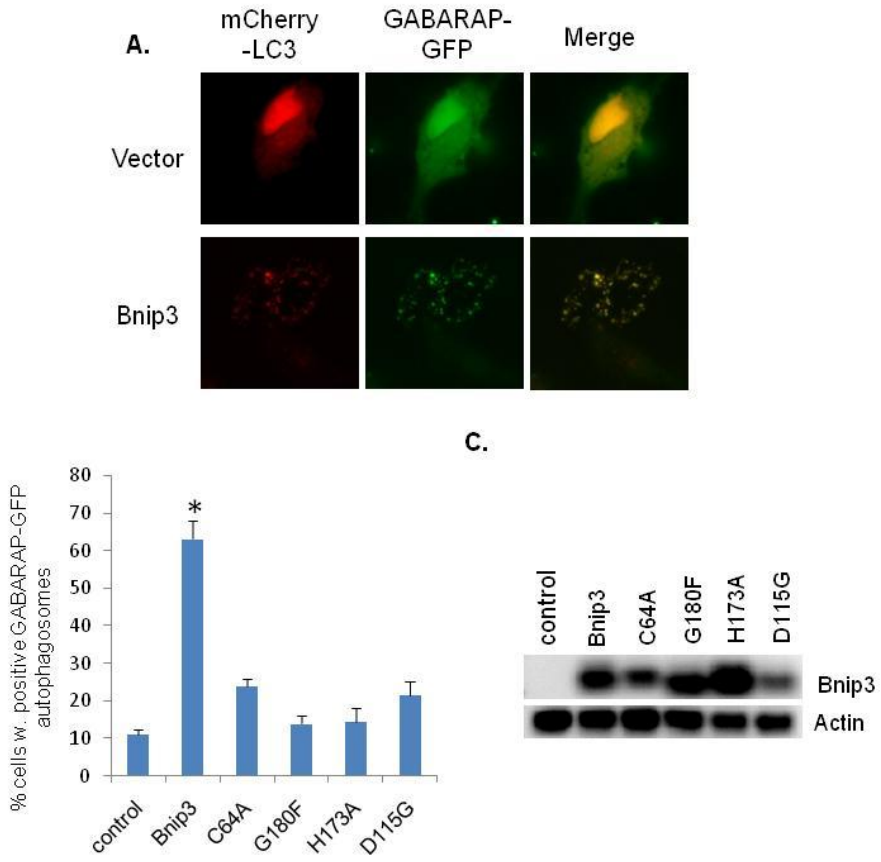

Figure 3. Bnip3 induces translocation of the LC3 homologue, GABARAP, to autophagosomes. HeLa cells were co-transfected with vector, Bnip3, or Bnip3 mutants plus GABARAP-GFP. **A.** GABARAP-GFP co-localizes with mCherry-LC3 positive autophagosomes in HeLa cells overexpressing Bnip3. **B.** HeLa cells were scored for the presence of GABARAP-GFP positive autophagosomes. Bnip3 induced significant autophagy compared to the mutants (*$p<0.05$, $n=4$). **C.** Expression of Bnip3 and mutants was verified by Western blot analysis. Actin was used as a loading control.

Chapter 2: Bnip3 Interacts with LC3 but not GABARAP

Introduction

Autophagy plays an important role in removing excess or damaged organelles (Klionsky et al., 2000). Our laboratory has previously found that overexpression of Bnip3 results in induction of mitochondrial autophagy (Quinsay et al., 2010, Rikka et al., 2011). However, the exact mechanism by which Bnip3 promotes the removal of mitochondria via autophagy is currently unknown. Recently, it was reported that Nix/Bnip3L, a Bnip3 homologue which also localizes to the mitochondria, interacts with LC3 and GABARAP, and that this interaction is used to link mitochondria to autophagosomes (Novak et al., 2010, Schwarten et al., 2009). Therefore, I investigated if Bnip3 also interacts with LC3 and GABARAP to promote removal of mitochondria via autophagy.

A. Homodimerization of Bnip3 is Important for the Interaction with LC3

First, I investigated if Bnip3 interacted with LC3 in HeLa cells. Co-immunoprecipitation experiments showed that Bnip3 interacted with LC3 when overexpressed in HeLa cells (Fig. 4A). Since my data suggest that the Bnip3 TM domain is essential for its ability to induce autophagy, I also investigated whether the TM domain was also important in the interaction between LC3 and Bnip3. As shown in figure 4A, Bnip3ΔTM did not co-immunoprecipitate with LC3, suggesting that an intact TM domain is important for its interaction with LC3. To further examine the role of homodimerization in the interaction with LC3, I

investigated whether Bnip3G180F and Bnip3H173A could bind to LC3. I found that both Bnip3G180F and Bnip3H173A had substantially reduced ability to interact with Bnip3 (Fig. 4B), suggesting that homodimerization of Bnip3 is important for its interaction with LC3.

Studies have reported that Nix interacts with both LC3 and GABARAP (Novak et al., 2010, Schwarten et al., 2009). Since I found that GABARAP translocates to autophagosomes in response to Bnip3, I investigated if Bnip3 interacted with GABARAP. HeLa cells were transfected with vector, Bnip3, or the different Bnip3 mutants plus GABARAP-GFP. GABARAP was immunoprecipitated using an antibody against GABARAP and the membrane was blotted with an antibody against Bnip3. Interestingly, I found that Bnip3 failed to co-immunoprecipitate with GABARAP (Fig. 4C), suggesting that Bnip3 only interacts with LC3. As expected, Bnip3G180F and Bnip3H173A did not interact with GABARAP (Fig. 4C).

B. Bnip3 Interacts with LC3 via its LC3 Interacting Region (LIR)

Both Nix and Bnip3 contains a conserved LC3 Interacting Region (LIR) in the N-terminus (Fig. 5A). The LIR has been shown to be the binding site for the Atg8 family members, and consists of the following sequence: W/YxxL/I (Kirkin et al., 2007). Studies have identified that the conserved tryptophan (W) in the LIR of Nix to be essential for the interaction between Nix and LC3/GABARAP (Novak et al., 2010, Schwarten et al., 2009). To investigate if this residue is also important for the interaction between Bnip3 and LC3, I used site-directed mutagenesis to

generate a Bnip3 mutant where the tryptophan was mutated to alanine. As shown in Figure 5B, co-immunoprecipitation experiments revealed that Bnip3W18A was unable to interact with LC3. Immunoblotting confirmed that Bnip3 and Bnip3W18A were expressed at equivalent levels when overexpressed in HeLa cells (Fig. 5C). This suggests that an intact LIR is important for the interaction between Bnip3 and LC3.

C. Bnip3W18A do not Affect Autophagy, but Reduces Mitophagy

The fact that Bnip3 interacts with LC3 on the autophagosome suggests that Bnip3 might function as an autophagy receptor that specifically targets mitochondria for removal by the autophagosomes. Thus, I investigated whether Bnip3W18A was able to induce mitochondrial autophagy in cells. First, I investigated whether the interaction between Bnip3 and LC3 was required for induction of general autophagy. HeLa cells were transfected with Bnip3 or Bnip3W18A plus GFP-LC3 to monitor formation of autophagosomes. At 48 h post-transfection, cells were fixed with 4% formaldehyde and analyzed by fluorescence microscopy. Quantitation of cells positive for GFP-LC3 autophagosomes showed that Bnip3W18A was as effective as wild type Bnip3 in inducing autophagy (Fig. 6A). Next, I measured whether the ability of Bnip3W18A to induce mitochondrial autophagy by analyzing co-localization between autophagosomes and mitochondria in cells overexpressing Bnip3 or Bnip3W18A. As shown in Figure 6B, mitochondrial autophagy was significantly reduced in cells overexpressing Bnip3W18A. Interestingly, mitochondrial autophagy was not

completely abrogated in cells overexpressing Bnip3W18A. This suggests that although Bnip3 might function as an autophagy receptor on mitochondria, there must be other mitochondrial proteins that can also function as autophagy receptors to ensure their removal.

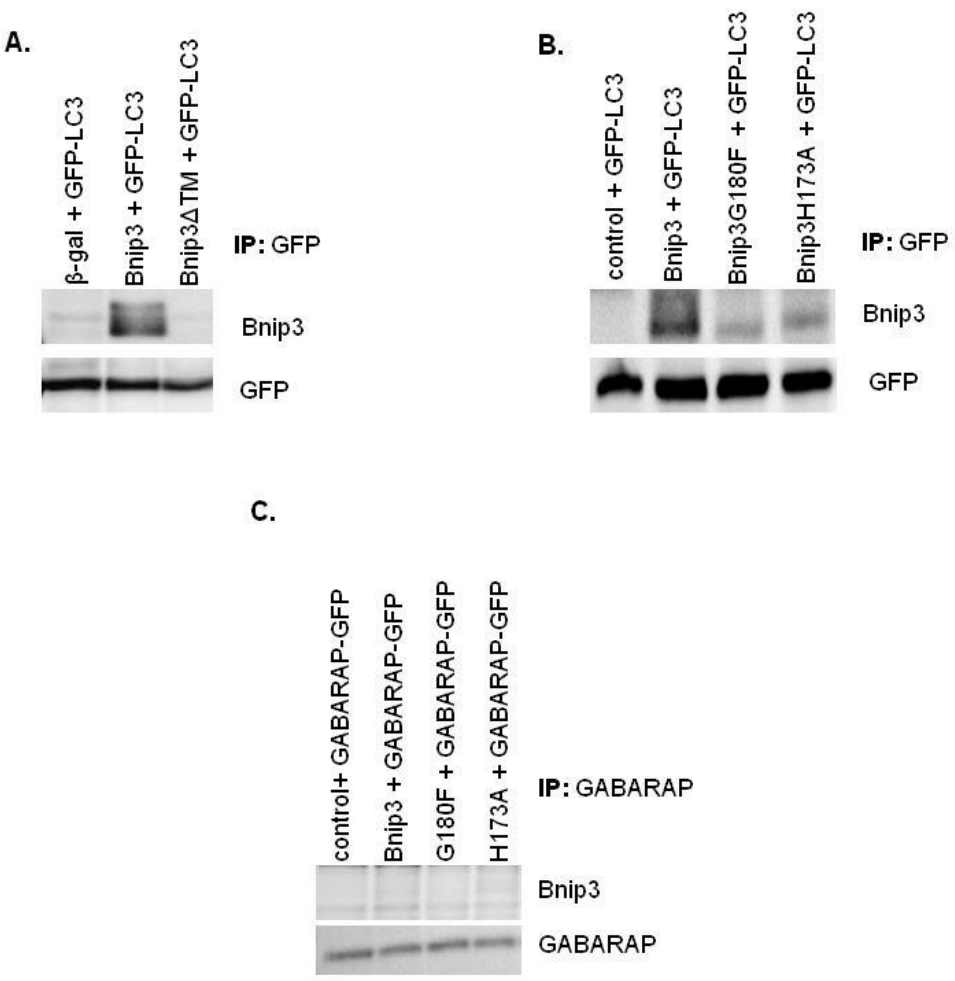

Figure 4. Bnip3 Interacts with LC3 but not GABARAP. **A.** Immunoprecipitation of GFP-LC3 using anti-GFP showed that Bnip3 but not Bnip3ΔTM interacted with LC3 **B.** Immunoprecipitation of GFP-LC3 using anti-GFP showed that Bnip3G180F and Bnip3H173A have reduced interaction with GFP-LC3. **C.** Immunoprecipitation of GABARAP-GFP using anti-GABARAP. Bnip3 did not co-immunoprecipitate with GABARAP.

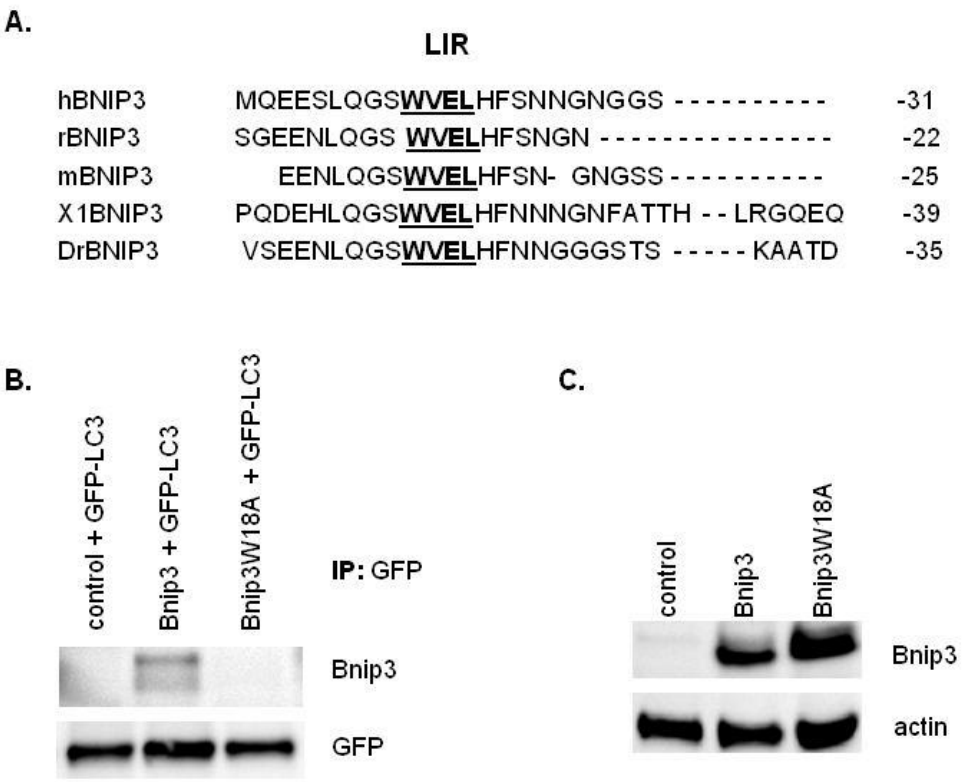

Figure 5. A point mutation in the LC3 Interacting Region (LIR) abrogates the interaction between LC3 and Bnip3. **A**. Bnip3 contains a conserved LIR sequence. **B.** Co-immunoprecipitation of GFP-LC3 from HeLa cells and Western blottting for Bnip3 and GFP. Only wild type Bnip3 co-immunoprecipitated with LC3. **C**. Equal expression levels of Bnip3 and Bnip3W18A were confirmed by Western blot analysis. Actin was used as a loading control.

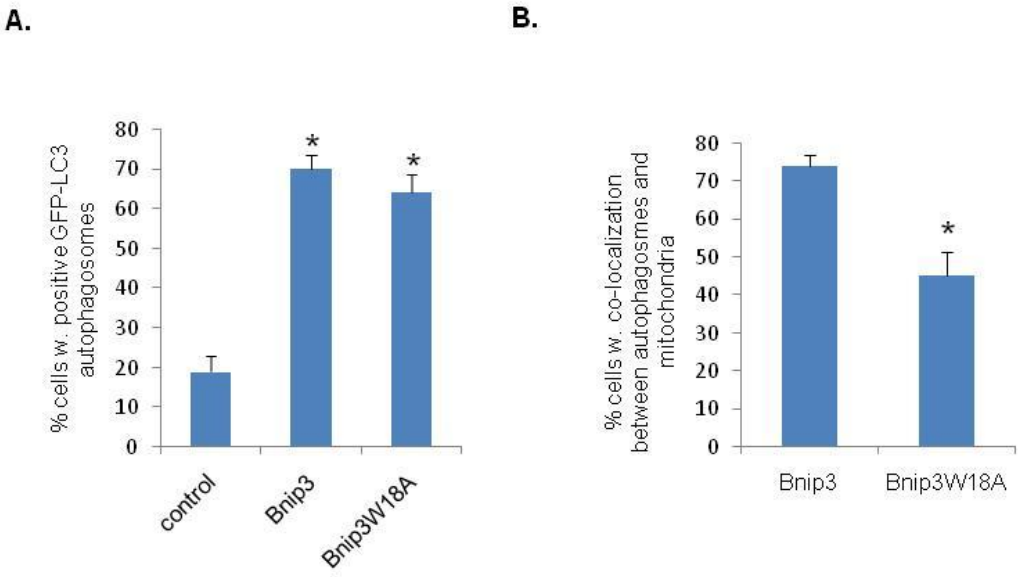

Figure 6. Bnip3W18A induced general autophagy in HeLa cells, whereas induction of mitophagy was reduced. HeLa cells overexpressing Bnip3 or a Bnip3W18A were scored for the presence of GFP-LC3 positive autophagosomes. **A.** Bnip3 and Bnip3W18A induced autophagy at similar levels in HeLa cells and significantly compared to control (*$p<0.05$, $n=3$). **B**. Quantitation of cells positive for mitochondrial autophagy. Mitophagy was determined by assessing co-localization between GFP-LC3 positive autophagosomes and TOM20 labeled mitochondria using fluorescence microscopy. Bnip3W18A induced significantly less mitophagy compared to Bnip3 (*$p<0.05$, $n=3$).

Chapter 3: ER and Mito-Targeted Bnip3

Introduction

Studies have found that several of the Bcl-2 proteins, including Bnip3 and Nix, are found in the endoplasmic reticulum (ER) where they perturb Ca^{2+} homeostasis (Ray et al., 2000, Ohi et al., 1999). Zhang et al. reported that specifically targeting Bnip3 to the ER induced cell death by increasing the release of Ca^{2+} from the ER with subsequent uptake by the mitochondria. The mitochondrial Ca^{2+}overload resulted in opening of the mPTP and loss of mitochondrial membrane potential (Zhang et al., 2009). Although it is clear that Bnip3 causes cell death even when it is localized at the ER, it is unknown whether it can induce autophagy from the ER. In this chapter, I have investigated if Bnip3 can induce autophagy when localized to the ER and whether ER-targeted Bnip3 interacts with LC3 to promote removal of ER.

A. Endogenous Bnip3 is localized to mitochondria and ER in Response to Hypoxia

First, I confirmed that endogenous Bnip3 is localized to the ER in HeLa cells. Bnip3 is expressed at very low levels under normal conditions in cells but is rapidly upregulated in response to hypoxia (Kubasiak et al., 2002, Regula et al., 2002). Therefore, I analyzed the cellular localization of Bnip3 in HeLa cells under normoxic and hypoxic conditions. Subcellular fractionation and western blot analysis of mitochondrial and ER fractions showed that Bnip3 was upregulated in HeLa cells after 24 h of hypoxia and that some of Bnip3 was localized to the ER (Fig. 7).

B. Mitochondria and ER-targeted Bnip3 Induce Autophagy and Interact with LC3.

To investigate if Bnip3 could induce autophagy from its ER location, we obtained Bnip3 constructs that were specifically targeted to the mitochondria or the ER. Bnip3 was targeted to the mitochondria or the ER by replacing the TM with sequence encoding ActA or cytochrome b5 (Cb5), respectively (Zhu et al., 1996). An increase in the number of autophagosomes could represent an increase in autophagic activity or accumulation of autophagosomes due to impaired fusion with lysosomes. Therefore, I investigated whether Bnip3 overexpression caused an increase in autophagic activity by incubating the cells with Bafilomycin A1 (Baf. A1), an inhibitor of the proton ATPase. Bafilomycin A1 inhibits fusion between autophagosomes and lysosomes and causes an accumulation of autophagosomes in the cell (Yoshimori et al., 1991). If there is no further increase in autophagy in response to Bnip3 in the presence of Bafilomycin A1, then flux is already inhibited in those cells. In contrast, if flux is not blocked, then the presence of Bafilomycin A1 will cause a further increase in autophagy. To investigate if Bnip3 enhanced autophagic activity or inhibited flux, HeLa cells were transiently transfected with vector, Bnip3, Bnip3Acta, or Bnip3Cb5 plus GFP-LC3 to monitor formation of autophagosomes with or without 50 nM Bafilomycin A1. The results showed that in the presence of Bafilomycin A1, autophagy was increased in cells overexpressing Bnip3, Bnip3Acta,or Bnip3C5b, suggesting that the increase in autophagy is due to increased autophagic activity and not to block in autophagy flux (Fig. 8A).

Since my data suggest that wild type Bnip3 interacts with LC3 on the autophagosome, I also investigated whether mitochondria and ER-targeted Bnip3 could interact with LC3. HeLa cells were transiently transfected with Bnip3Acta or Bnip3Cb5 and GFP-LC3. After 24 h, cell lysates were prepared and then incubated with anti-GFP to immunoprecipitate LC3. Subsequent immunoblotting for Bnip3 showed that both Bnip3Acta and Bnip3Cb5 interacted with LC3 (Fig. 8B). Although the TM domain has been replaced, Bnip3Acta and Bnip3Cb5 were still able to form homodimers (data not shown).

Next, I investigated if wild type Bnip3 promoted the removal of ER. HeLa cells were transiently transfected with vector, Bnip3, and Bnip3Cb5, plus GFP-LC3. Cells were fixed with 4% formaldehyde 48 h post transfection, and then stained for Calnexin to label ER. Using fluorescence microscopy, I analyzed co-localization between GFP-LC3 positive autophagosomes and Calnexin labeled ER. As shown in figure 8C, both wild type Bnip3 and Bnip3Cb5 induced significant ER autophagy compared to the control (Fig. 8C). This suggests that Bnip3 promotes removal of ER via autophagy.

C. Bnip3Cb5W18A Reduced ER Autophagy

Next, I investigated if mutation of the conserved tryptophan in the LIR of Bnip3Cb5 would disrupt the interaction with LC3. Using site-directed mutagenesis, I generated a Bnip3Cb5W18A mutant and assessed its ability to interact with LC3. Co-immunoprecipitation experiments showed that

Bnip3Cb5W18A was unable to interact with LC3 (Fig. 9A), confirming the importance of the Trp18 residue in the interaction between Bnip3 and LC3.

Next, I measured if Bnip3Cb5W18A induces ER autophagy by analyzing co-localization between autophagosomes and ER in cells overexpressing Bnip3Cb5 or Bnip3Cb5W18A. As shown in Figure 9B, ER autophagy was significantly reduced in cells overexpressing Bnip3Cb5W18A, suggesting that interaction with LC3 is important for ER removal via autophagy.

D. ER-targeted Bnip3 Promotes Mitophagy Independent of the mPTP

Opening of mitochondrial permeability transition pore (mPTP) is considered one of the signals for mitochondria to be removed by autophagosomes in cells (Elmore et al., 2001, Arrington et al., 2006, Teckman et al., 2004). Zhang et al. demonstrated that ER-targeted Bnip3 promoted opening of the mPTP in the mitochondria by causing release of Ca^{2+} from the ER (Zhang et al., 2009). Therefore, I investigated if Bnip3 induced mitochondrial autophagy when localized to the ER. HeLa cells were transiently transfected with vector, Bnip3Cb5, plus GFP-LC3 with or without Cyclosporine A (CsA), an inhibitor of the mPTP. Cells were fixed with 4% formaldehyde 48 h post transfection, and then stained for TOM20 to label mitochondria. Using fluorescence microscopy, I analyzed co-localization between GFP-LC3 positive autophagosomes and TOM20 labeled mitochondria. As shown in figure 10A, the induction of general autophagy was unaffected by the presence of CsA. Interestingly, Bnip3Cb5 induced significant mitophagy but the presence of CsA had no effect on

mitophagy (Fig. 10B). This suggests that Bnip3 at the ER can induce damage and removal of mitochondria independent of mPTP opening.

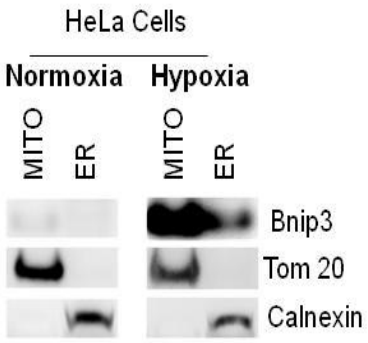

Figure 7. Bnip3 is upregulated in response to hypoxia and is localized to mitochondria and ER. HeLa cells were subjected to normoxia or hypoxia for 24 h and then analyzed by Western blotting for Bnip3

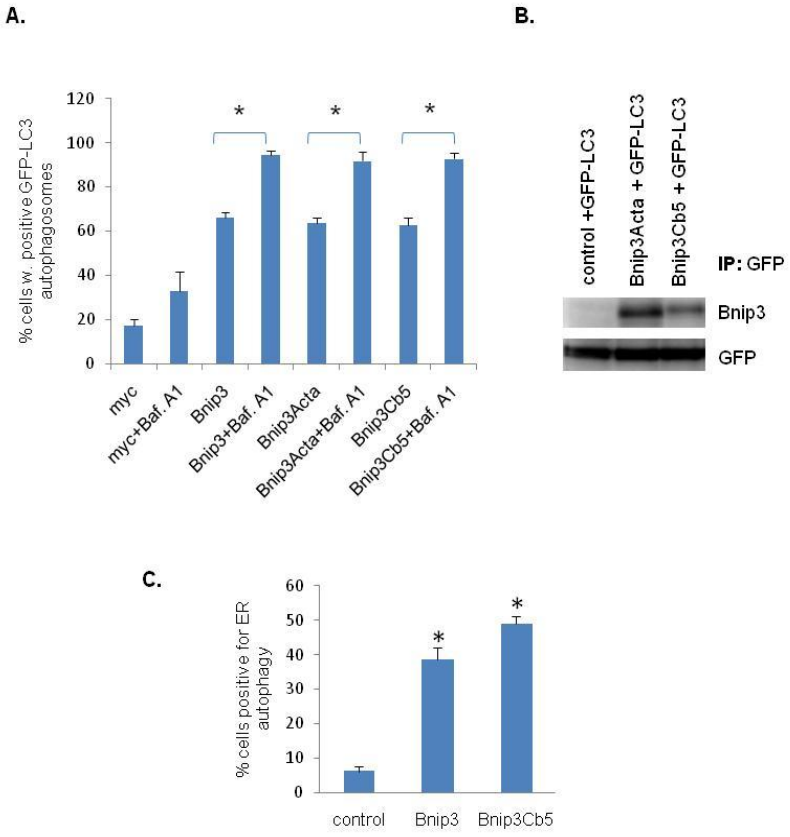

Figure 8. Mito and ER-targeted Bnip3 induce autophagy in HeLa cells. **A.** HeLa cells were transfected for 48 h and treated with 50nM Bafilomycin A1 (Baf. A1) for 2 h. Quantitation of autophagy in the presence and absence of Baf. A1 showed an increase in autophagy in the presence of Baf. A1 (*p<0.5, n=3). **B.** Mito- and ER-targeted Bnip3 interact with LC3. Co-immunoprecipitation of GFP-LC3 from HeLa cells and Western blottting for Bnip3 and GFP. Mito and ER-targeted Bnip3 co-immunoprecipitated with LC3. **C.** Quantitation of cells positive for ER autophagy. ER autophagy was determined by assessing co-localization between GFP-LC3 positive autophagosomes and Calnexin labeled ER . Both Bnip3 and Bnip3Cb5 induced significant ER autophagy compared to control (*p<0.05).

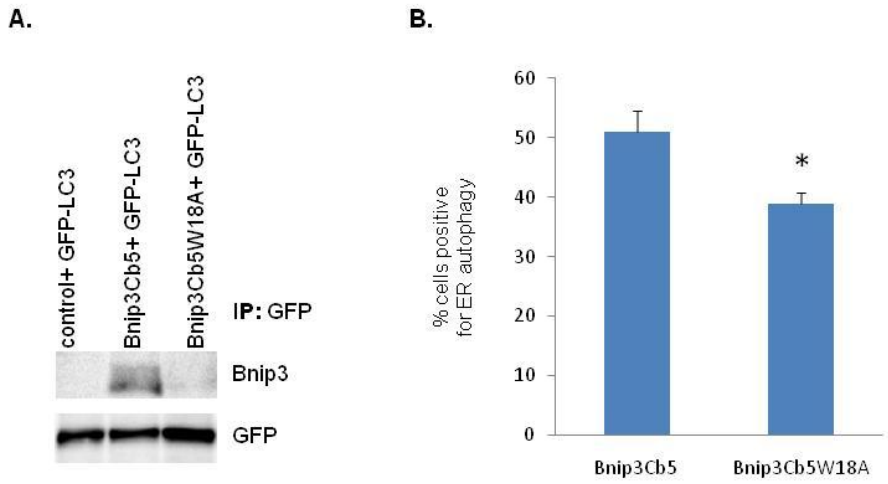

Figure 9. A point mutation in the LC3 Interacting Region (LIR) of Bnip3Cb5 disrupted the interaction with LC3. **A**. Co-immunoprecipitation of GFP-LC3 from HeLa cells and Western blottting for Bnip3 and GFP. Only Bnip3Cb5 co-immunoprecipitated with LC3. **B**. Quantitation of cells positive for ER autophagy. ER autophagy was determined by assessing co-localization between GFP-LC3 positive autophagosomes and Calnexin labeled ER using fluorescence microscopy. Bnip3Cb5W18A induced significantly less ER autophagy compared to Bnip3Cb5 (*$p<0.05$, $n=3$).

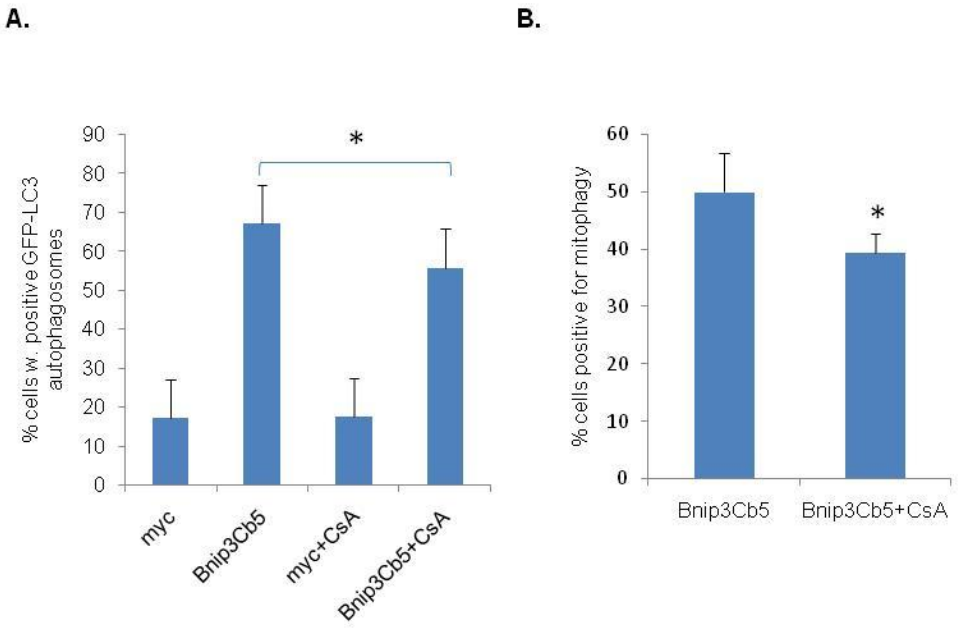

Figure 10. Cyclosporin A (CsA) treatment has no effect on Bnip3Cb5-induced autophagy. HeLa cells were transfected with vector or Bnip3Cb5, plus GFP-LC3 in the presence of 1μM CsA. **A.** CsA treatment did not affect induction of general autophagy compared to Cb5 alone (*p>0.05, n=3). Quantitation of cells positive for mitochondrial autophagy. Mitophagy was determined by assessing co-localization between GFP-LC3 positive autophagosomes and TOM20 labeled mitochondria. **B.** Bar graph shows that CsA did not reduce Bnip3Cb5-induced mitophagy (*p>0.05, n=3).

Chapter 4: Bnip3 and Autophagy in Vivo

Introduction

It is clear that autophagy is an important process in the heart and that defects in this process leads to development of heart failure. Many studies have demonstrated that autophagy is rapidly activated in response to ischemia in the heart. For example, Yan et al reported that autophagy is up-regulated in response to ischemia/reperfusion (Yan et al., 2005). Interestingly, another study reported that autophagy is inhibited during ischemia, but reactivated during reperfusion (Hamacher-Brady et al., 2007). Although studies have reported that autophagy is upregulated in cardiac myocytes in response to stress, it is unclear exactly if and when autophagy is upregulated in the heart during a myocardial infarction. In this chapter, I have examined whether autophagy is enhanced in the heart in response to permanent ligation of the left descending coronary artery (LAD).

A. Autophagy is Upregulated in the Myocardium after a Myocardial Infarction

In a time course experiment, I evaluated the upregulation of autophagy in hearts of mice that had been subjected to a myocardial infarction by ligation of the LAD. I analyzed autophagy in the border zone (BZ) as well as the remote zone (RZ) at different time points after the ligation. The BZ is the area right next to the infarcted area, whereas the RZ is a region distant to the infarct where the blood supply was not affected. Autophagy was analyzed by measuring changes

in LC3I and LC3II levels. LC3I is cytosolic and gets lipidated upon activation of autophagy to form LC3II. LC3II is recruited to the forming autophagosome. Thus, an increase the LC3II/I ratio indicates that autophagy is increased (Kabeya et al., 2000, Mizushima 2007). Western blot analysis for LC3II/I levels in the BZ at different time points revealed that autophagy was rapidly upregulated in the BZ (Fig. 11A). I found that the LC3II/I level increased as early as 4 h post ligation. In addition, Bnip3 has been reported to be upregulated in response to hypoxia in isolated myocytes (Kubasiak et al., 2002, Regula et al., 2002). To investigate the relationship between induction of autophagy and upregulation of Bnip3, I monitored changes in Bnip3 levels in the BZ. Interestingly, I found that Bnip3 was not upregulated until 24 h post-MI (Fig. 11B).

B. Upregulation of Parkin in the Ischemic Myocardium

Parkin is an E3 ubiquitin ligase that was recently shown to be recruited to dysfunctional mitochondria in cells which promoted their removal by autophagosomes (Narendra et al., 2008). Parkin is expressed in the heart (Kitada et al., 2000) but its functional role in the myocardium is currently unknown. To gain increased understanding of Parkin's role in the ischemic myocardium, I analyzed the expression of Parkin in the BZ and RZ after an infarct in mouse hearts. Western blot analysis revealed that Parkin was rapidly upregulated in the BZ (Fig. 12A). Parkin levels did not change in the RZ (Fig. 12B). These data suggest that Parkin might play an important role in the

adaptation to stress in the BZ. My laboratory is currently further exploring the functional role of Parkin in the myocardium.

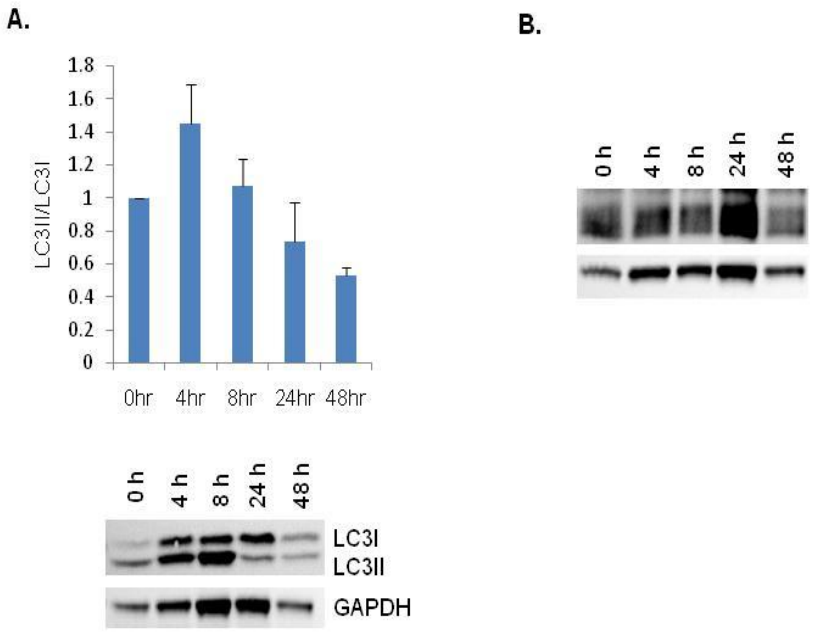

Figure 11. Autophagy is rapidly upregulated in the border zone of the infarct. **A.** Quantitation of LC3II/LC3I levels (n=4), and a representative Western blot for LC3, showing upregulation of autophagy at 4 h. **B.** Western blot for Bnip3 showing in increase in Bnip3 levels. GAPDH was used as a loading control.

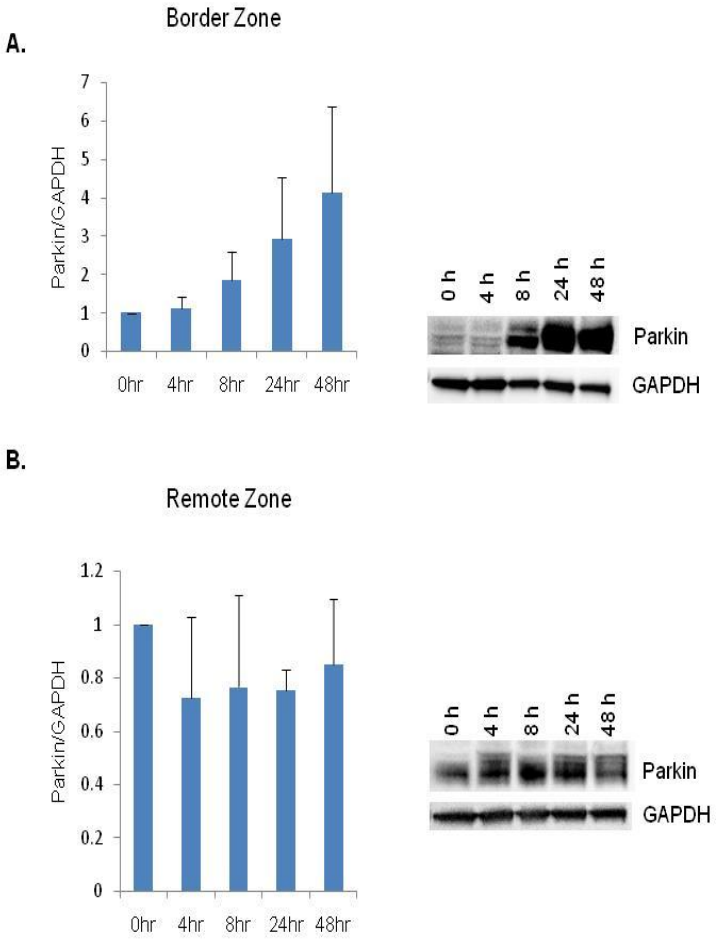

Figure 12. Rapid upregulation of Parkin in infarct. **A.** Quantitation of Parkin/GAPDH ratio (n=5) and a representative Western blot showing upregulation of Parkin expression in the border zone. **B.** Quantitation of the ratio of Parkin to GAPDH (n=5) and a representative Western blot illustrating Parkin expression in the remote zone.

Chapter 5: SUMMARY

The results from my study demonstrate the importance of the Bnip3 homodimerization in the induction of autophagy. Mutants that were unable to form homodimer were also defective in inducing autophagy. Moreover, my data show that the Bnip3 homodimer interacts with LC3 on the autophagosome. Scanning of the Bnip3 sequence revealed the presence of a conserved LIR domain (residues WVEL) in the N-terminus of Bnip3. I found that the tryptophan in the LIR of Bnip3 was important for the interaction with LC3. Interestingly, induction of general autophagy was not affected, but the level of mitochondrial autophagy was significantly reduced when the interaction between Bnip3 and LC3 was disrupted. I also found that Bnip3 localized to both the mitochondria and the ER and that Bnip3 induced autophagy from both locations. I discovered that ER-targeted Bnip3 interacted with LC3 and promoted removal of the ER via autophagy. In vivo experiments revealed that autophagy is rapidly upregulated in the BZ of the infarct. Bnip3 is also upregulated but at a later time-point.

Chapter 6: DISCUSSION AND CONCLUSION

Until recently, it was thought that autophagy was a non-selective process and that the autophagosomes randomly engulfed material in the cytosol. However, it is now clear that autophagy can be selective and specifically remove organelles such as mitochondria (Levine et al., 2008). For instance, my lab has found that Bnip3 specifically induces removal of mitochondria via autophagy in cardiac myocytes (Quinsay et al., 2010). It is still unknown how an autophagosome knows to remove a particular mitochondrion. It has been reported that the Bnip3 homologue Nix binds to LC3 and GABARAP on the autophagosome to promote removal of damaged mitochondria (Novak et al., 2010, Schwarten et al., 2009). Moreover, Nix has been shown to play an important role in the removal of mitochondria in erythroid maturation (Schweers et al., 2007). My data suggest that the Bnip3 homodimer can also interact with LC3 on the autophagosome and that disrupting the interaction results in reduced removal of mitochondria and ER suggesting that Bnip3 serves as an important cargo receptor that mediates selective autophagy and degradation of specific organelles (Fig. 13).

In contrast to Nix, I found that Bnip3 did not interact with GABARAP. In addition, LC3 consists of two different isoforms, LC3A and LC3B. Novak et al. reported that Nix interacted with LC3A but not LC3B (Novak et al., 2010). In my studies, I found that Bnip3 interacted with LC3B. Although the LIR is conserved

between Nix and Bnip3, they are only 56% homologous. This suggests that the non-homologous sequences are involved in determining the binding specificity. Also, my experiments in examining the interaction between Bnip3 and LC3 were limited to co-immunoprecipitation experiments. However, co-immunoprecipitation cannot distinguish between a direct or indirect interaction. Further, in vitro studies using isolated recombinant proteins are needed to confirm if there is a direct interaction between Bnip3 and LC3 or if they exist in a complex.

Although my data showed that disrupting the interaction between Bnip3 and LC3 by mutating the LIR significantly reduced removal of mitochondria, it did not completely abrogate mitochondrial autophagy. This suggests that Bnip3 is not the only “autophagy receptor” on the mitochondria. It is very likely that there are several other potential receptors that can bind to autophagy proteins to promote the removal of the mitochondria in response to Bnip3 overexpression. For instance, VDAC1 and mitofusin1 have both been shown to be subjected to ubiquitination by Parkin which then promotes removal of the mitochondria (Glauser et al., 2011, Geisler et al., 2010). p62 is a polyubiquitin-binding protein that also contains a LIR. p62 acts as an adaptor protein where it binds to ubiquitinated proteins on the mitochondria and then interacts with LC3 via its LIR on the autophagosome (Pankiv et al., 2007). This redundancy in “autophagy receptors” ensures removal of mitochondria upon injury even if one of the “autophagy receptors” is non-functional. Similar results were obtained in the study by Novak et al. which reported that disrupting the interaction between Nix

and LC3 or GABARAP through the mutation in the LIR caused only a partial inhibition of mitochondrial clearance (Novak et al., 2010).

My studies show that Bnip3 promoted removal of both mitochondria and ER in cells. Previous studies have reported that targeting Bnip3 to the ER causes cell death (Zhang et al., 2009, Zhu et al., 1996). Zhang et al. reported that Bnip3 caused release of Ca^{2+} from the ER and that this Ca^{2+} was taken up by the mitochondria. The Ca^{2+} overload caused opening of the mPTP and subsequent loss of mitochondrial membrane potential and cell death (Zhang et al., 2009). My experiments demonstrated that targeting Bnip3 to the ER still induced mitochondrial autophagy. Although opening of the mPTP has been reported to serve as a signal for mitochondrial autophagy, I found that mitochondrial autophagy by ER targeted Bnip3 was not reduced in the presence of the mPTP inhibitor CsA. Therefore, ER-targeted Bnip3 must damage or mark mitochondria for destruction in an mPTP independent manner. Bax and Bak are downstream pro-apoptotic effectors of Bnip3 and it is possible that Bax/Bak plays a role in mediating mitochondrial autophagy in response to ER-targeted Bnip3. Further studies are needed to determine the exact effects of ER-Bnip3 on the mitochondria.

My data show that autophagy is rapidly upregulated in the border zone after a myocardial infarction. This suggests that autophagy is important in salvaging myocytes in this area possibly by preserving amino acid and ATP levels and removing dysfunctional mitochondria. Dysfunctional mitochondria can be harmful to the cell and can cause activation of apoptosis. Although Bnip3 was

not upregulated until 24 h post-MI, there were still basal levels of Bnip3 that can be activated in response to the stress. In fact, Diwan et al. reported that Bnip3 knockout mice had less myocardial cell death in both border and remote zones, and had improved cardiac function after an MI compared to wild type mice (Diwan et al., 2007). Interestingly, my data suggest that autophagy is no longer enhanced at 24 h post-MI when Bnip3 is upregulated. My lab has found that inhibiting autophagy in cells overexpressing Bnip3 leads to cell death (data not shown). Therefore, if autophagy is no longer induced at 24 h, then Bnip3 will activate cell death.

Bnip3 has also been shown to be involved in the pathogenesis of heart failure, and is deregulated in pancreatic cancers, which is associated with the resistance to treatment (Erkan et al., 2005, Okami et al., 2004). Also, Murai et al. reported that Bnip3 inactivation in gastrointestinal cancer is essential in its progression (Murai et al., 2005). Thus, increased understanding of the mechanism and function of Bnip3 may be beneficial in understanding, treating, and preventing many diseases. Moreover, Bnip3 could serve as a great therapeutic candidate to treat and fully understand those diseases.

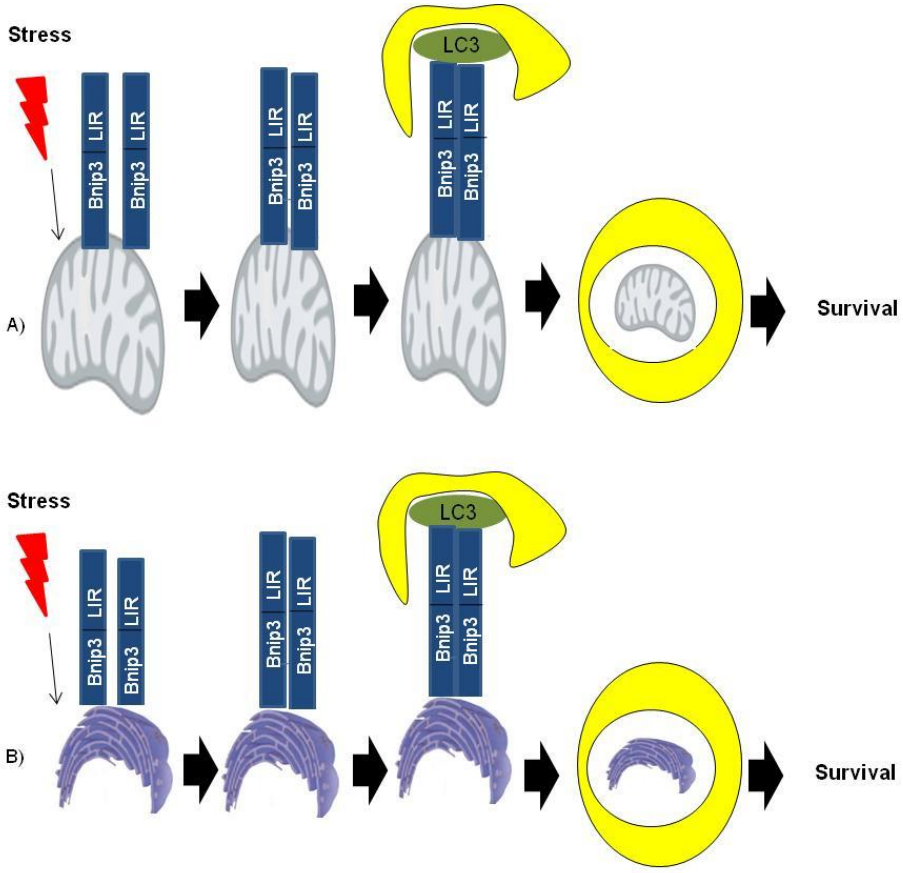

Figure 13. Bnip3 is a potential autophagy receptor. Bnip3 homodimer interacts with LC3 through LIR to promote selective mitochondrial (A) or ER (B) removal via autophagy.

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