p53 Mediates Autophagy and Cell Death by a Mechanism Contingent upon Bnip3

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Abstract

Autophagy is a process by which cells re-cycle organelles and macromolecular proteins during cellular stress. Defects in the regulation of autophagy have been associated with various human pathologies including heart failure. In the heart tumor suppressor p53 protein is known to promote apoptotic and autophagic cell death. We found p53 overexpression increased endogenous protein level of the hypoxia-inducible Bcl-2 death gene Bnip3 which leads to loss of mitochondrial membrane potential ($\Delta\Psi m$). This was accompanied by autophagic flux and cell death. Conversely, loss of function of Bnip3 in cardiac myocytes or Bnip3-/- mouse embryonic fibroblasts prevented mitochondrial targeting of p53 and autophagic cell death. These data provide the first evidence for the dual regulation of autophagic cell death of cardiac myocytes by p53 that is mutually dependent on Bnip3 activation. Hence, our findings may explain how autophagy and cell death are dually regulated during cardiac stress conditions where p53 is activated.

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List of Abbreviations

ANT - adenine nucleotide translocator

Atg - autophagy related genes

Bcl 2 - B-cell lymphoma 2

Bnip3- Bcl-2/adenovirus E1B 19-kDa interacting protein 3

BNIP3ΔTM- carboxyl terminal transmembrane deletion mutant of Bnip3

BNIP3Δexon3- spliced variant of Bnip3 generated by alternative splicing of exon3

Cyto C- cytochrome C

DMEM - Dulbecco's Modified Eagle's Medium

DNA - deoxyribonucleic acid

ER/SR-endoplasmic reticulum/sarcoplasmic reticulum

FBS - fetal bovine serum

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GFP- green fluorescent protein

HBSS- Hank's Balanced Salt Solution

HDAC - histone deacetylase

HPX - hypoxia

I/R - ischemia/reperfusion

IP3R- inositol triphosphate receptor

LC3- protein 1 light chain 3, a homologue of yeast Atg8

MFN - mitofusin

mPTP- mitochondrial permeability transition pore

mTOR - mammalian target of rapamycin

PBS - phosphate buffered saline

PI3K III - type III PI3 kinase

p53- tumor suppressor protein encoded by the TP53 gene

qPCR - quantitative polymerase chain reaction

SERCA - SR/ER calcium-ATPase

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

RNA - ribonucleic acid

ROS - reactive oxygen species

RT-PCR- reverse transcription polymerase chain reaction

TMRM- tetramethylrhodamine, methyl ester

VDAC- voltage-dependent anion channel

$\Delta\psi m$ - mitochondrial membrane potential

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I. INTRODUCTION

Heart failure is the major cause of death in North America and is on the rise as a global epidemic. The development of cardiovascular disease including heart failure is a result of reduction in the number of functioning and viable cardiac myocytes. As cardiac myocytes retain a limited regenerative capacity after birth, the functional loss of cardiac cells could have dramatic effect on cardiac performance. Understanding the complex underlying mechanisms of cardiac cell death is a major task for molecular cardiologists.

Autophagy provides a mechanism for the removal of accumulated macromolecular proteins and damaged organelles beyond proteasome activity during cellular crisis such as nutrient deprivation and oxidative stress. It has been described as the cell's major adaptive process in response to stressful stimuli. However, excessive autophagy may lead to cell death. This has become especially evident in post-mitotic terminally-differentiated cardiac myocytes, where defects in either too little or too much autophagy has been posited to contribute to pathological remodeling.

Autophagy is activated in response to the cellular stresses occurring in virtually all forms of heart disease. In some contexts, autophagy is an adaptive and compensative response, while in others, it is maladaptive and promotes disease progression. For example, in the context of ischemia-reperfusion injury, activation of autophagy during the early ischemic phase prior to reperfusion is considered protective while late or delayed activation of autophagy during reperfusion is detrimental[1].

The tumor suppressor protein p53 is a key mediator of cell response to a variety of stresses, including DNA damage and abnormal growth regulation[2-4]. In cardiomyocytes, p53 is best known for its role in maintaining cell cycle arrest, which might account in part for the failure of human hearts to regenerate adequately after injury. Another well-characterized feature of p53 is its ability to provoke apoptosis through transcription dependent and independent mechanisms. Loss of p53 function is associated with proliferative disorders and cancers underscoring its importance as a tumor suppressor protein. Recent studies reported that p53 is responsible for autophagy overload in certain transformed and immortalized cell lines. Although studies highlight the role of p53 in regulating mitochondrial-dependent autophagy, whether p53-induced autophagy in ventricular myocytes is adaptive or maladaptive, and involving cellular effectors is still poorly understood. Understanding the mechanisms by which p53 regulates cardiomyocyte cell death represents an attractive approach in supporting the survival of injured hearts[4-5].

Earlier work by our laboratory established that p53 localizes to mitochondria and triggers mitochondrial perturbations resulting in loss of mitochondrial $\Delta\Psi m$ and mPTP opening, which was inhibited by a Bcl-2-dependent mechanism[4]. Though the mode by which p53 triggered mitochondrial perturbations was undetermined, the fact that Bcl-2 suppressed the cytotoxic actions of p53, suggests that p53 likely impinges on one or more factors that regulate mitochondrial function[5-8].

p53 trans-activates a number of genes in response to cellular injuries[9]. Previous studies showed the pro-apoptotic Bcl-2 family member Bnip3 (Bcl-2/adenovirus E1B 19-kDa

interacting protein3) is among p53's downstreams targets[10]. Preliminary studies and sequence analysis revealed the presence of cis' acting elements for p53 within the human Bnip3 promoter, supporting the notion that Bnip3 may be transcriptionally regulated by p53. Bnip3 is sufficient to provoke mitochondrial defects leading to atypical programmed cell death and autophagy during ischemic and hypoxic injury. Therefore we reasoned that Bnip3 may be a putative downstream effector of p53-induced autophagic cell death.

In this dissertation, we examined the mechanisms by which p53 triggers autophagic cell death of ventricular myocytes. We provide new compelling evidence that p53 promotes autophagic cell death of ventricular myocytes and identified a novel mechanism that links p53-induced mitochondrial perturbations to autophagy via association with Bnip3.

II. LITERATURE REVIEW

1.0 Heart Failure

The heart is a pump receiving and delivering blood which carries oxygen and nutrients to the tissues of the body. The flow of blood which heart pumps to maintain metabolic requirements of the body is an important criterion to measure cardiac function. When the heart is not pumping blood efficiently, the clinical syndrome known as heart failure arises. Heart failure is a common condition that develops after the heart is damaged or weakened by diseases including heart attacks and other pathological conditions. It will be difficult for the body to function properly especially during stressful activities because the pumping action of the heart is not strong enough to supply corresponding metabolism.

Heart failure is a leading cause of morbidity in North America. It is estimated that there are about 500,000 Canadians living with heart failure[12]. Hospitalizations for heart failure among seniors is rising each year. Moreover, it is predicted to be a widespread cause of death worldwide by 2020[12].

There are many underlying risk factors for heart failure. Coronary artery disease, heart attack, and high blood pressure are the main causes of heart failure[13]. Thus the major cause for heart failure is due to damage to the heart muscle and the loss of functioning cardiac myocytes. This problem originates in the fact that cardiomyocytes lose the ability to divide shortly after birth[14]. Cardiac muscle has limited capacity for reparative mechanisms following injury caused by ischemia/reperfusion (I/R), oxidative stress, myocardial

infarction or inflammation[13]. Therefore, heart failure is irreversible. Moreover, when cardiomyocytes are lost, deposition of collagen occurs, which results in decreasing heart compliance, accompanied by increasing ventricular wall stress and impaired ventricular relaxation[15].

The molecular mechanism for heart failure involves numerous genetic pathways. Persistent responses at the cellular level, which might lead to organ-wide alterations, are highly correlated with cardiac dysfunction and heat failure. Much remains to be learned about the various mechanisms which modulate transition between compensatory responses in the heart and the development of heart failure[16]. Novel insights into pathophysiology and molecular mechanisms of heart failure will be significant for better understanding and developing novel therapeutic approaches.

2.0 Cell Death in the Heart

Cell death is a process which is essential for the development and homeostasis of multicellular organisms[17]. It is a highly orchestrated activity since either too much or too little cell death can contribute to pathogenesis[18]. Cardiac myocytes retain limited regenerative capacity after birth and if ongoing cell death occurs in the heart due to a variety of stimuli, such as ischemia, hypertension, or inflammation, the failing heart is unable to generate new cells to repair itself. Consequently dramatic effects on cardiac performance stem from the loss of functional cardiac cells. Thus, understanding the underlying mechanisms for cell death in the heart is critical in studying heart failure.

As there is no remedial therapy for heart failure caused by myocardial cell loss[20], one of most fascinating studies worldwide is myocardial regeneration by using progenitor cells to improve patients' cardiac function[21]. However, stem cell therapy is considered a novel tool with both substantial theoretical benefits and unpredictable side effects. It is equally critical to define which type of cell death the end-point is in different pathological conditions. Cardiac cell fate can be influenced by three major pathways of cell death: apoptosis, necrosis and autophagy, which are distinguished based on molecular underpinnings and morphologic features[22]. Necrosis and apoptosis both result in termination of functioning cardiac myocytes, whereas outcomes of autophagy could be dramatically different in specific cellular contexts[23].

2.1 Necrotic Cell Death

Necrotic cell death typically results from metabolic failure with rapid depletion of ATP. It is usually a response to acute stress or injury, such as ischemia. Necrosis has important biological consequences including the induction of the inflammatory response[17]. It was considered as a passive, unregulated type of cell death pathway. However, over the past decade it has been revealed to be well-regulated and can be programmed[24]. A recent study has shown that programmed necrosis can be stimulated by the same death ligands that activate apoptosis, such as tumor necrosis factor (TNF), Fas ligand (FasL), and TRAIL (TNF-related apoptosis-inducing ligand)[25]. In apoptosis, the central event in mitochondria is permeabilization of the outer mitochondrial membrane (OMM). In contrast, the triggering mito-chondrial event in necrosis is opening of mitochondrial permeability transition pore (mPTP)[28]. Loss of $\Delta\Psi m$, a primary feature of necrosis, often occurs in the late phase

of apoptosis. Thus cell death that begins as apoptosis might transit into necrotic morphology in the absence of disposal mechanisms[29]. The executed downstream signaling pathways lead to different modes of cell death.

As necrosis contributes to myocardial infarction and heart failure, the finding that cardiac necrosis can be programmed opens up the possibility of novel molecular and pharmacological approaches to prevent cell death in the heart and limit cardiac damage.

2.2 Apoptotic Cell Death

Apoptosis is a method by which unwanted or damaged cells or tissues are removed. It is an active, precisely regulated energy-requiring process[30]. Over the years, cardiac myocyte apoptosis has been studied as an important regulator of pathophysiological conditions, including heart failure. Apoptosis is characterized morphologically by membrane blebbing, externalization of phosphatidylserine, cell shrinkage, followed by DNA fragmentation, chromatin condensation, and rapid phagocytosis of apoptotic bodies by neighbouring cells in the absence of an inflammatory response.

Apoptosis can be induced by hypoxia, oxidative stress, ischemia-reperfusion and in patients with end-stage heart failure. Although it is known that apoptosis is involved in cardiac cell death, the mechanisms underpinning apoptotic execution. However, probably differ with various molecular stimuli. Apoptotic cell death can be activated through both the intrinsic mitochondrial pathway or the extrinsic death receptor pathway. The intrinsic apoptotic pathway is activated when mitochondria of the cell undergo functional changes

and release pro-apoptotic factors into the cytoplasm[33]. The extrinsic pathway is activated when cell-surface "death receptors" belonging to the TNF-receptor super family (TNFRSF) such as TNF-receptor 1 (TNFR1) [34] and death receptor 4 (DR4) are bound by extracellular death signals such as Fas ligand, tumor necrosis factor-α (TNFα) and TNF–related apoptosis-inducing ligand (TRAIL) .

2.3 Autophagic Cell Death.

Autophagy is a highly regulated process by which cells recycle organelles and macromolecular proteins during cellular stress. Although primarily an adaptive survival response, defects in the processes that govern autophagy can be maladaptive and trigger cell death. Autophagic cell death, or programmed cell death type II (PCD II), is caspase-independent and does not involve DNA fragmentation[35]. Autophagic cell death is characterized by the formation of autophagosomes, which degrade cellular components to a level where normal function is compromised and cell death occurs. Autophagy and autophagic cell death will be discussed as the main topic of this dissertation in the next section.

3.0 Autophagy

3.1 Autophagy Overview

The term "autophagy" is derived from the Greek words "auto" meaning self and "phagy" meaning eat. As indicated, autophagy is an evolutionarily conserved catabolic process that serves to degrade long-lived proteins or damaged organelles through a lysosomal-

mediated pathway as part of a normal homeostatic process. Autophagy provides a mechanism for generating substrates for ATP synthesis during cellular crisis such as nutrient deprivation or oxidative stress.

Autophagosomes, which are digestive vacuoles of autophagy that result from the fusion of a lysosome with an autophagic vacuole, have been observed by electron microscopy in mammalian cells since as early as the 1950s[37]. However, substantial progress in molecular studies has been made only in the past 15 years[38]. To date, genetic studies in yeast have identified 32 autophagy-related genes (Atg) involved in the autophagy cas-cade[39]. Since this initial discovery, several Atg yeast homologs have been identified and characterized in higher eukaryotes[40], demonstrating the evolutionary importance and conservation of this process.

Classically three types of autophagy have been detected in mammalian cells: chaperone-mediated autophagy, microautophagy, and macroautophagy. Chaperone-mediated autophagy sequesters proteins with a KFERQ-like motif through association with the Hsc70 complex, which are directly targeted to the lysosomes for degradation[41]. During microautophagy, the lysosomal membrane invaginates to engulf portions of the cytoplasm, which are consequently broken down by lytic enzymes once the entire structure is enclosed. During macroautophagy, specialized double-membraned vacuoles are formed for cargo transportation and removal. Though all three types of autophagy operationally exist in cells, macropahgy is the most commonly studied, and for simplicity will be the primary focus hereafter.

3.2 Autophagy in Regulating Cardiac Cell Fate

Cardiomyocytes display a baseline level of autophagy to maintain normal phenotype and function[42]. Efficient sequestration and clearance of unwanted or damaged cellular components, including organelles and ubiquitinated proteins, are crucial for cell survival, development and normal tissue homeostasis; however, excessive autophagy may lead to cardiac cell death[43].

This has become especially evident in post-mitotic terminally differentiated cardiac myocytes, where defects in either too little or too much autophagy have been posited to contribute to the underlying pathology, which is in agreement with studies in other cell types such as skeletal myocytes and neuronal cells. In the beginning of our study, we discovered that blocking autophagy with class III PI3K inhibitor 3-MA markedly aggravated cell death at 4 hrs which is considered the early phase of autophagy, but attenuated cell death at 18 hrs, which is considered the late phase of autophagy(see Figure 1). These results together with others indicate the outcome of autophagy is time-dependent. Early autophagy on a mild level is protective from cellular conditions of inadequate nutrient supply, whereas it eventually leads to cell death when excessive.

Autophagy is activated in response to the cellular stresses occurring in virtually all forms of heart disease[44]. In some settings, the autophagic response is beneficial and promotes survival; while in other contexts, it is maladaptive and promotes cell death. However, in contrast to apoptosis or programmed necrosis, no specific autophagic death pathway has been identified. The ensuing cell demise associated with the autophagic process may result from defects in autophagosome formation, fusion of the autophagosome with the ly-

sosome, or clearance of the autophagolysosomes, which collectively result in autophagic cell death[45]. Taken together, autophagy may act as both a temporary protective mechanism during a brief stressful episode, and a mode of cell death in response to prolonged injury.

3.3 Autophagy in Heart Disease

In the myocardium, autophagy at the basal level is crucial for maintaining tissue homeostasis[46]. Defects in autophagosome formation or clearance can cause cardiac dysfunction including heart failure[46]. One example highlighting the critical importance of autophagy is best illustrated in patients with Danon disease. These patients suffer from severe cardiomyopathy due to a defect in the autophagic pathway. Notably, Danon disease manifests as an excess accumulation of autophagic vacuoles within cardiac myocytes due to a mutation in lysosome associated membrane protein LAMP2, a membrane protein important for autophagosome-lysosome fusion[49]. The cardiomyopathy and heart failure occur as the consequence of autophagosome- lysosome fusion defect induced accumulation of cytotoxic proteins and damaged organelles.

Autophagy has also been detected in several other cardiac pathologies including ischemia-reperfusion, hypoxia and the myocardial infarct border zone[48]. In tissues from patients with end-stage heart failure, evidence of autophagy has also been reported[46-48]. However, it still remains unclear whether death from autophagy is a sign of a failed cardiomyocyte repair mechanism or a bona fide suicide pathway for discarding failing cardiomyocytes. Notably, conditional gene knockdown studies uncovered that severe cardiac contractile dysfunction develops after one week of Atg5 ablation[46]. Consistent with these findings,

defects in autophagy trigger classic hypertrophic signaling including increased cell size, activation of the fetal gene program and ultimately diminishing cell viability[46]. Similarly, autophagy has been detected in the heart following aortic banding-induced cardiac remodelling, where heterozygous deletion of the Beclin-1 gene not only abolished afterload-induced autophagy, but substantially diminished pathological remodelling of the heart as well[52].

Therefore, a critical balance of autophagy is essential in heart function. This notion is further exemplified in the context of ischemia-reperfusion injury, where activation of autophagy during ischemia is protective, while activation of autophagy during late reperfusion is detrimental which may shift cell fate to death[53]. Mechanistically, it has been recently proposed that early autophagy induced by ischemia or starvation activates a distinct cell signaling pathway from that of autophagy activated by reperfusion, and this might explain the reported differences observed in cell fate following different injuries in the heart[54].

The relative temporal and spatial relationship of the impending stimuli, as well as downstream signaling pathways activated by the autophagic stimuli, may ultimately determine whether autophagy prevents or promotes cardiac pathology.

3.4 Cross-Talk Between Autophagy and Apoptosis

Although apoptosis, necrosis, and autophagy are considered independent processes, there is growing evidence that different modes of cell death may be inextricably linked to each other. The physiological role of autophagic cell death in apoptotic-competent cells is not

well understood, and whether autophagy kills cells via an interaction with the apoptosis pathways or via an entirely independent pathway remains highly debated[58]. Indeed, there is crosstalk between apoptosis and autophagy in cell death regulation. In this regard, the mitochondrion is considered a major point of convergence, common to both apoptosis and autophagy. Recently, Atg12 was discovered to interact with Bcl-2 and Mcl-1 and inhibit their pro-survival effects to promote Bax-induced programmed cell death[61]. Although these findings demonstrate a molecular mechanism for the crosstalk between autophagic and apoptotic cell death, the role of this interaction in cardiac myocytes remains undetermined.

Current theories purport that basal levels of autophagy are essential for clearance of damaged mitochondria to avoid ROS production and inappropriate activation of the intrinsic mitochondrial death pathway. Notably, pig hearts subjected to repeated bouts of ischemia-reperfusion demonstrated that apoptosis was maximal after 3 ischemia-reperfusion cycles with apoptotic rates dropping at a time when autophagy was activated[63]. This supports our notion, and that of others, that autophagy may initially antagonize, or delay, programmed cell death by eliminating damaged mitochondria or misfolded proteins that may otherwise induce cell death.

Conversely, de-regulated or excessive autophagy are associated with cell death. For example, in a model of HIV infection in which T-cells expressing CD4/CXCR4 interact with cells expressing HIV-1-encoded envelope glycoproteins, autophagy appears to activate apoptosis[67].

4.0 Molecular Regulation of Autophagy

Autophagy is a tightly regulated process, where autophagosome formation is controlled by a number of evolutionary conserved Atgs. The autophagic cascade involves a complex series of stepwise events, including induction, cargo recognition and selection, vesicle formation, autophagsome-vacuole fusion, breakdown of the cargo, and finally the liberation and re-cycling of the degradation products into the cytosol[68]. The class III phosphatidylinositol 3-kinase (PI3K) complex mediates nucleation of an intracellular membrane fragment termed the phagophore, which can be derived from pre-autophagosomal structures coming from the plasma membrane and other organelles, such as the endoplasmic reticulum or mitochondria[69.] By association with the Atg proteins, namely Atg5, Atg12 and Atg16, the isolated membrane curves and begins to engulf cytosolic proteins, protein aggregates, and targeted organelles (such as mitochondria)[70]. The gene product of LC3 is cleaved by Atg4, becoming LC3-I, and conjugated with phosphatidylethanolamine (PE), to become LC3-II[71]. LC3-II is recruited to the pre-autophagosome membrane, together with the transmembrane protein Atg9, facilitating phagophore expansion. LC3-II remains tethered to the pre-autophagosome throughout maturation until degradation by the lysosome. The membrane invaginates and closes, forming autophagosome[72]. Upon vesicle completion, most of the Atg proteins are dissociated from the autophagosome, allowing autophagosome-lysosome fusion and cargo degradation by lysosomal proteases. The contents are catabolized by lysosomal enzymes, and thereby provide the cell with amino acids and other small molecules that can be utilized for ATP production or recycled in future anabolic reactions.

4.1 Signal Transduction Pathways of Autophagy

Under basal states the occurrence of autophagy is relatively low[48], but it is rapidly induced in response to changes in nutrient status or metabolic stress conditions that compromise the cells' energy status[75], such as starvation, hypoxia and ER stress. Furthermore, autophagy has also been shown to be activated by pharmacological agents (e.g. the mTOR inhibitor rapamycin), innate immune signals, and in disease states such as bacterial, viral, and parasitic infections.

4.1.1 Starvation

The most potent physiological inducer of autophagy is nutrient starvation[76], which dramatically induces autophagosome formation. In cardiac myocytes, prolonged starvation is observed to induce accumulation of autophagosome which eventually leads to cell death (see Figure 2). Two well-characterized signaling cascades that regulate autophagy during nutrient stress include the mTOR and AMPK pathways[77]. Under basal states mTOR typically inhibits autophagy by phosphorylating and inactivating Atgs, such as ULK1 and Atg13, which ultimately inhibits the Beclin-1/Vsp34/class III PI3K complex[77]. In contrast, inhibition of mTOR during nutrient stress from loss of class I PI3K-Akt activity, promotes autophagy[78]. Moreover, declining ATP levels during nutrient or metabolic crisis activates AMPK which phosphorylates the tuberous sclerosis complex (TSC) 1/2, which further inhibits Rheb-dependent mTOR activity[79]. Hence, this highly regulated signaling pathway for activating autophagy provides a mechanism for generating ATP and cell survival during nutrient stress. However, persistant autophagic responses eventually

compromises cellular metabolism and leads to cell death due to loss of functioning mitochondria.

4.1.2 ER Stress

Endoplasmic reticulum (ER) stress is another common stress stimulus inducing autophagy. The ER is a key compartment in the cell that facilitates folding of newly synthesized proteins and initiates pathways of vesicular movement of membrane and proteins to various organelles and the plasma membrane[56]. A number of factors, for example, glucose deprivation, hypoxia and oxidative stress[75] and Ca^{2+} efflux from the ER[56], lead to the accumulation of unfolded proteins within the ER lumen and exceed its folding capacity[56]. Once the unfolded protein response is activated, ER stress stimulates autophagy through multiple pathways, including the PERK-eIF2α pathway and the IRE1-JNK1 pathway. In non-muscle mammalian cells, the ER serves as the major intracellular Ca^{2+} reservoir. Calcium-activated calmodulin-dependent kinase kinase-β (CaMKKβ) is stimulated by increases in the intracellular Ca^{2+} level and further activates AMPK, and autophagy.

4.1.3 Hypoxia

Accumulating evidence suggests that hypoxia also induces autophagy in mammalian cells[82]. This particular area of study is in its infancy, with the signaling pathways responsible for autophagy induction contingent on the given cell-type studied[83]. Hypoxia-inducible factor-1 (HIF-1) appears to be the dominant transcription factor acutely induced by hypoxic conditions in most cell types[84]. In multiple cell lines, mitochondria are re-

moved by mitophagy in response to hypoxia, which is dependent on HIF-1. Enhanced mitochondrial autophagy during hypoxia was suggested to be an adaptive response, reducing the levels of reactive oxygen species (ROS) and protecting cell integrity, whereas prolonged hypoxia mediates autophagic cell death[87]. However more recent works substantiate the role of autophagy in promoting cell death under hypoxia. In an early study of autophagy in neonatal cardiac myocytes death and survival, we observed excessive cell death after 18 hours hypoxia which was simultaneous with the peak of autophagic flux, demonstrating autophagy in neonatal cardiac cells corresponds to cell death under hypoxia (see Figure 2).

5.0 Molecular Regulators of Autophagy

Autophagy has long been considered to be a non-selective bulk degradation pathway, however several signaling pathways have been proved to regulate autophagy selectively in mammalian cells. The induction of autophagy by mTOR inhibition under starvation conditions is a well-established autophagic pathway in mammalian cells[92]. In addition various pathways and small molecules regulating autophagy via mTOR and mTOR-independent mechanisms have been identified in recent years[93]. Regression of cardiac autophagy is accompanied by activation of a unique set of genes, including fetal-type genes and genes involved in protein degradation[94].

5.1 Regulation of Autophagy by p53

The prototypical tumor suppressor gene, p53 has been extensively studied as an inducer of programmed cell death[95].Among the best characterized features of p53 is its ability to

provoke apoptosis through transcription dependent and independents mechanisms. p53 can act as a DNA-bound transcription factor to promote the expression of pro-apoptotic genes, such as the multi-domain Bcl-2 family member Bax and the BH3-only protein PUMA[96]. p53 was found to play a cytoplasmic role by targeting mitochondria directly to induce outer mitochondrial membrane permeabilization and intrinsic cell death[97]. Work by our laboratory has demonstrated that a transcriptionally inactive mutant of p53 is nearly as potent at inducing cell death as the wild-type transcriptionally competent p53 in cardiac myocytes[46]. Recently, p53 has also been reported to promote autophagy in certain transformed and immortalized cell lines[4]. As recently reviewed by Guido Kroemer's group, nuclear p53 is capable of inducing an autophagic response by activating pro-autophagic Bcl-2 family members or target genes that inhibit mTOR signaling. During metabolic stress, several molecular cascades promote autophagy via the stabilization of p53, as well as eliciting signaling pathways by extracellular ligands through the mitogen activated protein kinase (MAPK) family, including c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinases (ERKs). Furthermore, the ability of p53 to induce autophagy is attenuated by mutants of p53 that have lost their ability for transactivation or ability to interact with Bcl-2 family members[98].

The underlying mechanism is not well understood and it remains to be evaluated in cardiac myocytes under relevant pathological conditions. Distinct from the role of p53 in programmed cell death, whether autophagy induced by p53 during cellular stress in ventricular myocytes is adaptive, maladaptive or involves cellular effectors common to apoptosis is poorly defined. Earlier work by our laboratory established that p53 localizes to

mitochondria triggering loss of mitochondrial ΔΨm and mPTP changes that were inhibited by a Bcl-2 dependent mechanism[4]. Though the mode by which p53 triggered mitochondrial perturbations was not undetermined, the fact that Bcl-2 suppressed the cytotoxic actions of p53, suggests p53 likely impinges on one or more factors that regulate mitochondrial function. This dissertation is focused on p53-induced autophagy.

5.2 Regulation of Autophagy by the Bcl-2 Gene Family

The Bcl-2 family members are characterized by the presence of one to four Bcl-2 homology (BH) domains, and are functionally classified as either pro-survival or pro-death. The Bcl-2 family members are crucial mediators of cell fate decision. In addition to being considered as regulators of apoptosis, Bcl-2 proteins also participate in the regulation of autophagy and autophagic cell death.

The BH3-only protein Beclin-1, the mammalian ortholog of Atg6, serves as an essential regulator of autophagy[90]. Beclin-1 activates autophagy when complexed with class III PI3K by inducing autophagosome formation. Beclin-1 was originally discovered as an interacting protein of the prototypical antiapoptotic protein Bcl-2[103], and subsequent studies demonstrated that Bcl-2 and/or Bcl-XL antagonize autophagy when complexed with Beclin-1[104]. The functional regulation of the Beclin-1 and Bcl-2/Bcl-XL complex appears to involve the BH3 domain of Beclin-1 and BH3 receptor domain of Bcl-2 and Bcl-XL[104]. Physiologically, the interaction between Beclin-1 and Bcl-2/Bcl-XL is stabilized under nutrient-rich conditions, likely through alterations in Akt/PI3K and AMPK-mTOR axis; whereas, nutrient deprivation results in the disruption of the interaction between Beclin-1

and Bcl-2/Bcl-XL, dissociating Beclin-1 from Bcl-2 inhibition ostensibly. Notably, Bcl-2 was found to only inhibit autophagy when localized to the ER and not mitochondrion[35] The interaction between Beclin-1 and Bcl-2/Bcl-XL takes place at the inositol triphosphate receptor (IP3R)[90].

The functional regulation of the Beclin-1 and Bcl-2/Bcl-XL complex appears to occur from competition between the BH3 domain of Beclin-1 and other BH3-only proteins for the BH3-receptor domain on Bcl-2 and Bcl-XL. Previous studies suggest a model whereby starvation and/or hypoxia actives BH3-only proteins of the Bcl-2 family, such as Bad, Bnip3, or Bnip3L/Nix[105-107]. Upon activation, these BH3-only proteins serve to disrupt the inhibitory interaction between Beclin-1 and Bcl-2/Bcl-XL and activate apoptosis, autophagy or both.

5.3 Regulation of Autophagy by Bnip3

The hypoxia-inducible death protein Bnip3 (for Bcl-2/adenovirus E1B 19-kDa interacting protein3) is an unique BH3-only pro-apoptotic family member, as it does not require the BH3-like domains to induce programmed cell death[108]. Bnip3-induced mitochondrial perturbations and cell death are dependent on its carboxyl-terminal transmembrane domain (TM). The Bnip3 promoter is strongly repressed under normal basal conditions but highly induced during hypoxic or ischemic stress[86]. In response to hypoxia, HIF-1 dependent Bnip3 expression was found to induce mitochondrial turnover[109]. HIF-1 independent Bnip3 was also reported under stress conditions[110]. Several additional stimuli are known to induce Bnip3 expression, although the specific transcriptional regulators involved are unknown.

Bnip3 up-regulation and mitochondrial localization have been implicated in activation of apoptosis, autophagy and necrosis, depending on the cell type and surrounding stimulus. On one hand, Bnip3 is responsible for loss of cardiac myocytes by triggering mitochondrial permeabilization, swelling, and cytochrome c release[123,124.] On the other hand, Bnip3 is sufficient to induce autophagy/mitophagy by recruiting LC3II to depolarized mitochondria[126]. The precise mechanism by which Bnip3 regulates autophagy has yet to be fully understood. An earlier study shows that Bnip3 promotes autophagy by liberating Beclin-1 from Beclin1-Bcl2 interaction. Bnip3 may also indirectly promote autophagy by causing mitochondrial dysfunction. Mitochondrial ROS, which is produced after loss of mitochondrial membrane potential induced by Bnip3, are potent stimuli of autophagy[125]. The removal of damaged mitochondria through selective mitophagic pathway is thus signaled[127].

How is it possible for Bnip3 to induce both apoptosis and autophagy, both of which seemingly evoke diametrically opposing actions on cell survival? Considering that the major mode of Bnip3-induced cell death is to perturb mitochondrial function, we propose a model whereby following Bnip3 gene activation, mitophagy may be initially induced via Bnip3's established ability to induce mitochondrial permeability transition pore opening. Presumably, mitochondria that have undergone permeability transition would then be removed from the cell by autophagy, postponing or delaying the induction of Bnip3-mediated programmed cell death and limiting cellular injury. Autophagy, if activated early, may initially serve as a protective response to Bnip3-mediated death signaling[11,128].

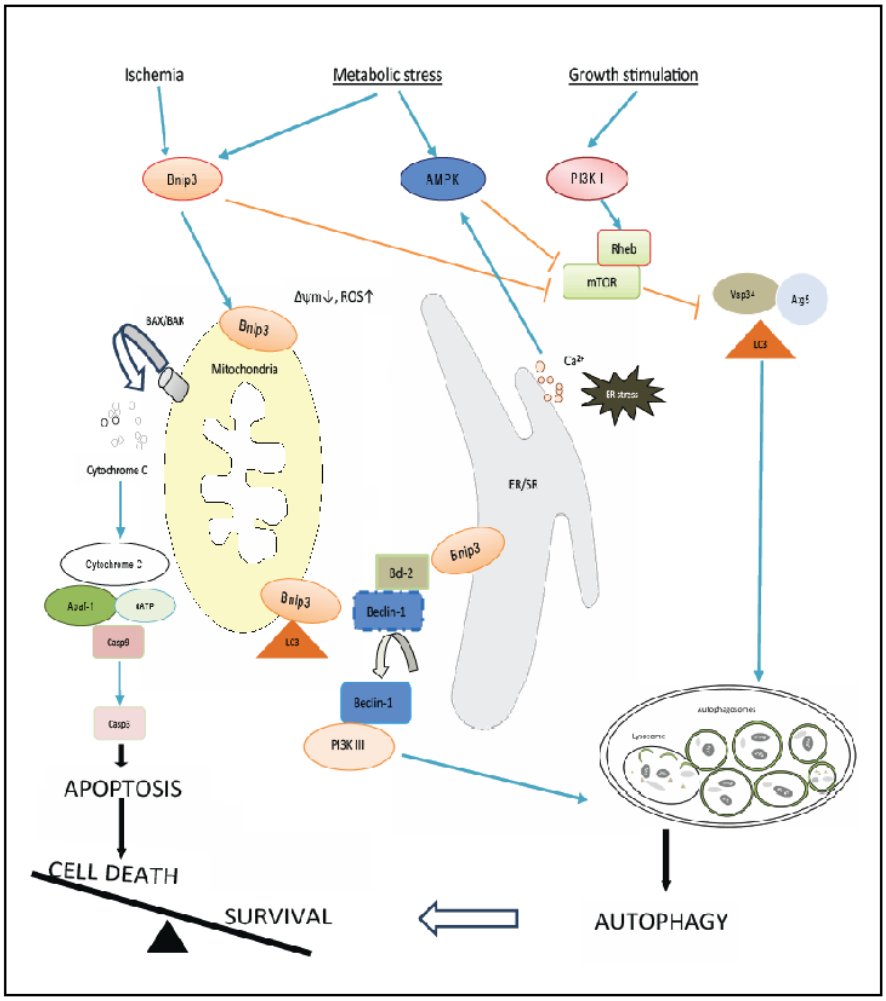
However, excessive autophagy and/or failure to discard dysfunctional mitochondria would eventually lead to cell death.

Taken together, Bnip3 activates apoptotic and autophagic cell death through mitochondrial dysfunction, by several different mechanisms which have not yet been fully elucidated. Notably, our preliminary studies revealed a marked induction of Bnip3 gene expression by p53 in postnatal cardiac myocytes. We reasoned that Bnip3 may be a putative down-stream effector of p53 in the autophagic pathway.

6.0 Alternative Splicing of Bnip3

Recently, studies by our laboratory identified a novel alternatively spliced variant of Bnip3 which acts as an endogenous inhibitor of programmed cell death in cardiac myocytes in response to hypoxic injury[117]. This spliced variant lacks the third exon (Bnip3Δex3), while fusion of exon 2 to exon 4 results in a frame-shift and subsequent premature stop codon. Bnip3Δex3 physically interacts with full-length Bnip3 and blocks its integration into the mitochondria to circumvent Bnip3-induced mitochondrial perturbations and cell death. Although largely unknown at this time, it is of great potential importance to uncover a novel cellular defensive mechanism, whereby alternative splicing of Bnip3 can dictate cell fate by curtailing the mitochondrial defects, autophagy, and programmed cell death associated with hypoxia and ischemic injury in ventricular myocytes.

7.0 Model Illustrating Autophagy pathways in Cardiac Myocytes



Supplemental Figure 1. Schematic Representing the Autophagy Pathways in Cardiac Myocytes.

Upon acute ischemic or metabolic stress, the mitochondrion is a major point of convergence to apoptosis and autophagy. An adaptive autophagy response is activated through an AMPK/mTOR-mediated signaling pathway. However, reperfusion injury or chronic afterload induces a maladaptive autophagy response dependent on Beclin-1 that ultimately leads to cell demise. The role of the BH3-only Bcl-2 family member Bnip3 is also illustrated, where BH3-only proteins can disrupt the inhibitory interaction between Bcl-2 and Beclin-1 at the endoplasmic reticulum, provoke mitochondrial perturbations, and promote mitochondrial fission and mitophagy by recruiting LC3 to mitochondrial membranes. On the other hand, Bnip3 is responsible for intrinsic apoptosis by triggering mitochondrial permeabilization, swelling, and cytochrome c release. Whether autophagy kills cells via an interaction with the apoptosis pathways or via an entirely independent pathway remains undetermined. Current theories purport that basal autophagy is essential for clearance of damaged mitochondria to avoid activation of the intrinsic apoptotic pathway, whereas excessive autophagy is associated with cell death.

III. MATERIALS AND METHORDS

Neonatal Myocytes Isolation and Cell Culture

Neonatal ventricular myocytes were isolated from two-day-old Sprague-Dawley rat hearts[113]. Myocytes were submitted to primary culture and plated at a density of 1 x 10^6 cells per 35mm plate. After overnight incubation at 37^{o}C in Dulbeco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (DF) supplemented with 17mM HEPES, 3mM $NaHCO_3$, 2mM L-glutamine, 50μg/mL gentamicin and 10% fetal bovine serum (FBS), the myocytes were transferred to DF serum-free (DFSF) media as previously described[113]. Wild-type mouse embryonic fibroblasts (MEFS) and Bnip3-/- MEFS were cultured as previously reported[4]. Myocytes were maintained in DFSF medium and MEFs were maintained in DMEM + 10% FBS medium. All cell cultures were harvested 18hrs after genetic interference.

Protein Detection by Western Blot Analysis

For detecting total protein, neonatal myocytes were harvested in RIPA lysis buffer containing protease inhibitors. Protein cell lysates (20-25 ug) were denatured for 5 minutes at 100^{o}C and resolved on a 12-18% sodium dodecyl sulfate polyacrylamide gel at 80 volts for 20 minutes followed by 100 volts for 1 hour. The protein lysates were electrophoretically transferred to a Thermo Scientific Pierce PVDF (Polyvinylidene difluoride) membrane at 100 volts for one hour at 4^{o}C or at 20 volts overnight at room temperature. Membranes were subjected to Ponceau staining to visualize protein bands and blocked

for 1 hr in 5% skim milk in TBS-TWEEN (50mM Tris-HCl, 150mM NaCl, 0.3% Tween-20, pH 7.4) at room temperature. Membranes were incubated with primary IgG antibodies directed against p53 (Cat# 9282, Cell Signaling), p62/SQSTM1 (Cat#5114, Cell Signaling), VDAC (Cat #4661, Cell Signaling), adenine nucleotide translocator (SC-11433, Santa Cruz), GAPDH (SC-32233, Santa Cruz), or Bnip3 (developed in-house) respectively at a specified dilution overnight at 4^{o}C, as previously reported[116]. Following incubation, membranes were washed three times with 1x TBS-TWEEN for 10 mins each and incubated with specific secondary antibodies for 1 hr at room temperature. A chemiluminescence reaction using horseradish peroxidise-conjugated antibody with enhanced chemiluminescence (ECL) reagents (Pharmacia Inc) was used to detect bound proteins. To detect equal protein loading, membranes were probed with α–actin (Sigma, sarcomeric) at a dilution of 1:1000 in 2.5% BSA. Cytoplasmic S-100 and mitochondrial fractions of cardiac myocytes were prepared as previously reported[117]. Compartment specific markers GAPDH (cytosol) and VDAC (mitochondria) verified the integrity of cell fractionation.

Detection of Transcripts by Real-Time PCR (qPCR)

Total RNA (1μg) from ventricular myocytes following interventions was isolated with the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and reverse-transcribed with oligo dT20 (Invitrogen). Primer sets were designed to detect Bnip3 mRNA, forward 5'-TGCACTTCAGCAATGGGAAT-3', reverse 5'-ACATTTTCTGGCCGAATTGA-3' (Sigma, Accession number: NM_053420) and L32 mRNA as a house-keeping control gene: forward 5'-TAAGCGAAACTGGCGGAAAC-3', reverse 5'-

GCTGCTCTTTCTACGATGGCTT-3' (Sigma, Accession number; XO 6483). Real-time PCR was performed using the iQ5 multicolor real-time PCR detection system (Bio-Rad), fold change in gene expression was calculated by ΔΔCT method.

Recombinant Adenoviruses

Replication defective adenoviruses were propagated using HEK293 cells that express the genes necessary for adenoviral replication (E1A and E1B). The adenoviruses were harvested, tittered and purified as previously reported[118]. Myocytes and MEFs were infected with adenoviruses encoding wild-type p53, Bnip3FL, Bnip3ΔTM or Bnip3Δex3 at multiplicity of infection (MOI) of 10 which achieves gene delivery of >90% to cells[119] for 18hrs in serum free media. To control the effects of viral infection alone, the adenovirus designated Ad-CMV that contains the CMV enhancer-promoter with an empty expression cassette was used.

Cell Viability Assay

Cell viability was determined by fluorescent dyes and assessed by epifluorescent microscopy. Cells were stained with the vital dyes calcein acetoxymethylester and ethidium homodimer (each 2 μM) (Molecular Probes, Eugene, Oregon) to visualize live and dead cells respectively[120]. Calcein AM stained the live cells as green and ethidium homodimer-1 stained the dead cells as red. After genetic manipulation of the neonatal ventricular myocytes on glass coverslips, the cells were washed with PBS and incubated in the dark with DFSF media containing both fluorescent dyes at 37^{o}C for 30 minutes. Myocytes were washed again with PBS and maintained in fresh DFSF media before being visual-

ized. The glass coverslips were inverted onto glass sides and visualized using Olympus AX-70 Research fluorescence microscope. At least 200 cells from n=3-4 independent myocyte isolations were tested for each condition.

Autophagosome Formation and Autophagic Flux

To assess autophagy in cardiac myocytes, cells were infected with the GFP-LC3 expression vector as reported[121]. Myocytes were washed with PBS and fixed with 70% ethanol for 20 mins in room temperature. Cells were washed again with PBS for 3 times. Ultimately cells were mounted with florescent reagent and examined with an Olympus AX-70 Research fluorescence microscope to assess GFP-LC3 localization. The number of green puncta in cells was counted as an index for autophagic flux compared to control cells as previously reported[86]. To assess autophagic flux, cells infected with GFP-LC3 were incubated in the absence and presence of chloroquine for another 18hrs before intervention. At least 180 cells from n=3-4 independent myocyte isolations were tested for each condition.

Mitochondrial Membrane Potential ΔΨm

Mitochondrial membrane potential (ΔΨm) in cells was assessed by epifluorescence microscopy. Cells were pre-incubated with 50 nM tetra-methylrhodamine methyl ester perchlorate (TMRM)[121]. Cells were mounted with florescent reagent and examined with an Olympus AX-70 Research fluorescence microscope. At least 180 cells from n=3-4 independent myocyte isolations were tested for each condition.

Statistical Analysis

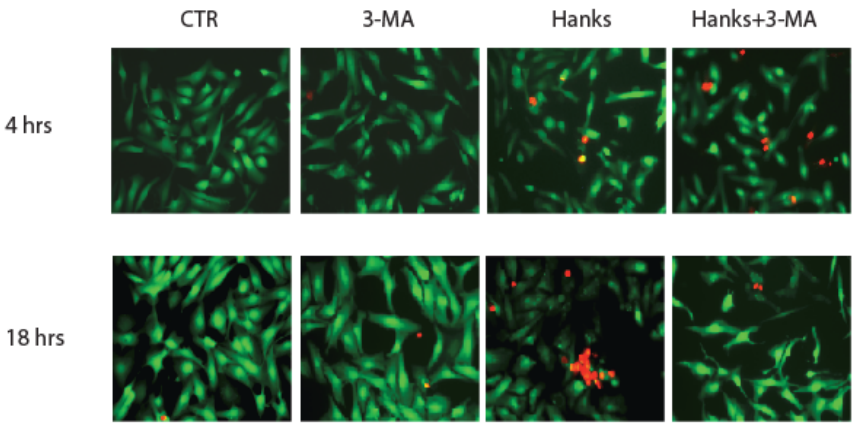
Multiple comparisons between groups were determined by one-way ANOVA. A two-way ANOVA was specifically used for cell viability assessment of MEF wild-type and Bnip3-/- mouse embryonic fibroblasts. Bonferroni post-hoc tests were used to determine the difference between specific means. Unpaired two-tailed student t-test was used to compare mean difference from control. Statistically significant differences were considered at a level of $p < 0.05$. In all cases, the data was obtained from at least n=3 to 4 independent myocyte isolations using n=3 replicates for each condition tested.

IV. RESULTS

Autophagy Regulates Cell Fate in a Time-Dependent Manner

To determine the role of autophagy in regulating cell fate under stress, viability was determined after blocking autophagy with class III PI3K inhibitor 3-MA. Live-dead assay was performed after 4hrs which is considered as early phase of autophagy and 18hrs which is considered as late phase of autophagy. Blocking autophagy with 3-MA markedly aggravated cell death at 4 hrs but attenuated cell death at 18 hrs, shown in Figure 1.

A


CTR
3-MA
Hanks
Hanks+3-MA
4 hrs
18 hrs

B

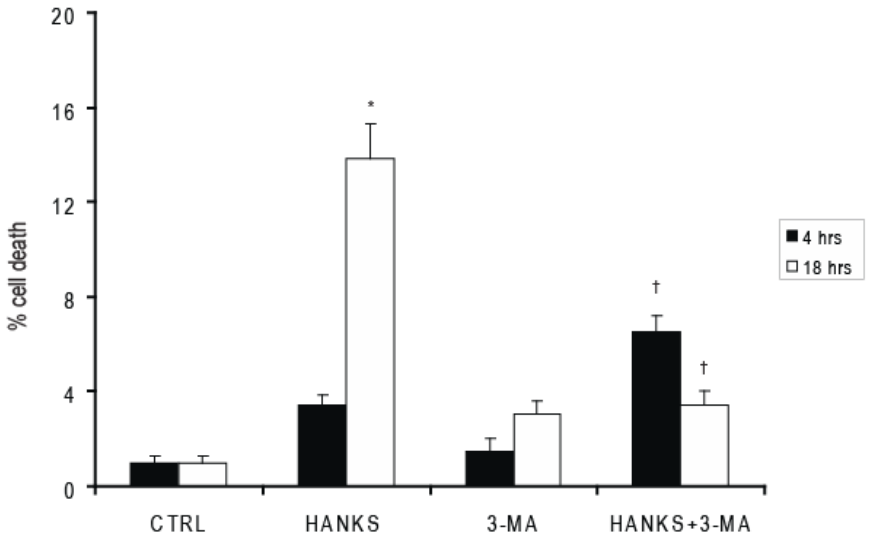

20
16
12
8
4
0
% cell death
*
†
†
4 hrs
18 hrs
CTRL
HANKS
3-MA
HANKS+3-MA

Figure 1. Autophagy Regulates Cell Death and Survival in a Time-Dependent Manner

Panel A, Vital Staining by epifluorescence of neonatal myocytes in normal and HANKS's media with and without 3-MA (10 mM) at 4hrs and 18 hours incubation; **Panel B,** Histogram represents quantitative data for panel A. Data are expressed as mean±S.E. from n=3-4 independent myocyte isolations, counting > 200 cells for each condition tested; * denotes $p<0.01$ compared to control; † denotes $p<0.01$ compared to Hanks condition.

Autophagic Cell Death is Induced by Hypoxia and Starvation in Neonatal Cardiomyocytes.

To test if autophagy is inducible in neonatal cardiac myocytes, neonatal cardic myocytes are subjected to hypoxic condition for 18 hours in an air-tight chamber under serum-free culture conditions continually gassed with 95% N2, 5% CO2, and po2 ≤5 mm Hg at 37 C^{o} as previously reported[123]; nutrient stress is induced by incubating in Hank's balanced salt solution for 18 hours. Results showed excessive cell death after 18 hours hypoxia and starvation which was simultaneous with autophagosome accumulation, demonstrating autophagy is inducible under metabolic stress in neonatal cardiac cells and prolonged autophagy leads to cell death.

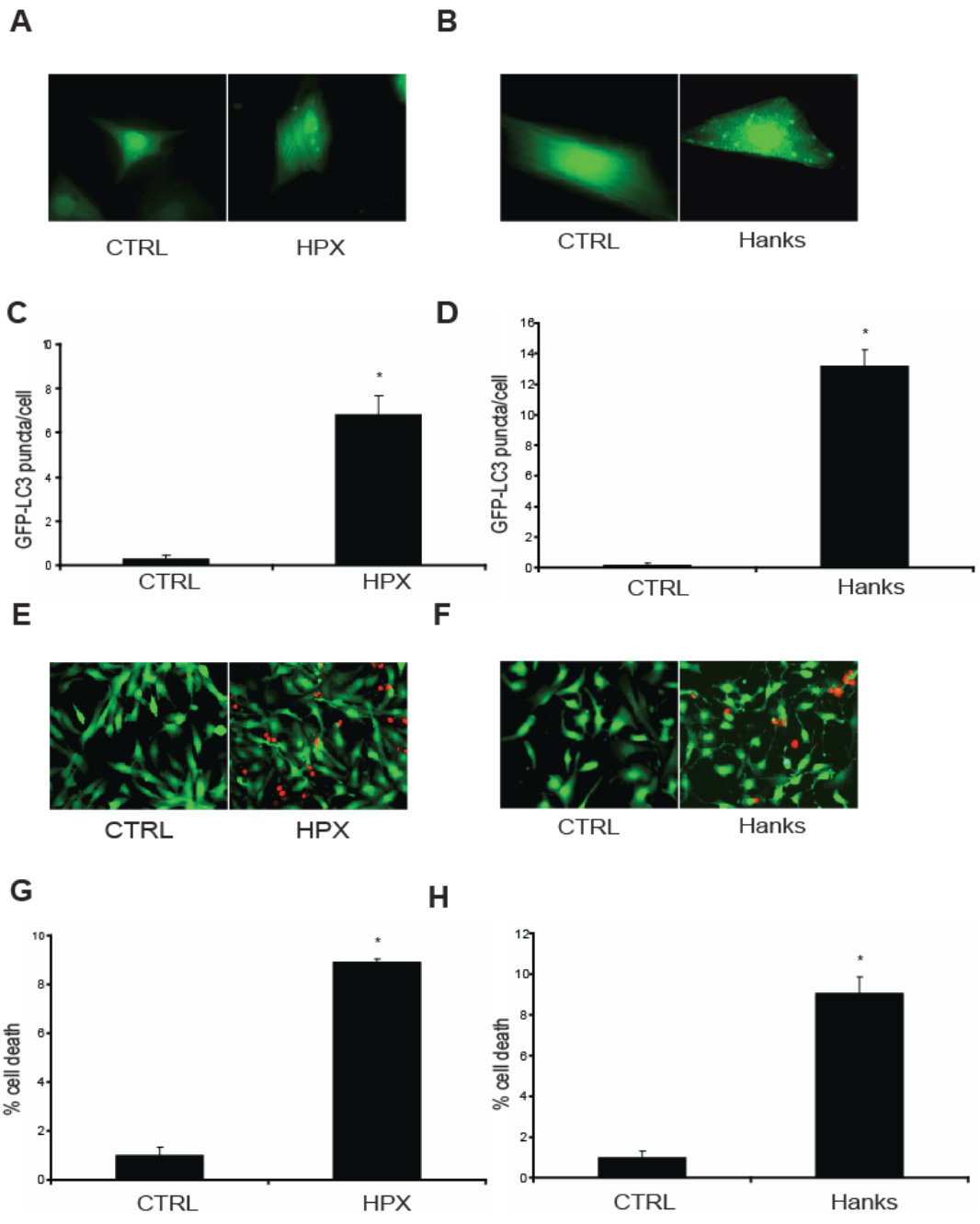

A
B
CTRL
HPX
CTRL
Hanks
C
D
GFP-LC3 puncta/cell
CTRL
HPX
GFP-LC3 puncta/cell
CTRL
Hanks
E
F
CTRL
HPX
CTRL
Hanks
G
H
% cell death
CTRL
HPX
% cell death
CTRL
Hanks

Figure 2. Autophagy is Induced by Hypoxia and Starvation in Neonatal Cardiomyocytes.

Panel A and B, Primary ventricular myocytes under control and hypoxia(A) or starvation conditions(B) were infected with adenovirus encoding an LC3-GFP fusion protein and Imaged by standard fluorescence techniques. Green puncta indicate autophagosome formation. Representative fluorescent images show increasing level of autophagy under stimulations; **Panel C and D** : Histogram represents quantitative data for panel A and B. Data are expressed as mean±S.E. from n=3-4 independent myocyte isolations counting > 180 cells for each condition tested; * denotes $p<0.01$ compared to control; **Panel E and F**: Epifluorescence microscopy of cardiac myocytes stained with vital dyes calcein -AM and ethidium homodimer-1 to identify the number of live cells (green) and dead cells (red) respectively for conditions shown in panel A and B; **Panel G and H:** Histogram represents quantitative data for panel E and F. Data are expressed as mean±S.E. from n=3-4 independent myocyte isolations counting > 200 cells for each condition tested; * denotes $p<0.01$ compared to control.

Autophagy and Cell Death in Ventricular Myocytes is Induced by p53

To establish whether p53 activates autophagic processes in post-natal ventricular myocytes, we assessed autophagic flux in cardiac myocytes over-expressing p53. In contrast to vector control cells, a marked increase in GFP-LC3 puncta was observed in cells expressing p53 (Figure 1 panel A-D). The finding verifies that p53 increases autophagy in cardiac myocytes. In addition, vital staining of cells revealed p53 induced autophagy of ventricular myocytes was accompanied by a significant increase in cell death compared to vector control cells (Figure 1 panel E-H). Interestingly, inhibition of autophagy with either 3-MA (3-methyl adenine), or with shRNA to knockdown Atg7 suppressed autophagy and cell death of ventricular myocytes induced by p53.

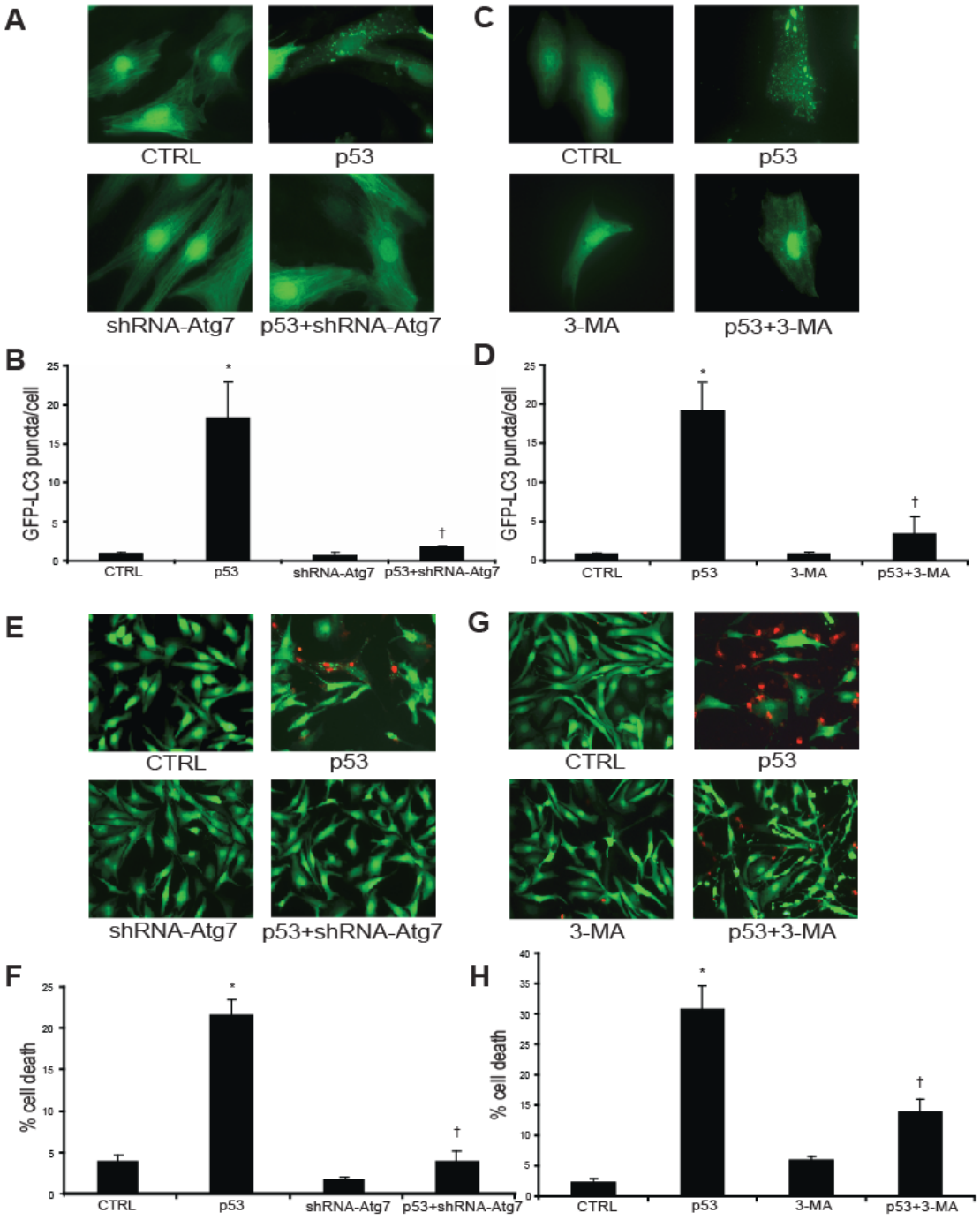

A
CTRL
p53
shRNA-Atg7
p53+shRNA-Atg7
C
CTRL
p53
3-MA
p53+3-MA
B
GFP-LC3 puncta/cell
CTRL
p53
shRNA-Atg7
p53+shRNA-Atg7
D
GFP-LC3 puncta/cell
CTRL
p53
3-MA
p53+3-MA
E
CTRL
p53
shRNA-Atg7
p53+shRNA-Atg7
G
CTRL
p53
3-MA
p53+3-MA
F
% cell death
CTRL
p53
shRNA-Atg7
p53+shRNA-Atg7
H
% cell death
CTRL
p53
3-MA
p53+3-MA

Figure 3. p53 Induces Autophagy and Cell Death in Ventricular Myocytes.

Panel A: Epifluorescence microscopy of ventricular myocytes infected with autophagy reporter GFP-LC3 under control and p53 over-expression conditions with and without shRNA directed against Atg7. The number of green puncta were counted as an index of autophagosome formation compared to control cells; **Panel B:** Histogram represents quantitative data shown in panel A, data are expressed as mean±S.E. puncta/cell, from n=3-4 independent myocyte isolations counting > 180 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53; **Panel C:** GFP-LC3 puncta in cardiac myocytes under control and p53 over-expression conditions with and without 3-methyl adenine (3-MA, 10mM), using methodologies described in panel A; **Panel D:** Histogram represents quantitative data for panel C, data expressed as mean±S.E. puncta/cell, from n=3-4 independent myocyte isolations counting > 180 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53; **Panel E:** Epifluorescence microscopy of cardiac myocytes stained with vital dyes calcein-AM and ethidium homodimer-1 to identify the number of live cells (green) and dead cells (red) respectively for conditions shown in panel A; **Panel F**: Histogram represents quantitative data for panel E. Data are expressed as mean ±S.E. from n=3-4 independent myocyte isolations counting > 200 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53; Panel G: Vital staining of cardiac myocytes by epifluorescence microscopy for conditions shown in Panel C; **Panel H:** Histogram represents quantitative data for panel G. Data are expressed as mean±S.E. from n=3-4 independent myocyte isolations counting > 200 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53.

Bnip3 is Up-Regulated by p53

Since earlier work by our laboratory demonstrated the ability of Bnip3 to trigger autophagy and cell death of ventricular myocytes, we reasoned that Bnip3 may be a transcriptional target of p53. To test this possibility we monitored Bnip3 expression in cardiac myocytes in the absence and presence of p53. As shown in Figure 4, in contrast to vector control cells, a marked increase in endogenous Bnip3 mRNA and protein expression levels were observed in cells expressing p53. These findings suggest that Bnip3 is up-regulated by p53.

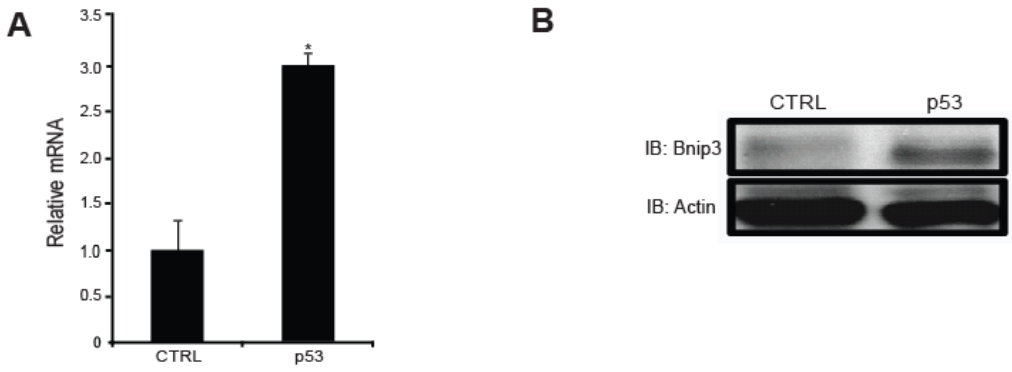


Figure 4. p53 Up-Regulates Bnip3 in Ventricular Myocytes.

Panel A: Real time PCR analysis of Bnip3 mRNA level in cardiac myocytes in the absence and presence of p53; **Panel B:** Western blot analysis of cardiac cell lysate in the absence and presence of p53. The membrane was probe with an antibody directed against Bnip3 as previously reported[116]: Antibody directed against α-Actin (sarcomeric) was used for loading control.

p53-Induces Mitochondrial Defects Dependent upon Bnip3

Since earlier work by our laboratory demonstrated that p53 localizes to mitochondria in cardiac myocytes[116], we reasoned that mitochondrial perturbations induced by p53 may underlie autophagy and cell death. As shown by Western blot analysis (Figure 5 panel C), p53 was detected in the mitochondrial fractions of cardiac myocytes following p53 over-expression. Notably, this was accompanied by a marked reduction in mitochondrial $\Delta\Psi m$ (Figure 5 panels A-B). These findings support the notion that mitochondrial perturbations induced by p53 promote autophagy and cell death of ventricular myocytes.

Based on these observations, we reasoned that p53 may provoke mitochondrial perturbations in a manner dependent upon Bnip3. To test this possibility, we rendered cardiac myocytes defective for Bnip3 with shRNA directed against Bnip3. Notably, previous work by our laboratory verified the specificity of the shRNA used to knock-down Bnip3 in ventricular myocytes for these studies. As shown in Figure 5 panels A-B, a marked reduction in mitochondrial $\Delta\Psi m$ was observed in cells expressing p53, in contrast however, knock-down of Bnip3 completely abrogated the loss in $\Delta\Psi m$ induced by p53. To verify these findings and the involvement of Bnip3 in p53 mediated mitochondrial defects, we tested the effects of p53 in the presence of a carboxyl terminal transmembrane domain mutant of Bnip3 (Bnip3ΔTM), previously reported by our laboratory to be defective for integrating into mitochondrial membranes in cardiac myocytes[123]. As shown in Figure 5 panels A-B, in contrast to vector control cells p53 mediated loss of mitochondrial $\Delta\Psi m$ was suppressed by the Bnip3 ΔTM mutant defective for mitochondrial targeting - a finding concordant with our shRNA knock-down data for Bnip3. Interestingly, we

observed a marked reduction in mitochondrial associated p53 in cells in the presence of the Bnip3ΔTM (Figure 5 panel C). Taken together these findings strongly suggest the involvement of Bnip3 in mitochondrial defects induced by p53.

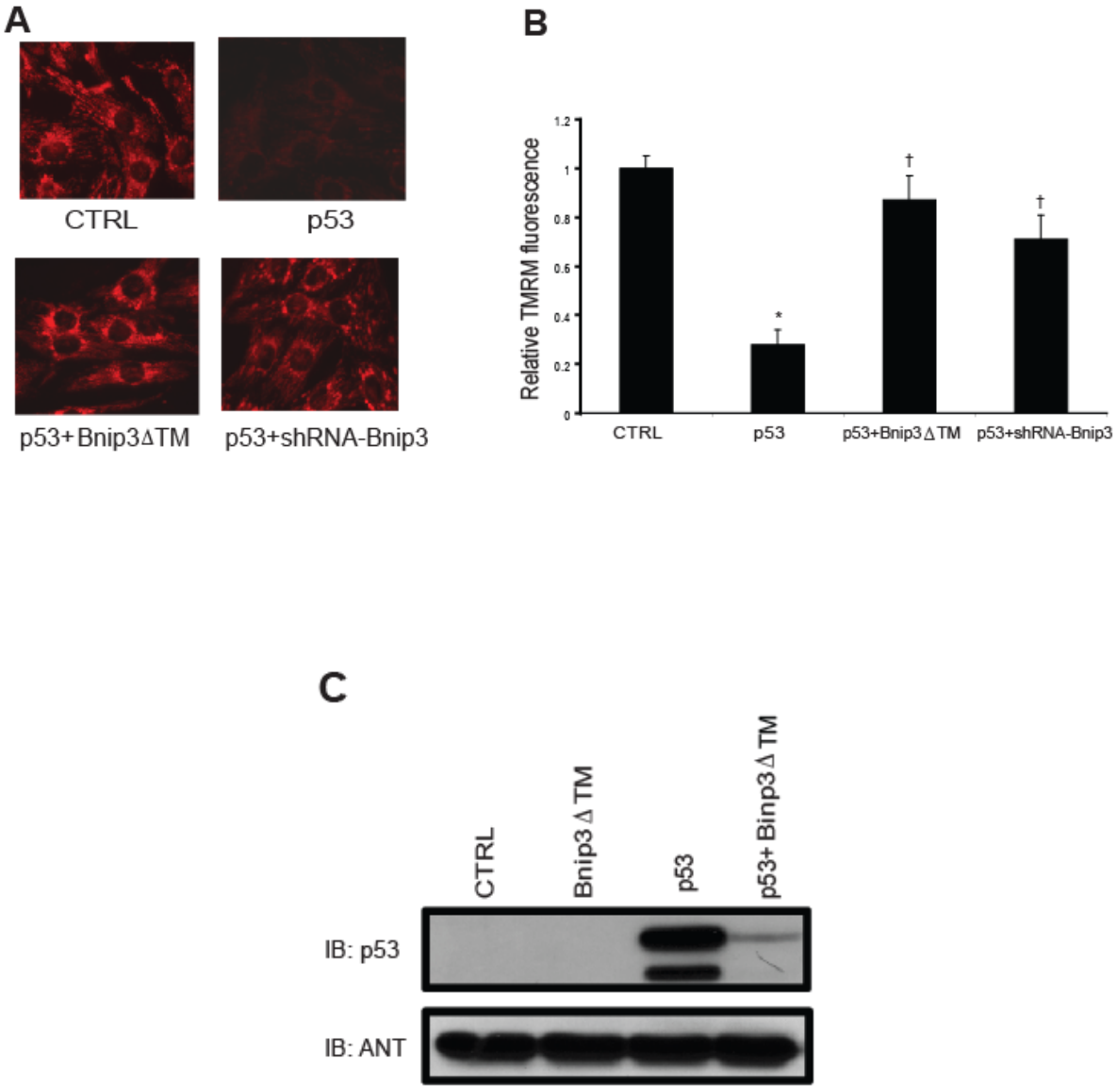
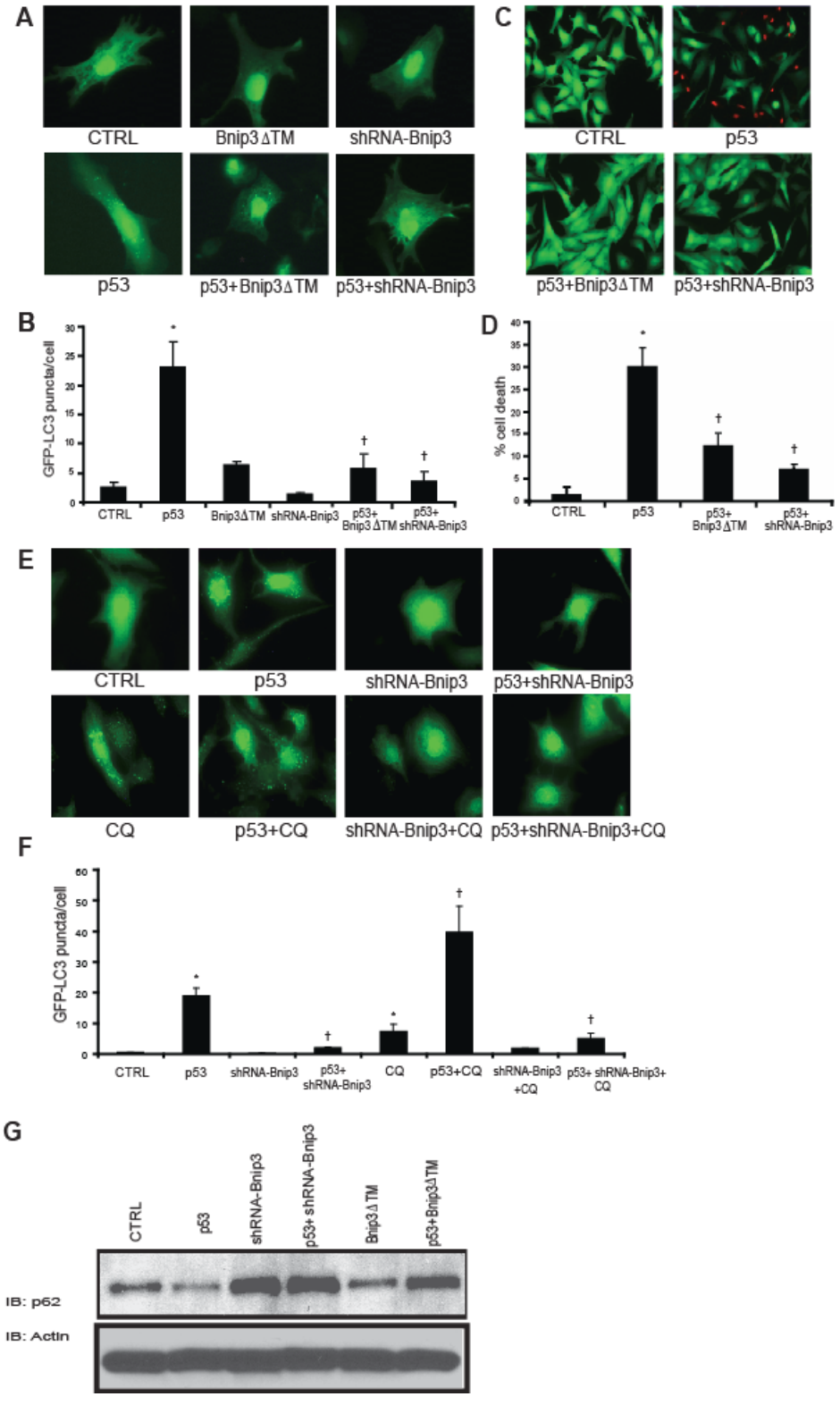

A
CTRL
p53
p53+Bnip3ΔTM
p53+shRNA-Bnip3
B
Relative TMRM fluorescence
1.2
1
0.8
0.6
0.4
0.2
0
*
†
†
CTRL
p53
p53+Bnip3ΔTM
p53+shRNA-Bnip3
C
CTRL
Bnip3ΔTM
p53
p53+ Binp3ΔTM
IB: p53
IB: ANT

Figure 5. p53-Induces Mitochondrial Defects Dependent upon Bnip3

Panel A: Mitochondrial membrane potential was assessed by fluorescence microscopy in cardiac myocytes with 50 nM tetra-methylrhodamine methyl ester perchlorate (TMRM)[116], (Molecular Probes, Eugene Oregon), under control and p53 over-expression conditions with and without shRNA directed against Bnip3 or Bnip3 mutant defective for mitochondrial membrane integration, (designated Bnip3ΔTM)[116]; **Panel B:** Histogram represents quantitative data shown in Panel A. Data are expressed as mean±S.E. fold change from control, from n=3-4 independent myocyte isolations counting > 200 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53; **Panel C:** Western Blot analysis of mitochondrial fractions derived from cardiac myocytes under control and p53 over-expression conditions with and without Bnip3ΔTM. Antibody directed against ANT (Adenine nucleotide translocator) was used as control for mitochondrial fraction.

p53-Induces Autophagy and Cell Death Dependent upon Bnip3

To establish whether Bnip3 underlies autophagy and cell death of ventricular myocytes induced by p53, we assessed autophagic flux and cell viability in cells in which Bnip3 was impaired or inhibited. Notably, autophagosome formation was further increased in cells over-expressing p53 in the presence of chloroquine (Figure 6 panel E- F). Inhibiting clearance of autophagsome by chloroquine slightly increased basal level of autophagy, but greatly augmented autophagy after p53 over-expression, meaning accumulation of autophagsomes is caused by promoting autophagosome formation instead of inhibiting their lysosomal clearance. As shown in Figure 6 panels A-B and E-F, in contrast to control cells, autophagic flux induced by p53 was completely suppressed in cells following Bnip3 knock-down or by the Bnip3ΔTM. Accompanied reduction in p62 expression levels was observed in cells over-expressing p53. However, genetic knock-down and dominant-negative mutant of Bnip3 suppressed the degradation of p62 protein induced by p53 (Figure 6 panel G). Further, inhibition of Bnip3 by shRNA knock-down or by dominant-negative inhibition independently suppressed cell death induced by p53 (Figure 6 panels C-D) - a finding concordant with our autophagy data.


A
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p53+shRNA-Bnip3
C
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p53
p53+Bnip3ΔTM
p53+shRNA-Bnip3
B
GFP-LC3 puncta/cell
CTRL
p53
Bnip3ΔTM
shRNA-Bnip3
p53+ Bnip3 ΔTM
p53+ shRNA-Bnip3
D
% cell death
CTRL
p53
p53+ Bnip3 ΔTM
p53+ shRNA-Bnip3
E
CTRL
p53
shRNA-Bnip3
p53+shRNA-Bnip3
CQ
p53+CQ
shRNA-Bnip3+CQ
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CQ
p53+CQ
shRNA-Bnip3 +CQ
p53+ shRNA-Bnip3+ CQ
G
CTRL
p53
shRNA-Bnip3
p53+ shRNA-Bnip3
Bnip3Δ TM
p53+Bnip3Δ TM
IB: p62
IB: Actin

Figure 6. p53-Induces Autophagy and Cell Death Dependent upon Bnip3.

Panel A: Epifluorescence microscopy of ventricular myocytes infected with autophagy reporter GFP-LC3 under control and p53 over-expression conditions with and without shRNA directed against Bnip3 or Bnip3ΔTM; **Panel B:** Histogram represents quantitative data for panel A. Data are expressed as mean±S.E. puncta/cell from n=3-4 independent myocyte isolations counting > 180 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53; **Panel C:** Vital staining of cells by epifluorescence for conditions in Figure 5 panel A, using methodologies described in Figure 1; **Panel D:** Histogram represents quantitative data for panel C. Data are expressed as mean±S.E. from n=3-4 independent myocyte isolations counting > 200 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53; **Panel E:** p53 induces autophagic flux in cardiac myocytes. Autophagic flux of GFP-LC3 puncta was measured in the presence of chloroquine (8μM) under control and p53 over-expression conditions with and without shRNA directed against Bnip3; **Panel F:** Histogram represents quantitative data for panel E. Data are expressed as mean±S.E. puncta/cell, from n=3-4 independent myocyte isolations counting > 180 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53; **Panel G:** Western blot analysis of cardiac cell lysate under control and p53 over-expression conditions with and without shRNA directed against Bnip3, or Bnip3 mutant defective for mitochondrial membrane integration(Bnip3ΔTM). The membrane was probed with an antibody directed against p62/SQSTM1. Antibody directed against α-Actin (sarcomeric) was used for loading control.

p53 Induced Autophagy and Cell Death is Abrogated in Cells Deficient for Bnip3

To conclusively demonstrate the involvement of Bnip3 in autophagy and cell death induced by p53, we tested the impact of p53 in wild type and Bnip3-/- mouse embryonic fibroblasts (MEFs). As shown in Figure 7 panels A-D, in contrast to wild type cells, which readily displayed the presence of autophagosomes and reduced cell viability in the presence of p53, p53 failed to induce autophagy or promote death in Bnip3-/- cells. Notably, repletion of Bnip3 into the Bnip3-/- deficient background completely restored p53's ability to provoke autophagy and cell death equivalent to that of wild type cells. Notably, autophagy and cell death induced by p53 correlated with its mitochondrial targeting in wild type cells but not Bnip3-/- cells (Figure 7 panel E). We prepared mitochondrial and cytoplasmic fractions from wild type and Bnip3 -/- MEFs for Western Blot analysis for p53. p53 was expressed to comparable levels in wild type and Bnip3-/- cells. p53 protein was readily detectable in mitochondria of MEF wild type cells, concordant with its ability to provoke profound cell death with intervention. However, p53 was not detected in the mitochondria of Bnip3-/- cells, which is consistent with the inability of p53 to provoke mitochondria defects in cells rendered defective for Bnip3. Collectively, these findings strongly support our contention that p53 promotes autophagy and cell death of ventricular myocytes through a mechanism involving Bnip3.

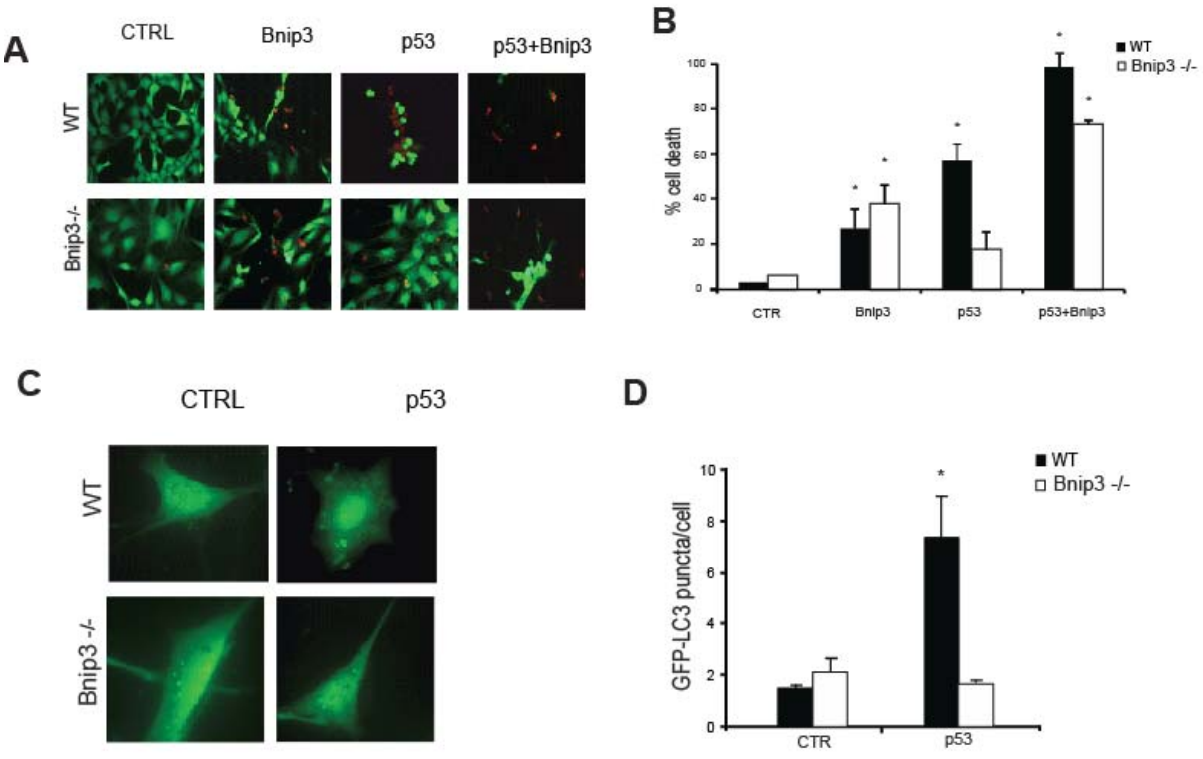

A
CTRL
Bnip3
p53
p53+Bnip3
WT
Bnip3-/-
B
% cell death
WT
Bnip3 -/-
CTR
Bnip3
p53
p53+Bnip3
C
CTRL
p53
WT
Bnip3 -/-
D
GFP-LC3 puncta/cell
WT
Bnip3 -/-
CTR
p53
E
Mito
Cyto
WT
Bnip3-/-
WT
Bnip3-/-
IB: p53
IB: VDAC
IB: GAPDH

Figure 7. Cells Deficient for Bnip3 are Resistant to p53-Induced Autophagy and Cell Death.

Panel A: Vital staining by epifluorescence microscopy of wild type and Bnip3-/- mouse embryonic fibroblasts (MEFs) expressing p53 with and without Bnip3 reconstitution (see text for details) by methodologies described in Figure 1; **Panel B:** Histogram represents quantitative data for panel A. Data are expressed as mean±S.E. from n=3-4 independent myocyte isolations counting > 200 cells for each condition tested; * denotes $p<0.01$ compared to control cells; **Panel C:** Epifluorescence microscopy of GFP-LC3 puncta in wild type and Bnip3 -/- MEF cells under control and p53 over-expression conditions, as described in Figure 1; **Panel D:** Histogram represents quantitative data for panel C. Data are expressed as mean±S.E. puncta/cell from control, from n=3-4 independent myocyte isolations counting > 180 cells for each condition tested; * denotes $p<0.01$ compared to control cells. **Panel E:** Western Blot analysis of mitochondrial(mito) and cytoplasmic(cyto) fractions derived from wild type and Bnip3-/- mouse embryonic fibroblasts. The filter was probed with an antibody directed against p53. Antibody directed against VDAC (Voltage-dependent anion channel) and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) were used as controls for mitochondrial and cytoplasmic fractions respectively.

P53 Induced Autophagic Cell Death is Prevented by Bnip3 Alternative Spliced Variant Bnip3Δex3

Earlier studies by our laboratory identified the alternatively spliced variant of Bnip3, Bnip3Δex3, as an endogenous inhibitor of programmed cell death. Based on the result that p53-induced autophagic cell death is dependent on Bnip3-mediated mitochondria perturbation, we studied whether Bnip3Δex3 plays a defensive role in p53-induced autophagic cell death. We monitored GFP-LC3 puncta in cells over-expressing p53 in the absence and presence of Bnip3Δex3 introduction. Notably, a marked decrease in GFP-LC3 puncta was observed in cells co-expressing Bnip3Δex3 and p53 (Figure 8 panel A-B), suggesting autophagy is extensively blocked. Moreover, over-expression of Bnip3Δex3 greatly elevates the reduction in mitochondrial ΔΨm caused by p53 (Figure 8 panels C-D). This was further accompanied by an evident remedy in cell viability (Figure 8 panels E-F). These findings support the conclusion that mitochondrial perturbations induced by p53 is dependent upon Bnip3-mitochondria targeting and provide an intrinsic defensive mechanism.

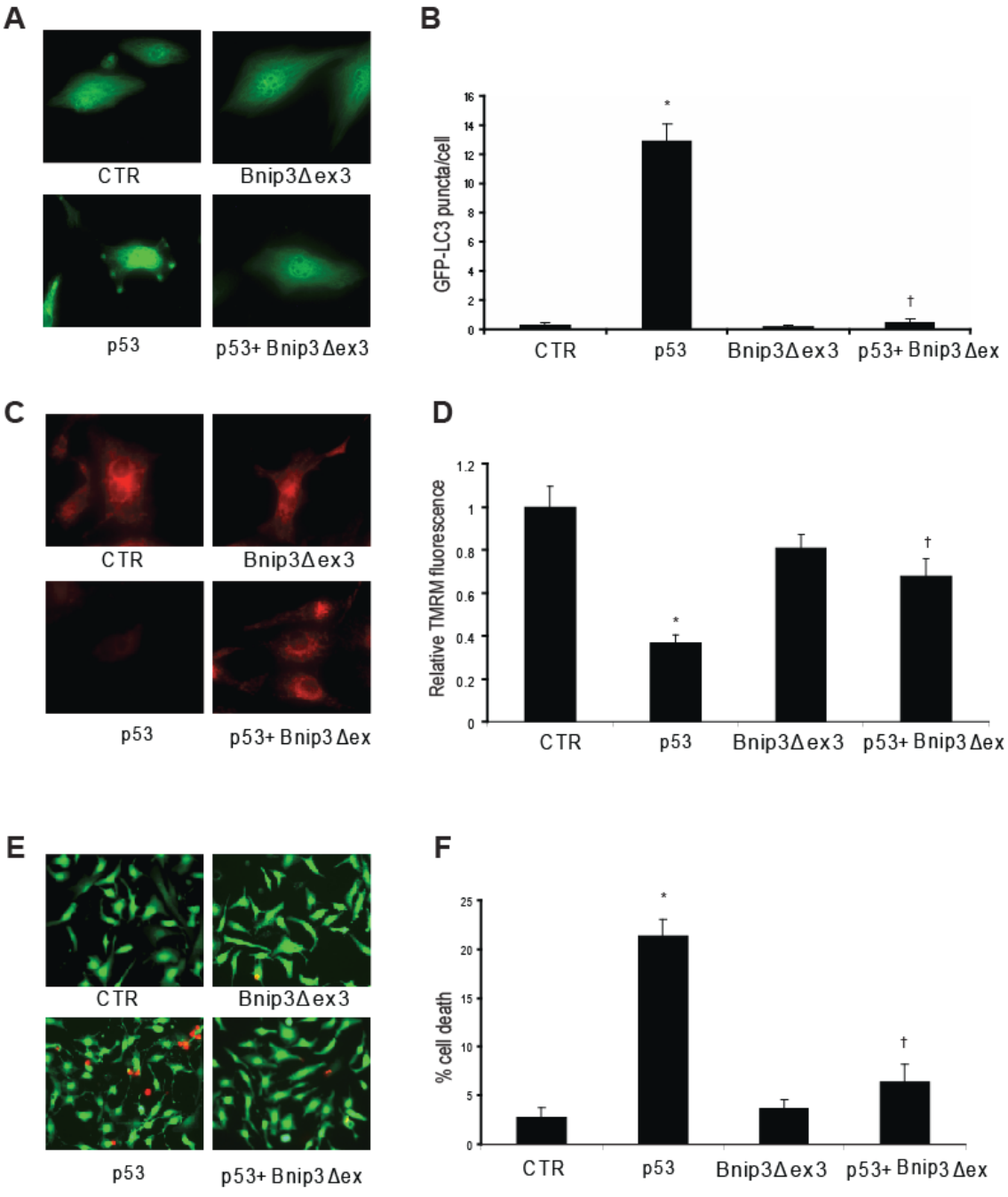

A
CTR
Bnip3Δex3
p53
p53+ Bnip3Δex3
B
GFP-LC3 puncta/cell
16
14
12
10
8
6
4
2
0
*
†
CTR
p53
Bnip3Δex3
p53+ Bnip3Δex
C
CTR
Bnip3Δex3
p53
p53+ Bnip3Δex
D
Relative TMRM fluorescence
1.2
1
0.8
0.6
0.4
0.2
0
*
†
CTR
p53
Bnip3Δex3
p53+ Bnip3Δex
E
CTR
Bnip3Δex3
p53
p53+ Bnip3Δex
F
% cell death
25
20
15
10
5
0
*
†
CTR
p53
Bnip3Δex3
p53+ Bnip3Δex

Figure 8. p53 Induced Autophagic Cell Death is Prevented by Bnip3 Alternative Spliced Variant Bnip3Δex3.

Panel A: Epifluorescence microscopy of ventricular myocytes infected with autophagy reporter GFP-LC3 under control and p53 over-expression conditions with and without introduction of Bnip3 alternative spliced variant Bnip3Δex3; **Panel B:** Histogram represents quantitative data shown in panel A, data are expressed as mean±S.E. puncta/cell, from n=3-4 independent myocyte isolations counting > 180 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53; **Panel C:** Mitochondrial membrane potential was assessed by fluorescence microscopy in cardiac myocytes with 50 nM tetra-methylrhodamine methyl ester perchlorate (TMRM) for conditions shown in panel A; **Panel D:** Histogram represents quantitative data shown in Panel C. Data are expressed as mean±S.E. from n=3-4 independent myocytes isolations counting > 200 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53; **Panel E**: Vital staining of cardiac myocytes by epifluorescence microscopy for conditions shown in Panel A; **Panel F:** Histogram represents quantitative data for panel E. Data are expressed as mean±S.E. percent change from control, from n=3-4 independent myocyte isolations counting > 200 cells for each condition tested; * denotes $p<0.01$ compared to control; †denotes $p<0.01$ compared to p53.

V. DISCUSSION

Autophagy is a "self-eating" program which has essential roles in survival, development and homeostasis by providing a mechanism for generating substrates for ATP synthesis during cellular crisis. It was generally thought to be a protective response to maintain homeostasis. However, irregular or excess autophagy has been shown to be maladaptive and incompatible with life. Basal autophagy is beneficial for regulating cellular homeostasis and accumulation of toxic effects of protein aggregates. However, prolonged or excessive autophagy, beyond a given threshold, can exacerbate cell injury and result in the demise of the cells[9]. This is supported by cell viability analysis of autophagy in cardiac myocytes by different time points (showed in Fugure 1).

Our lab has previously reported independent death pathways mediated by the tumor suppressor p53 and BCL-2 19 kDa interaction protein Bnip3 in ventricular myocytes stimulated with hypoxia and ischemic conditions. In this dissertation we provide novel evidence that p53 induces ventricular myocytes death as an autophagy stimulator dependent upon Bnip3.

Consistent with reports showing autophagic cell death triggered by p53 in other cell lines, there is ongoing autophagy and cell death are induced by p53 in cardiac myocytes. Cell death induced by p53 was suppressed by Atg7 knock-down or pharmacological inhibition of autophagy with 3-methyl adenine (Figure 3). Thus by highlighting the fact that cell death induced by p53 was suppressed by autophagy inhibition, our study indicates that autophagy induced by p53 in this context is maladaptive.

The fact that Bnip3 mRNA and protein expression were increased by p53 is compelling and identifies Bnip3 as a putative down-stream effector of p53 (Figure 4). Supportively, mitochondrial defects, autophagy and cell death induced by p53 were abrogated by dominant-negative inhibition of Bnip3 or Bnip3 knock-down (Figure 5 and 6). Western blot also confirmed that genetic knock-down of Bnip3 completely suppressed the degradation of p62 protein induced by p53, which indicates initiation of autophagy overload (Figure 6).

We further determined the impact of Bnip3 on p53-induced autophagy in a knock-out tissue culture model. Bnip3-knockout cells exhibit significant reduction of autophagy and cell death mediated by p53, indicating that Bnip3 presence is crucial for cell death mediate by p53. Moreover, p53 protein was readily detectable in mitochondria of MEF wild type cells, concordant with its ability to provoke profound cell death with intervention. In contrast, p53 was absent in the mitochondria of Bnip3-/- MEFs (Figure 7). This finding is consistent with the inability of p53 to provoke mitochondria defects in cells rendered defective for Bnip3. Collecitvely, we demonstrate by not one, but several independent approaches that p53-triggers autophagy and cell death of cardiac myocytes in a Bnip3-dependent manner.

Recently, we have identified a novel splice variant of Bnip3, named Bnip3ΔEx3, which lacks the third exon, in which fusion of exon 2 to exon 4 results in a frame-shift and sub-sequent premature stop codon. Both full-length Bnip3 (Bnip3FL) and Bnip3ΔEx3 share a common N-terminus, while Bnip3ΔEx3 has a divergent C-terminus[117]. Bnip3ΔEx3 acts as an endogenous inhibitor of programmed cell death in cardiac myocytes under hypoxia.

Unlike the mitocondrial-targeting full length isoform, earlier work from our lab demonstrated that Bnip3ΔEx3 predominantly localizes to the endoplasmic reticulum (ER) to promote cell survival. We found Bnip3ΔEx3 notably blocks mitochondria perturbation, autophagy and cell death induced by p53, providing an adaptive mechanism that promotes survival (Figure 8). Thus further proved p53-Bnip3 induced autophagic cell death is dependent upon domain-mediated mitochondrial defects.

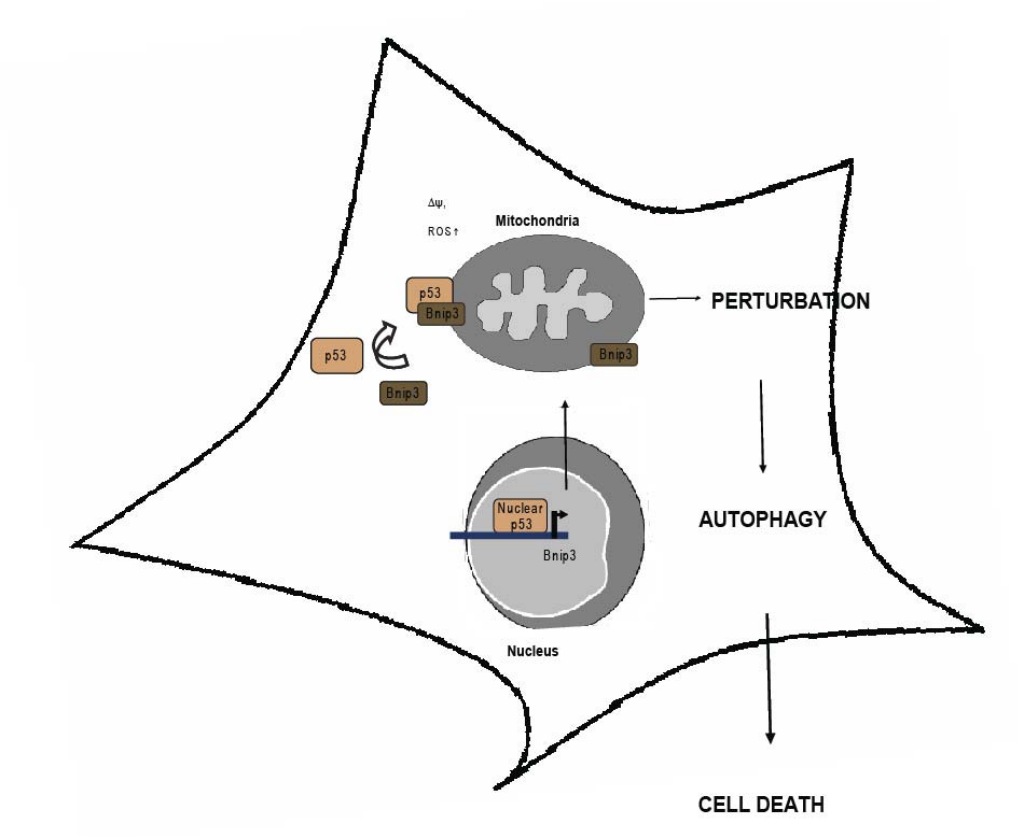
These findings provide the first direct evidence to mechanistically link mitochondrial perturbations induced by p53 to Bnip3 as an effector of autophagy and cell death of ventricular myocytes. This may explain more fundamentally how p53 dually regulates autophagy and cell death during cardiac stress.Though protein-protein associations were not determined here, it is tempting to speculate that p53 anchors to mitochondrial membranes in a Bnip3 dependent manner. This notion is concordant with the reduction of mitochondrial associated p53 in the presence of Bnip3ΔTM and the resistance of cardiac cells rendered deficient for Bnip3 or Bnip3-/- cells to the cytotoxic actions of p53. One hypothesis is by a physical interaction between p53 and Bnip3 protein exists at the mitochondria membrane.

Our data strongly supports a model by which p53 provokes mitochondrial permeability changes dependent upon Bnip3 following p53 induction, Bnip3 is over expressed and translocated to mitochondria causing loss of mitochondrial membrane potential and opening of transition pore. Mitophagy is initially induced via Bnip3's established ability to induce mitochondria perturbation. Presumably, damaged mitochondria that have undergone permeability transition would then be removed from the cell by autophagy.

The lack of sufficient mitochondria attribute to a persistant autophagic response eventually compromise cellular metabolism and lead to cell death (Supplemental Figure 2).

Therapeutic interventions that modulate p53 expression may prove beneficial in curtailing maladaptive autophagy and cell death after injury. A better understanding of the signaling pathways and molecular effectors that mediate autophagy under normal and pathological conditions would be of tremendous clinical benefit toward the long-term goal of developing novel therapeutic agents for averting cardiac cell death during disease conditions.

VI. Model Illustrating the Dual Role of p53 During Autophagy.



Supplemental Figure 2. Schematic Illustrating the Dual Role of p53 During Autophagy.

In response to cell stress and following p53 induction, Bnip3 is over expressed and translocated to mitochondria causing loss of mitochondrial membrane potential and opening of the transition pore. Mitophagy is initially induced via Bnip3's established ability to induce mitochondria perturbation. Presumably, damaged mitochondria that have undergone permeability transition would then be removed from the cell by autophagy. The lack of sufficient mitochondria attribute to persistant autophagic response eventually compromise cellular metabolism and lead to cell death. Taken together p53 promotes autophagy and cell death of ventricular myocytes through a mechanism requiring Bnip3.

VII. FUTURE DIRECTIONS

Given the dual nature of autophagy and its crosstalk with other types of cell death, there has been considerable interest in exploring the potential mechanisms by which the interrelationship between these processes may be coordinated. However, many outstanding scientific questions require investigation. First, much of the current knowledge regarding autophagy is derived from studies utilizing transformed cell lines and human cancer models. These cells contain fundamental defects in the programmed cell death pathways, and many harbor mutations in critical tumor suppressors, such as p53. Therefore, it seems reasonable to suspect cell-type specific differences in the autophagy response and the mechanisms by which autophagy could be terminated once an initial stress is removed. Second, mechanistic data regarding the transition from adaptive autophagy that promotes cell survival to maladaptive autophagy leading to cell demise remains fascinating subject in the field of cardiac biology. Finally, how autophagy is regulated in cardiac myocytes by alternatively splicing of Bcl-2 family members, such as Bnip3, Nix, and Bcl-X, remains an open area of study. These future studies, along with others, will define the molecular basis of cardiac autophagy, and how selective targeting of autophagy by novel therapies will attenuate or even reverse cardiac pathologies.

VIII. CONCLUSION

In this report, we provide novel evidence that p53 induces autophagy of cardiac myocytes which shifts the cell viability balance toward death. We show that p53 localizes to the mitochondrion and provokes mitochondrial defects in a manner dependent on the Bcl-2 death protein, Bnip3. Loss of Bnip3 function completely abrogated autophagy and cell death induced by p53. Hence, our findings reveal a novel signaling axis between mitochondrial localization of p53 and Bnip3 for autophagy and cell death that may explain more fundamentally how p53 dually regulates autophagy and cell death of ventricular myocytes during cardiac stress.

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