REGULATION OF ENDOPLASMIC RETICULUM STRESS BY TRANSCRIPTION FACTOR E2F-1 IN VENTRICULAR MYOCYTES

BY

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A Thesis Submitted to the Faculty of Graduate Studies of the University of Manitoba

In Partial Fulfillment of the Requirements of the Degree of

MASTER OF SCIENCE

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ABSTRACT

E2F-1 is a transcription factor that is involved in cellular growth, proliferation and regulates the transition between G1 and S phase during the cell cycle. In comparison to the other 8 members of the E2F family, E2F-1 also plays a unique role in regulating cell death in cardiac myocytes. However, the molecular mechanisms by which E2F-1 regulates endoplasmic reticulum (ER) stress in cardiac myocytes remain poorly defined. Evidence suggests a critical role of the signaling molecules involved in the unfolded protein response (UPR) that get activated with the onset of ER stress and the role of BH3-only pro-apoptotic proteins. The effect of E2F-1 on the relationship between ER stress and cell death has not been formerly investigated in neonatal myocytes.

Methods: Ventricular myocytes were induced to express E2F-1 activity and assessed for its effect during ER stress as triggered by tunicamycin or thapsigargin. Gene transcription of UPR signaling genes was assessed by real-time reverse transcriptase polymerase chain reaction and protein expression was detected by western blot analysis. The effect of E2F-1 regulation of ER stress on cell fate was assessed by cell viability assay as monitored by fluorescent microscopy imaging. Following over expression of Bnip3Δex3 (a splice variant of Bnip3), the effects on mitochondrial defects, cardiac apoptosis and cardiac cell survival were examined.

Results: E2F-1 repressed mRNA expression of UPR signaling molecules during ER stress triggered by tunicamycin or thapsigargin and repressed CHOP protein expression in cardiac myocytes. E2F-1 sensitized ventricular myocytes to apoptotic cell death triggered by thapsigargin induced ER stress. E2F-1 regulated ER stress induced

cell death was inhibited in Bnip3 null fibroblasts. Bnip3Δex3 rescued E2F-1 regulated thapsigargin induced cardiac apoptosis and promoted cell survival. Thapsigargin-induced cell death was mediated by opening of the mitochondrial permeability transition pore which was blocked by Bnip3Δex3 to promote cell survival and restore mitochondrial function. Furthermore, Bnip3Δex3 rescued hypoxia and thapsigargin induced cardiac cell death.

Conclusions: To our knowledge this study provides evidence that E2F-1 sensitizes neonatal myocytes to ER stress induced apoptosis 1) by repressing the UPR; 2) that is Bnip3 dependent; and 3) mediated by mitochondrial dysfunction. The present study has also identified a method for rescuing these effects to promote cell survival by over expression of Bnip3Δex3.

ACKNOWLEDGEMENTS

I wish to thank Dr. Kirshenbaum for his guidance, support and mentorship throughout my graduate training. His wisdom and enthusiasm for science gave me the opportunity to explore scientific research, provoked scientific curiosity within myself and motivated me to always ask questions. I greatly appreciate his understanding and drive for excellence that has motivated me to achieve my goals. I would like to express how grateful I am to my committee members, Drs. Singal, Dhalla, Czubryt and Duhamel, for their guidance, helpful feedback and encouragement throughout my degree.

I would like to thank the Kirshenbaum lab members, both past and present. They have been incredible in sharing their expertise, technical assistance, constructive criticism, wisdom, and have been more like family to me than coworkers. I would sincerely like to thank Mrs. Floribeth Aguilar, Dr. Rimpy Dhingra, Mrs. Victoria Margulets, Dr. Joseph Gordon, Ms. Pamela Lowe, Dr. Yaron Aviv, Mr. Donald Chapman, Ms. Erika Wang, Dr. Agnieszka Biala, Mrs. Hongying Gang, Mr. Tong Zhang and Ms. Kairee Ryplanski. Their hard work and support made my experience in the lab a fun place. Getting our game faces on for the Halloween pumpkin carving competitions was always a great way to do science at a non-science level. I am grateful for our friendships and couldn't have done it without your help. I would also like to thank the summer students that have come through the lab.

Thank you to my family and friends for all their support and for pretending to understand my detailed scientific explanations regarding my research. I would like to thank my sister Madiha for coming to the lab on the weekends and late at night so I wouldn't have to be working alone. No matter what the difficulties I had to face, you

always had a way to make me smile. You have always been there for me and I appreciate it a lot. I want to also thank Mr. Muhib Chughtai for his support, strength and inspiration.

Additionally, I would like to thank other members of the Institute of Cardiovascular Sciences, who took time out to share their advice and expertise with me including the late Mr. Kalwant Dhalla and Mr. Vijayan Elimban. Lastly, I would like to express my gratitude to the Manitoba Health Research Council and the Institute of Cardiovascular Sciences for providing me with financial support during my graduate training to obtain my degree.

LIST OF ABBREVIATIONS

Δψm – mitochondrial membrane potential

AIF – apoptosis inducing factor

APAF– apoptotic protease activating factor

ASK – apoptosis signal-regulating kinase

ATF – activating transcription factor

Atg – autophagy related genes

ATP – adenosine triphosphate

Bcl-2 – B-cell lymphoma 2

BH – Bcl-2 homology

BiP/GRP78 – binding immunoglobin protein/glucose-regulated protein 78

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

Bnip3L – Bcl-2/adenovirus E1B 19 kDa interacting protein 3-like

Calcein-AM – calcein-acetoxymethylester

CHOP – CCAAT/enhancer-binding protein homologous protein

dATP – deoxyadenosine triphosphate

DED – death effector domain

DIABLO – direct IAP-binding protein with low isoelectric point

DISC – death inducing signal complex

DMEM – Dulbecco's modified Eagle's medium

DNA – deoxyribonucleic acid

DR – death receptor

ECL – enhanced chemiluminescence

eiF2α – eukaryotic translation initiation factor 2-alpha

Endo G – endonuclease G

ER – endoplasmic reticulum

ERAD – endoplasmic reticulum associated degradation

FADD – Fas associated via death domain

FasL – Fas ligand

FBS – Fetal Bovine Serum

GFP – green fluorescent protein

HDAC – histone deacetylase

HPX – hypoxia

I/R – ischemia followed by reperfusion

IAP – inhibitor of apoptosis

IKK – inhibitor of kappa light chain gene enhancer in B cells, kinase of

IL – interleukin

IMM – inner mitochondrial membrane

IRE – inositol-requiring kinase

IκBα – inhibitor of kappa light chain gene enhancer in B cells

LAMP2 – lysosomal-associated membrane protein 2

LC3 – light chain 3

LPS – lipopolysaccharide

MOI – multiplicity of infection

mPTP – mitochondrial permeability transition pore

mTOR – mammalian target of rapamycin

NFκB – nuclear factor-kappa B

Nix – Nip3-like protein X

NLS – nuclear localization signal

OMM – outer mitochondrial membrane

PBS – phosphate buffered saline

PERK – protein kinase-like ER kinase

PI3K – class III phosphatidylinositol-3 kinase

Puma – p53-upregulated modulator of apoptosis

qPCR – quantitative polymerase chain reaction

Rb – retinoblastoma

RIP – receptor interacting protein

RNA – ribonucleic acid

ROS – reactive oxygen species

RT-PCR – reverse transcription PCR

SERCA – SR/ER calcium-ATPase

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

Smac – second mitochondrial activator of caspases

SR – sarcoplasmic reticulum

TAB – TAK binding protein

TAK – transforming growth factor beta activated kinase

TBS – tris buffered saline

Thaps – thapsigargin

TM – transmembrane

TNFR – TNF receptor

TNFRSF – TNF receptor superfamily

TNFα – tumor necrosis factor alpha

TRADD – TNFR1 associated DEATH domain

TRAF – TNF receptor associated factor

TRAIL – TNF-related apoptosis-inducing ligand

UPR – unfolded protein response

XBP – X-box binding protein

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I. INTRODUCTION

Shortly after we are born, our cardiomyocytes are unable to actively participate in cell division. Since cardiac myocytes do not contain cell cycle machinery capable of regrowth after suffering an injury, the number of functioning and viable cardiac myocytes is very critical in maintaining proper cardiac performance (1). This aspect of myocyte physiology contributes to detrimental effects once the myocardium is injured and leads to the development of cardiovascular disease including heart failure. As cardiac disease is on the rise as a global epidemic (2), understanding the complex underlying mechanisms of apoptosis resulting in cardiac cell death is a major task at hand for both scientists and clinicians for treating heart failure patients.

The process of apoptosis has been extensively studied in many animal models identifying numerous evolutionarily conserved genes and proteins that underlie the apoptotic signaling mechanisms (3). Physiological embryonic development of tissues and organs during fetal growth requires apoptosis to remove excess cells no longer needed to maintain cellular homeostasis. However, excess or improper regulation of apoptosis can disturb the balance between cell life and cell death, resulting in irreversible cellular damage (4). The ability of apoptotic death to signal detrimental consequences for the cell is well recognized and has been documented in human pathologies including proliferative cancers, neurodegenerative and cardiovascular disease (5).

Apoptosis is a programmed mode of cell death with regulatory molecules and death signals that mediate activation of apoptotic pathways. As an energy-dependent process, activation of apoptosis can be induced by two signaling mechanisms involving cell surface membrane receptors and the mitochondria, both resulting in caspase-induced

cell death specific to apoptosis. The extrinsic arm of apoptosis is triggered by external death signals that activate a ligand-receptor pathway at the membrane which initiates activation of cytoplasmic signaling molecules. The intrinsic apoptotic pathway involves disruption of mitochondrial functionality including permeabilization of the outer mitochondrial membrane and opening of the mitochondrial permeability transition pore. These result in loss of membrane potential, matrix swelling and release of mitochondrial intermembrane proteins that trigger caspase activity, committing the cell to an apoptotic demise (4, 6, 7).

Mitochondrial-mediated apoptosis is influenced by the ratio of pro-apoptotic to anti-apoptotic members of the Bcl-2 family (8). Bnip3 is a pro-apoptotic member that triggers mitochondrial dysfunction by insertion into the mitochondrial membrane during hypoxic mediated apoptosis in cardiac myocytes (9,10). Nuclear transcription factors are key regulatory molecules that control Bnip3 expression. Overexpression of E2F-1 promotes Bnip3 upregulation during hypoxia and increases cell death but in contrast, nuclear factor kappa B (NFκB) represses Bnip3 gene expression during normoxia (10,11,12). Epigenetic control of Bnip3 is influenced by histone deacetylase 1 (HDAC1) that forms an inhibitory complex with NFκB, that creates steric hindrance once bound to the Bnip3 promoter sequence and prevents E2F-1 driven Bnip3 transcription (12,13).

The endoplasmic reticulum (ER), which lies within close proximity to the mitochondria, is a membranous organelle that plays a role in triggering apoptotic death as documented in hypoxic and ischemia/reperfusion (I/R) conditions (15,16,17). Ubiquitously expressed in eukaryotic cells, the physiological role of the ER includes synthesizing proteins, posttranslational protein modifications and managing calcium

(Ca^{2+}) levels. This multifunctional organelle is made up of a reticulated network of cisternae and tubules that organizes the ER into two morphologically distinct structures: rough ER and smooth ER. The rough ER is studded with ribosomes and serves a major role in protein synthesis, modification and transport. In contrast, the smooth ER lacks ribosomes and contributes to lipid and steroid synthesis, detoxification and calcium signaling. The smooth ER has also been known to exist as a separate entity in muscle, the sarcoplasmic reticulum (SR), which plays a specialized role in cell contraction and relaxation based on Ca^{2+} regulation in excitable cells including cardiac myocytes.

The SR is responsible for sequestering a high concentration of Ca^{2+} through SR/ER Ca^{2+}-ATPase (SERCA) that pumps Ca^{2+} from the cytosol into the SR lumen, thus decreasing cytosolic Ca^{2+} and inducing relaxation. The release of Ca^{2+} through intracellular ligand-gated receptors such as ryanodine receptor (RyR) and inositol triphosphate receptor (IP_3R) raises Ca^{2+} levels within the cytoplasm and stimulates contraction. As cardiac cells continue to contract and beat throughout their limited life cycle, these highly specialized cells contain both ER and SR to maintain healthy cellular function.

ER stress is triggered during ER dysfunction that impairs vital processes including protein synthesis, lipid production and calcium management. When the maintenance of cellular homeostasis becomes compromised, ER stress activates the unfolded protein response (UPR) and removes irregular proteins through ER-associated degradation (ERAD) (18,19). Activated ER molecular chaperones mediate nuclear and cytosolic signaling pathways to restore ER homeostasis. ER stress can be induced by agents such as tunicamycin and thapsigargin that trigger stress by independent

mechanisms. Tunicamycin raises the accumulation of unfolded proteins while thapsigargin decreases Ca^{2+} uptake into the ER/SR lumen by inhibiting SERCA, thus increasing cytosolic Ca^{2+}.

ER stress has been implicated in many diseases and understanding the crossroads involved in ER stress and apoptotic signaling mechanisms can further our understanding of heart failure. The role of key apoptotic regulators of cardiac cell death in association with ER stress and its association with mitochondria remains poorly defined. Therefore, the goals of my research are to 1) verify if E2F-1 regulates ER stress in ventricular myocytes; 2) identify the signaling pathways and molecular mechanisms involved in ER stress-induced apoptosis as mediated by E2F-1. The specific aims of my study are to:

1) assess the impact of E2F-1 on ER stress gene transcription in ventricular myocytes;
2) evaluate the impact of ER stress regulated by E2F-1 on cellular viability;
3) determine if ER stress regulated by E2F-1 provokes apoptosis and the role of Bnip3;
4) assess the impact of mechanisms employed to rescue ER stress induced apoptosis.

II. LITERATURE REVIEW

1.0 Heart Failure

Cardiovascular disease is on the rise and is a major concern to public health in terms of treatment costs and management. In 2003, cardiovascular disease was the most costly disease in Canada, responsible for 33% of all deaths (20). The loss of functioning cardiac myocytes results in cardiac impairment and is a detrimental consequence of heart failure. Understanding how cardiac cells die and the underlying molecular mechanisms thereof has great potential to bring about the development of therapeutic strategies in order to treat this pervasive epidemic. According to the American Heart Association, cardiovascular disease is expected to increase in prevalence during the next ten years due to an aging population (2), indicating the importance of improving cardiovascular health through research and the potential benefits preventative therapy may have for the growing number of heart failure patients (20,21).

The heart pumps blood and delivers oxygen to the tissues of the body. On average, the heart pumps 5L of blood per minute to meet metabolic requirements at rest. This flow of blood, known as cardiac output, is a useful way to measure the functionality of the heart muscle (22). The heart's capacity to maintain sufficient cardiac output is determined by the number of effectively functioning cardiac myocytes it possesses; these serve as the contractile units responsible for its pumping function. Cardiac myocytes are specialized, terminally differentiated cells which do not possess the ability to actively divide (1,23). In the failure of cardiac myocytes to function collectively, the subsequent decrease in cardiac output results from improper delivery of oxygen to the myocardium (22). Upon cardiac output deviation and cardiovascular injury from factors such as

myocardial infarction, ischemia, ischemia/reperfusion (I/R) and oxidative stress, the cardiac muscle has a limited capacity to undergo reparative mechanisms (24,25,26). Extensive loss of healthy cardiac myocytes at the site of injury is indicative of cardiac cell death (10), which leads to a decline in cardiac performance. The injured heart responds with the formation of scar tissue in place of viable cardiac myocytes, which, due to lack of contractility, are unable to support proper cardiac function; this ultimately leads to altered cardiac structure (23) and ventricular remodeling (28,29).

One common factor contributing to cardiovascular disease is myocardial ischemic injury. As the chambers of the heart pump blood, nutrients are delivered to the body but not directly to the heart itself. The heart possesses its own circulation consisting of coronary vessels that branch off to provide oxygen to the cardiac tissue (23). However, a blockage within a coronary artery results in a decrease in blood flow towards the target tissue, which leads to myocardial ischemia (30). The restriction in blood flow alters the relationship between oxygen demand and oxygen supply. In hypoxic conditions where oxygen levels are insufficient, cardiac cells cannot maintain cardiac function (31), resulting in a myocardial infarction or heart attack. Prolonged hypoxic conditions result in irreversible cellular damage and myocardial infarctions can occur in any region of the myocardium wall (31). The removal of a coronary obstruction leads to the sudden burst of oxygen towards the injured tissue, or reperfusion, delivering nutrients to the damaged cells. Ironically, this further adds to the detrimental consequences of cardiac remodeling and results in cardiac cell death (31,32). Many signaling pathways within the injured myocardium regulate adaptive and maladaptive biological processes that are important for understanding the multiple mechanisms in mediating cell death in failing hearts.

2.0 Cell Death

Cardiac cell fate can be influenced by at least two recognized pathways of cell death that are "programmed": necrosis and apoptosis; both of which result in the death of functioning cardiac myocytes. Autophagy is a cellular process that provides a mechanism for cells to survive under conditions of nutrient starvation but excessive autophagy has been associated with cardiac disease including heart failure. However, a recognized pathway or conclusive evidence is not known to exist that underlies autophagic cell death. As these processes are defined by morphological characteristics and signaling pathways, identifying the underlying mechanisms that determine how a cell may commit to a specific cell death pathway is ongoing.

3.0 Autophagy

Autophagy is defined as a process in which intracellular organelles and proteins are engulfed by an autophagosome, a double membrane vesicle, and degraded by lysosomal enzymes as the cell's natural way of recycling. The removal of unnecessary material is important to maintain homeostasis. Cells experience basal autophagy, but nutrient shortage during cellular stress further activates autophagy in order to sustain increased metabolic requirements. Excess or deregulated activation of autophagy has been implicated as a trigger of cell death (33,34,35) and implicated in heart failure (36). Irregular autophagic activity has been documented as a contributor towards many diseases, including neurodegenerative disorders, aging, cancer, diabetes and cardiovascular disease, as reviewed by Essick and Sam (37) and Beau et al (38).

Over the last 30 years, the role of defective lysosomal machinery has been documented in many diseases (39). This is most evident in patients with Danon disease,

which develop cardiomyopathy due to a deficiency in lysosomal-associated membrane protein 2 (LAMP2) (40,41). Mice deficient in LAMP2 displayed large aggregates of autophagosomes in isolated cardiac cells (42) and went on to develop contractile impairment (43). Maladaptive autophagy in the myocardium has been shown to be detrimental in various diseases, including pressure overload-induced cardiac hypertrophy (44,45), oxidative stress (46) and myocardial I/R (47,48), all of which contribute to the progression of heart failure.

The process of autophagy is regulated by the autophagy related genes (Atg) (49) that have been evolutionarily conserved from yeast to mammals (50) and identified as important regulators during the four stages of autophagy: induction, elongation and autophagosome formation, docking/fusion and lysosomal degradation (51,52). The initial stage consists of Atg proteins that are responsible for vesicular elongation and recruitment of microtubule light chain 3 (LC3) to the complex. Proteolytic cleavage of LC3 produces LC3-I and a lipid conjugation system converts LC3-I to an autophagic membrane-associated form, LC3-II (53). This forms an autophagosome, a double membrane structure consisting of engulfed material. Disassembly of Atg proteins allows fusion of the autophagosome with a lysosome, resulting in lysosomal degradation of the vesicular contents (54).

Fluorescent microscopy analysis of autophagosome formation utilizes expression of LC3 fused to a green fluorescent-protein (GFP), GFP-LC3 (55, 56). Basal level cells express GFP-LC3 in a uniform manner, but when autophagy is induced, cells display a punctate pattern of bright green dots as GFP-LC3 becomes incorporated into the membrane during autophagosome formation (55); in addition the extent of GFP-LC3 dots

correlates to the degree of autophagy (57). Another biochemical technique detects the LC3-I and LC3-II protein bands by western blot analysis in which the LC3-II to LC3-I ratio of protein expression correlates with the amount of autophagosomes formed (55,58).

The process of autophagy is under tight regulation: the activated form of mammalian target of rapamycin (mTOR) interferes with autophagosome formation (59, 60) while induction occurs by activation of a protein complex comprised of class III phophatidylinositol-3 kinase (PI3K) and Beclin1 (47,61). The activity of Beclin1 is known to be suppressed when bound to Bcl2, an anti-apoptotic factor, which reduces the ability of Beclin1 to induce autophagy (62,63) but disruption of the Beclin1/Bcl-2 complex restores induction (64). Beclin-1 in contrast, cannot inhibit the anti-apoptotic function of Bcl-2 (65). Recent studies have demonstrated inactivation of autophagy by caspase-mediated cleavage of Beclin1 (66,67), in which caspases are key players of apoptotic death. Such interaction involving Beclin1 with key regulators of cell death promotes crosstalk between autophagy and apoptosis. Cellular conditions resulting in deregulated autophagy and the inability to maintain metabolic requirements of the cell may lead to the activation of apoptotic cell death.

4.0 Apoptosis

In a healthy organism, programmed cell death is an integral part of the tissue renewal process. In humans, the outer layer of epidermal cells are lost and replaced by a new layer daily. When the body suffers physical injury, it activates a program to specifically choose and eliminate damaged cells to be replaced with fully functional cells. Physiological apoptosis serves an essential homeostatic role. Apoptosis during embryogenesis removes excess cells, such as in the case of the loss of Mullerian

structures in the fetus for male organ development (5) and it is also important for developmental stages during tadpole tail regression (68). However, apoptotic inhibition during embryonic limb development leads to the emergence of a "webbed" phenotype due to excess tissue formation between fingers and toes (69). Significant work conducted in *Caenorhabditis elegans* and other animal models have identified many evolutionarily conserved genes, regulatory mechanisms and the specificity of apoptosis during certain biological processes (3).

Defined as a highly regulated form of cell death during the 1970s (70), apoptosis is considered to be a response to external and internal stimuli activating separate signaling cascades resulting in cell death (4). A defect in the apoptotic machinery results in deregulated or abnormal cell death, disturbs cellular homeostasis and contributes to human pathologies. It is well documented that apoptosis occurs in the myocardium during hypoxia, I/R, myocardial infarction and heart failure (71,72,73). Although apoptosis plays a homeostatic role during development, studies suggest the possibility that mechanisms involved in apoptotic execution may be tissue specific and regulated differently during development and disease.

Apoptosis is characterized by several factors: the presence of membrane blebbing without the loss of membrane integrity, formation of apoptotic bodies, chromatin condensation, cell shrinkage, DNA fragmentation and lack of inflammation (74). After cells are genetically manipulated or exposed to conditions such as hypoxia, they can be stained with Hoechst 33258 to visualize nuclei (71); healthy cells display well rounded nuclei with definite shape, while cells undergoing apoptosis exhibit condensed and abnormally shaped shrunken nuclei (75). DNA laddering is another biochemical

technique used to detect fragmented DNA which is a result of the cleaving action of nucleases during apoptosis that create DNA fragments of distinct sizes (76). Caspase activity is another key hallmark unique to apoptosis; caspase signaling cascades are activated by apoptotic triggers not otherwise observed in other forms of cellular death (77). Caspases are believed to work in a hierarchal manner in which upstream initiator caspases including caspase-8,-9,-10 activate downstream executioner caspases such as caspase-3,-6 and -7 (78). External and internal stimuli activate receptor-mediated or mitochondrial mediated-apoptosis.

4.1 Extrinsic Death Pathway

External death ligands activate the extrinsic pathway mediated by transmembrane receptors at the surface of the cell membrane. Extracellular death signals such as Fas ligand (FasL) (79), tumor necrosis factor-α (TNFα) (80) and TNF–related apoptosis-inducing ligand (TRAIL) (81) are received by the death receptors belonging to the TNF-receptor super family (TNFRSF) such as TNF-receptor 1 (TNFR1) (82) and death receptor 4 (DR4) (83). Ligand-receptor binding induces receptor trimerization which recruits cytosolic adaptor molecules such as Fas-associated death domain (FADD) (84) and TNFR-DEATH domain (TRADD) (85); these molecules assemble the death inducing signal complex (DISC). This protein complex is responsible for the recruitment and activation of initiator caspases that proteolytically cleave downstream executioner caspases, committing the cell to an apoptotic fate.

4.2 Intrinsic Death Pathway

In comparison to ligand-induced death, the intrinsic pathway of apoptosis becomes activated by various signals both of external and internal sources including

ultraviolet radiation, treatment with toxic agents, hypoxic conditions and genetic damage. Also termed as the mitochondrial pathway, the apoptotic mechanisms are mediated through the "powerhouse" of the cell in which the mitochondria undergo functional changes and release pro-apoptotic factors into the cytoplasm. The cardiac mitochondria are responsible for providing the cellular energy to myocytes; managing cellular metabolic demands by generating adenosine triphosphate (ATP) is accomplished through the mitochondrial respiratory chain and oxidative phosphorylation (7). Healthy mitochondria consist of an impermeable inner membrane that creates an electrochemical gradient between the matrix and intermembrane space established by the electron transport chain. ATPase, an enzyme localized in the inner mitochondrial membrane (IMM), pumps protons and is responsible for generating the mitochondrial membrane potential ($\Delta\psi m$). This is essential for generating ATP to drive cellular biological processes.

Although mitochondria are the site of energy production, the organelle is also the source of reactive oxygen species (ROS) generation that contributes to oxidative stress and triggers mitochondrial dysfunction. Under certain circumstances, mitochondrial disturbances involve opening of the mitochondrial permeability transition pore (mPTP) and selective permeabilization of the outer mitochondrial membrane (OMM) resulting in impairment of oxidative phosphorylation machinery (7). Permeabilization of the OMM by Bax/Bak, the pro-apoptotic proteins belonging to the Bcl-2 family, leads to a lethal dissipation of the $\Delta\psi m$, contributing to the initial stages of apoptotic cell death including release of pro-apoptotic factors (86). With the rupture of the outer membrane, mitochondria release intermembrane proteins such as cytochrome c (87), second

mitochondria derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein with low pI (Smac/DIABLO) (88), apoptosis inducing factor (AIF) (89), and endonuclease G (endo G) (90). The IAPs (91) repress apoptosis by preventing the activation of mature caspases. Following a death signal, the release of Smac/DIABLO in the cytoplasm prevents the inhibitory function of IAPs, thus promoting apoptotic death (92). Endo G translocates from the cytoplasm to the nucleus which activates DNA fragmentation while AIF promotes progression of apoptotic damage (90). Cytochrome c gets recruited to an apoptosome consisting of apoptotic protease activating factor 1 (APAF1) (93), deoxyadenosine triphosphate (dATP) (94) and pro-caspase-9 (95) that is responsible for activating downstream caspases. It is thought the cell's "choice" to signal activation of apoptosis may be determined by the balance between pro-apoptotic and anti-apoptotic proteins within a cell.

5.0 Regulators of Apoptosis

5.1 Bcl-2 family

The balance between cardiac cell survival and cardiac cell death involves the relative ratio of specific proteins belonging to the B-cell lymphoma 2 (Bcl-2) family in which members are both pro-apoptotic and anti-apoptotic (96,97). Abundance of pro-apoptotic Bcl-2 family members will inhibit life promoting Bcl-2 family members and result in mitochondrial damage by disruption of the mitochondrial membrane.

As a hypoxia-inducible member of the Bcl-2 family, Bcl-2/Adenovirus E1B 19 kDa protein-interacting protein-3 (Bnip3) has been identified as a pro-apoptotic protein in tissue cultures including isolated cardiac myocytes, sharing only the BH3 domain with Bcl-2 (9). Recent work has reported abnormal Bnip3 activation associated with

neurodegenerative diseases such as Huntington's (98) and Parkinson's (99), neuroexcitotoxicity (100), rheumatoid arthritis (101) and endometrial cancer (102). Studies by the Kirshenbaum lab have documented Bnip3 as a unique protein that becomes activated under hypoxic injury, mediates mitochondrial defects consistent with mPTP opening, loss of $\Delta\psi m$ in cardiac myocytes and is upregulated in ischemic cardiac models (9). Loss of the transmembrane (TM) domain of Bnip3 (Bnip3ΔTM) prevented hypoxia-induced mitochondrial dysfunction and reduced cell death mediated by apoptosis in neonatal myocytes (11) demonstrating that Bnip3 integration into mitochondrial membrane is critical in triggering mitochondrial disruption. Along with hypoxic injury, Bnip3 has been recognized as an important molecular player during myocardial I/R (103). Therefore, pro-apoptotic members of the Bcl-2 family such as Bnip3 play a critical role in human pathologies and contribute to activation of mitochondrial mediated apoptotic cell death in cardiac myocytes.

5.2 NFκB

Nuclear transcription factor, NFκB, is ubiquitously expressed in eukaryotic cells with a role in different biological processes including innate immunity (104), inflammation (105) proliferation and cell survival (106,107) . However, deregulation has been associated with many diseases including breast and lung cancer (108,109), periodontitis (110) and other metabolic diseases (111). NFκB exists as a dimeric protein consisting of different subunits including p50/p105, p65 (Rel A), p54 (NFκB2)/p100, RelB and c-Rel. Homodimer or heterodimer combinations of NFκB result in different transcriptional functions that mediate specific biological processes as reviewed by Hayden and Ghosh (112). Specific to the myocardium, our laboratory has focused on the

role of p65 subunit of NFκB in regulating pro-survival mechanisms. In neonatal ventricular myocytes, the Kirshenbaum lab was among the first to establish the importance of NFκB signaling in mediating cell survival in which the functional loss of p65 increased TNFα-induced apoptotic cell death (113).

During non-stimulated conditions, NFκB is found localized in the cytoplasm. The inactive form of NFκB is tightly controlled by inhibitor of kappa B (IKBα) that prevents translocation to the nucleus by masking the nuclear localization signal (NLS) (114). Stimulation from ionizing radiation (115), hypoxia (116) and inflammatory cytokines such as interleukin-6 (IL-6) and TNFα (117) trigger NFκB activation. In the heart, TNFα stimulation activates ligand-receptor activation of TNFR1 followed by the recruitment of adaptor proteins including TRADD, TNFR-associated factor (TRAF2) (85), TRAF5 and receptor interacting protein 1 (RIP1) to form complex I (118), a complex essential for the activation of NFκB.

Ubiquitin chains bind at site Lys-377 of RIP1, resulting in a polyubiquinated status essential for the induction of survival genes (119). RIP1 acts as a scaffold for assembling the transforming growth factor-β activated kinase 1 (TAK1)-binding protein 2 or 3 (TAB2/3) complex that further recruits the inhibitor κ kinase (IKK) complex consisting of IKKα, IKKβ and IKKγ (120,121). Phosphorylated IKKβ functions to phosphorylate IKBα at sites Ser-32 and Ser-36, signaling ubiquitylation and 26S proteosomal degradation of IKBα resulting in the release of NFκB (122). The NLS is unmasked and the p65 subunit of NFκB can translocate into the nucleus to initiate transcription of its target genes. Functional loss of p65 has rendered cells more susceptible to apoptotic death induced by TNFα (123). Studies have shown IKKβ is

essential for NFκB activation such that IKKβ deficient mouse embryos died (124). The Kirshenbaum lab also reported repression of IKKβ increased susceptibility to apoptosis in ventricular myocytes after TNFα stimulation (113,125). Furthermore, it was shown that NFκB mediated cell survival in neonatal ventricular myocytes by preventing hypoxia-induced cell death (126).

5.3 E2F-1

Another molecule involved in regulating cell growth is E2F-1, a member of the E2F family of nuclear transcription factors involved with cell cycle control in mammalian cells (127). In contrast to the other 8 identified members of the E2F family (128), E2F-1 activation regulates the transition between G1 and S phase during the cell cycle and uniquely promotes apoptotic death (129). Transcriptional control of E2F-1 is regulated by the retinoblastoma gene product (Rb) (130), a tumor suppressor protein that inhibits the progression of the cell cycle when faulty mechanisms such as DNA damage have been recognized to signal growth arrest. Under normal cellular division, Rb binds to E2F-1 in the cytoplasm and prevents transcriptional function (131). Cyclin D, a member of the cyclin family involved with cell cycle control, inactivates Rb upon phosphorylation (132) which allows E2F-1 to translocate to the nucleus to regulate gene transcription. Overexpression of E2F-1 has been known to induce apoptotic cell death (133,134) and regulate apoptotic molecular players such as Bnip3 (9). A tumor study led by Gomez-Gutierrez and coworkers designed a truncated form of the E2F-1 gene lacking the transactivation domain which was shown to greatly induce apoptosis and decrease tumor size (135). Thus, balanced E2F-1 activity is essential in regulating underlying mechanisms of apoptotic cell death.

6.0 Epigenetic Regulation

Understanding the molecular regulatory mechanisms involved in intrinsic apoptotic cell death required investigating epigenetic regulation of Bnip3. Promoter sequence analysis identified binding site cis-elements for transcription factors NFκB and E2F-1 that were shown to regulate Bnip3 gene expression (11,12,13). An NFκB-HDAC1 inhibitory complex recruited to the Bnip3 promoter silenced gene transcription in cardiac myocytes during normoxia while hypoxia caused a decrease in NFκB activity, restoring Bnip3 promoter activity and apoptotic cell death. Additionally, overexpression of E2F-1 was sufficient to activate Bnip3 gene transcription and induce cell death (11). A cardiac model produced by Shaw and colleagues illustrated that E2F-1 bound to Bnip3 promoter released the inhibitory brake of NFκB-HDAC1, relieving steric hindrance and enhancing Bnip3 expression during hypoxia while NFκB activation inhibited E2F-1 driven Bnip3 expression (13). These studies highlight the transcriptional control of Bnip3 in mediating apoptosis and signify how activity of histone-modifying proteins can influence cardiac gene expression involving epigenetic regulation of key pro-apoptotic factors (14).

Novel evidence has documented cellular events that can regulate cell survival and cell death mediated by an endogenous inhibitor. Bnip3Δex3, a novel isoform of full length Bnip3 generated by alternative splicing during hypoxia, was reported by the Kirshenbaum laboratory to promote cell survival and repress hypoxia-induced cell death mediated by Bnip3. Studies in ventricular cardiac myocytes demonstrated that Bnip3Δex3 reduced mitochondrial dysfunction driven by Bnip3 and the alternative splicing machinery event was specific to hypoxia (136). The study by Gang and colleagues was the first report to illustrate how the alternative splicing machinery of a

Bcl-2 pro-apoptotic family member rescued mitochondrial-mediated apoptosis during hypoxic injury in cardiac cells. This novel finding further implicates the importance of the underlying mechanisms regulating life and death processes in the heart.

7.0 Endoplasmic Reticulum Stress

Although mitochondrial defects are implicated in cardiac dysfunction, other organelles have been documented to also play a functional role. Over the last decade, advances in research have emphasized the role of endoplasmic reticulum (ER) in cardiac cell death. The ER is an organelle involved in performing diverse functions in mammalian cells including lipid production, regulation of Ca^{2+} handling, protein synthesis and post-translational modification prior to protein export (137). Accumulation of unfolded proteins or disruption of ER function activates the unfolded protein response (UPR) (138,139) to restore ER functionality and remove irregular proteins through ER-associated degradation (ERAD) (18,19). The unique ER lumen consists of an oxidizing environment essential for the formation of disulfide bonds but that also contributes to oxidative stress resulting in apoptosis (140) and myocarditis (141) (Figure 1).

Furthermore, chronic or severe ER stress during I/R and hypoxia can activate apoptotic cell death (142,143,144) as it is implicated in neurodegenerative disorders (145,146), metabolic disease (147) and cardiovascular disease (148,149). Tissue cultures can be stimulated by substances involved in altering the oxidative environment, managing calcium levels and proper protein folding such as dithiothreitol, tunicamycin and thapsigargin to be used as experimental tools to understand the UPR (150,151). Tunicamycin increases accumulation of misfolded proteins by inhibiting the

glycosylation of proteins while thapsigargin alters Ca^{2+} regulation by blocking SERCA and raising cytosolic Ca^{2+}.

UPR activation involves an ER resident molecule, binding immunoglobin protein/glucose-regulated protein 78 (BiP/GRP78) that senses ER stress (152). Under normal conditions, GRP78 is tethered to ER transmembrane proteins and UPR activation releases GRP78 into the lumen where it corrects the conformation of unfolded proteins (153). The three integral ER transmembrane proteins that activate the UPR are protein kinase-like ER kinase (PERK), inositol-requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6) (138). Dissociation of the transmembrane proteins from GRP78 induces cytoplasmic and nuclear signaling pathways (154,155) (Figure 2).

Auto-phosphorylation and dimerization of PERK, a serine/threonine kinase, leads to phosphorylation of eukaryotic translation initiation factor 2 alpha (eiF2α) resulting in inhibition of protein translation (154). Increased PERK activation leads to expression of ATF4 that regulates CCAAT/enhancer-binding protein homologous protein (CHOP), a proapoptotic transcription factor (156). Similar to PERK, phosphorylation and dimerization of IRE1 activates its endoribonuclease domain (155), critical in mediating the mRNA splicing event of X-box binding protein-1(XBP1) mRNA that yields a transcriptionally active form of XBP1 involved in the UPR and ERAD (155). Cleavage-mediated activation of the third transmembrane protein, ATF6, also functions in regulating CHOP and XBP1 mRNA (157) (Figure 2). In failing hearts, ER stress activation of XBP1 was found to regulate cardiac brain natriuretic peptide, an important molecule used in diagnosing heart failure (158).

Ischemic injury in cardiac myocytes has been shown to activate ERAD (159), upregulate BiP/GRP78 expression (15,160) and caspase -12 (16), indicating that ER stress contributes to cardiac apoptosis. In a heart failure animal model, activation of BiP/GRP78 and CHOP were significantly increased in induced chronic myocardial ischemic hearts (17). Recent work by Miyazaki and colleagues found ER stress induced by myocardial I/R in CHOP-deficient mice inhibited myocycte apoptosis and repressed cardiac injury while the expression of inflammatory cytokines induced by thapsigargin was also suppressed in CHOP-deficient myocytes (161), identifying CHOP as a molecular player contributing to myocardial injury. Earlier it was reported that the p65 subunit of NFκB repressed CHOP promoter activity, inhibited tunicamycin induced ER stress-mediated cell death and increased cell survival in breast cancer cells (162).

Research suggests that CHOP may mediate apoptosis by disrupting the balance between pro-apoptotic and anti-apoptotic Bcl-2 members including Bcl-2 itself (163) and Bim (164,165). Furthermore, blocking the activation of CHOP/p53 upregulated modulator of apoptosis (Puma) signaling pathway inhibited ER stress-induced apoptosis in cardiomyocytes (149), supporting work of another independent study (166). Such reports suggest CHOP plays a critical role in mediating ER stress-induced apoptotic death in cardiac myocytes.

Furthermore, ER-induced stress mediated by IRE1 involves interaction between the α-isoform of IRE1 with pro-apoptotic Bcl-2 members, Bax and Bak, in amplifying the apoptotic signal (167). IRE1 recruits TRAF2 that activates a death mechanism by recruiting apoptosis signal-regulating kinase 1 (ASK1) (148,168,169) and caspase-12 (170), contributing to cardiac apoptosis.

Activation of the different arms of the UPR in response to severe ER stress results in irreversible cardiac damage by activating the apoptotic signaling machinery (Figure 3). These findings suggest ER stress in non-UPR pathways may be mediated by other mechanisms as interest in ER and mitochondria communication has greatly increased. Along with inducing UPR signaling molecules, the ER lumen holds the highest concentration of Ca^{2+} in the cell. Mitochondrial Ca^{2+} is utilized in energy production and fluctuations in ER Ca^{2+} released by pro-apoptotic Bcl-2 family members have been shown to promote apoptosis (171). Therefore, disruption of ER-mitochondrial signaling interplay contributes to ER stress induced apoptotic cell death (172,173) and understanding such a close organelle relationship can identify potential therapeutic targets for preventing the progression of cardiovascular disease.

Therefore, the goals of this research are to 1) verify if E2F-1 regulates ER stress in ventricular myocytes and 2) identify the signaling pathways and molecular mechanisms involved in ER stress-induced apoptosis as mediated by E2F-1. The specific goals of this project are to:

1) assess the impact of E2F-1 on ER stress gene transcription in ventricular myocytes;
2) determine the impact of ER stress regulated by E2F-1 on cellular viability;
3) determine if ER stress regulated by E2F-1 provokes apoptosis and the role of Bnip3;
4) assess the impact of mechanisms employed to rescue ER stress induced apoptosis.

III. Materials and Methods

Cell Culture

Neonatal ventricular myocytes from two day old Sprague-Dawley rat hearts were isolated as previously reported (1). Myocytes were submitted to primary culture and plated at a density of 1 x 10^6 cells per 35mm plate. After overnight incubation at 37C in Dulbeco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (DF) supplemented with 17mM HEPES, 3mM NaHCO3, 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 (1). Wild-type mouse embryonic fibroblasts (MEFS) and Bnip3$^{-/-}$ MEFS were cultured as previously reported (174,175). Myocytes were maintained in serum-free medium and MEFs were maintained in DMEM + 10% FBS medium and all cell cultures were harvested 48hr after genetic manipulation.

ER stress and Hypoxia

To induce ER stress, cells were exposed to tunicamycin T7765 (1µg/ml; Sigma-Aldrich,Oakville, Ontario) or thapsigargin T9033 (100ng/ml; Sigma-Aldrich, Oakville, Ontario) for a 18hr incubation after genetic manipulation. Post-natal ventricular myocytes were subjected to 18hr in an air tight chamber under serum free culture conditions continually gassed with 95% N_2 plus 5% CO_2, pO_2<10mmHg as previously described (12,176).

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, titered and purified as previously reported (8). Myocytes and MEFs were infected with adenoviruses encoding wild-type E2F-1, Bnip3FL and Bnip3Δex3 at multiplicity of infection (MOI) of 10 which achieves gene delivery of >90% to cells (177). 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.

Western Blot Analysis

For detecting total protein, neonatal myocytes were harvested in NP-40 lysis buffer containing protease inhibitors. Protein cell lysates (20-25μg) were denatured for 5 minutes at 100C and resolved on a 4-12% sodium dodecyl sulfate polyacrylamide gel at 80 volts for 20 minutes followed by 100 volts for 1hr. The protein lysates were electrophoretically transferred to a nitrocellulose (Amersham Biosciences, Baie d'Urfe Quebec) membrane or a polyvinylidene difluoride (PVDF) membrane at 100 volts for one hour at 4C or at 20 volts overnight at room temperature. Membranes were subjected to Ponceau-S staining to visualize protein bands and blocked for 1 hr in 5% powdered skim milk in TBS-TWEEN (150mM NaCl, 50mM Tris-HCl, 0.3% Tween-20, pH 7.4) at room temperature. Membranes were incubated with primary IgG antibodies directed toward a protein of interest at a specified dilution overnight at 4C. Following incubation, membranes were washed three times with 1 x TBS-TWEEN for 10 minutes each and incubated with specific secondary antibodies for one hour at room temperature. A chemi-

luminescence reaction using horseradish peroxidase conjugated antibody with enhanced chemi-luminescence (ECL) reagents (Amersham Biosciences) was used to detect bound proteins. For detection of CHOP protein, a mouse anti–CHOP antibody (L63F7, Cell Signal, Danvers, Massachusetts) at a dilution of 1:1000 in 2.5% bovine serum albumin (BSA) was used. To detect equal protein loading, membranes were probed with α–actin (Sigma) at a dilution of 1:1000 in 2.5% BSA.

Cell Viability and Apoptosis

Cell viability was determined by fluorescent dyes to assess cardiac gene expression and epifluorescent microscopy. This technique consisted of staining cells with 4μM calcein acetoxymethyl ester (AM) and 2μM ethidium homodimer-1 (Molecular Probes). Calcein AM stained the live cells green and ethidium homodimer-1 stained the dead cells 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 37C for 30 minutes. Myocytes were washed with PBS and fresh DFSF media was added. The glass coverslips were inverted onto glass sides and visualized. Cells were analyzed from at least n=3-4 independent myocyte cultures counting ≥ 300 cells for each condition tested (75). Data are expressed as mean±S.E percent dead cells from control.

Fluorescent microscopy was utilized to analyze nuclear morphological changes associated with apoptotic cells. Cardiac neonatal myocytes were grown on coverslips and after experimental manipulation, myocytes were fixed in cold 70% ethanol and stained with Hoechst dye 33258 (Molecular Probes, Eugene, Oregon) for 3-5 minutes in

PBS. Coverslips were washed with PBS and mounted onto glass slides. Individual cells were assessed for nuclear morphological changes such as nucleosomal DNA fragmentation as characterized by pyknotic hyperchromatic nuclei (8,75). All fluorescent images of cells were captured using an Olympus AX-70 research fluorescence microscope. Cells were analyzed from at least n=3-4 independent myocyte cultures counting ≥ 300 cells for each condition tested (75). Data are expressed as mean±S.E percent apoptotic cells.

Mitochondrial Permeability Transition Pore

Mitochondrial PTP opening was determined by loading myocytes with 5μM calcein-AM (Molecular Probes) in the presence of 5mM cobalt chloride ($CoCl_2$) and incubated for 30 min at 37C (9, 12,178). Calcein-AM will stain mitochondria of viable cells and in the presence of $CoCl_2$, this signal is quenched. The change in integrated fluorescent intensity is an index of PTP opening. Viable cells will be visualized as green organelles against a dark background while PTP opening will leak the calcein-AM from the mitochondria and organelles will appear colourless. Myocytes plated on coverslips were washed with PBS and fresh DFSF media was added. Myocytes were visualized and captured by using an Olympus AX-70 research fluorescence microscope and analyzed using Image J software to generate a histogram of corrected fluorescence signals for each condition relative to control. Data are expressed as mean±S.E percent change from control.

Real-Time Quantitative PCR

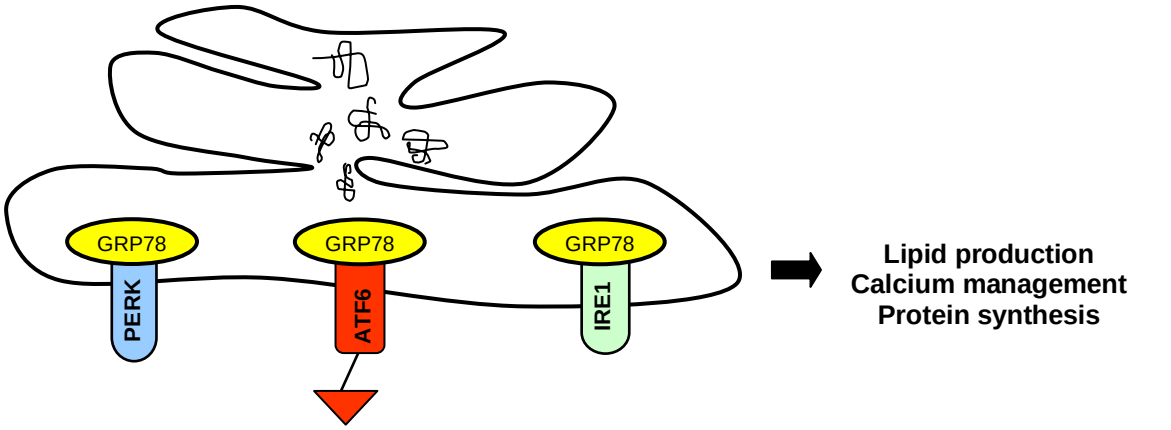
Total RNA (1μg) from neonatal cardiac myocytes was isolated with the GenElute^{TM} Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and reverse-transcribed with oligo dT_{20} (Invitrogen). One-twentieth of the reaction was amplified with gene-specific primers for Bnip3, Bnip3ΔEx3, GRP78, CHOP, IRE1, ATF6 and house keeping control gene L32. Real-time RT-PCR was performed using the iQ5 multicolor real-time PCR detection system (Bio-Rad) using the ΔΔCT method to calculate the fold change in gene expression. Data are expressed as mean±S.E as relative fold change from control.

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 value <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

A



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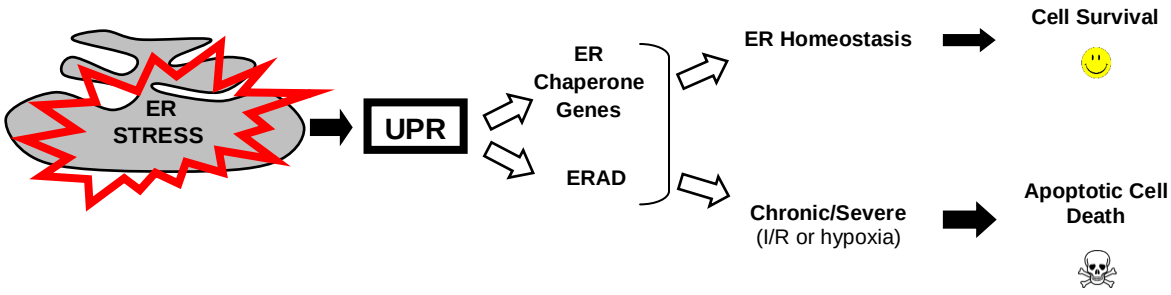

Figure 1: ER function and activation of ER stress.
A. Under homeostatic conditions, the ER resident protein GRP78/BiP, is bound to the three integral ER transmembrane proteins responsible for the UPR signaling pathways. The ER is responsible for lipid production, regulating Ca^{2+} levels, protein synthesis and post translational modification. **B.** With the onset of ER stress, the UPR results in upregulation of ER chaperone genes and ERAD to remove irregular proteins and to correct for the accumulation of unfolded proteins. Activation of the UPR pathway is to restore ER homeostasis and promote cell survival. However, if homeostasis is not reestablished, chronic or severe ER stress caused by I/R or hypoxia can trigger apoptotic cell death in myocytes.

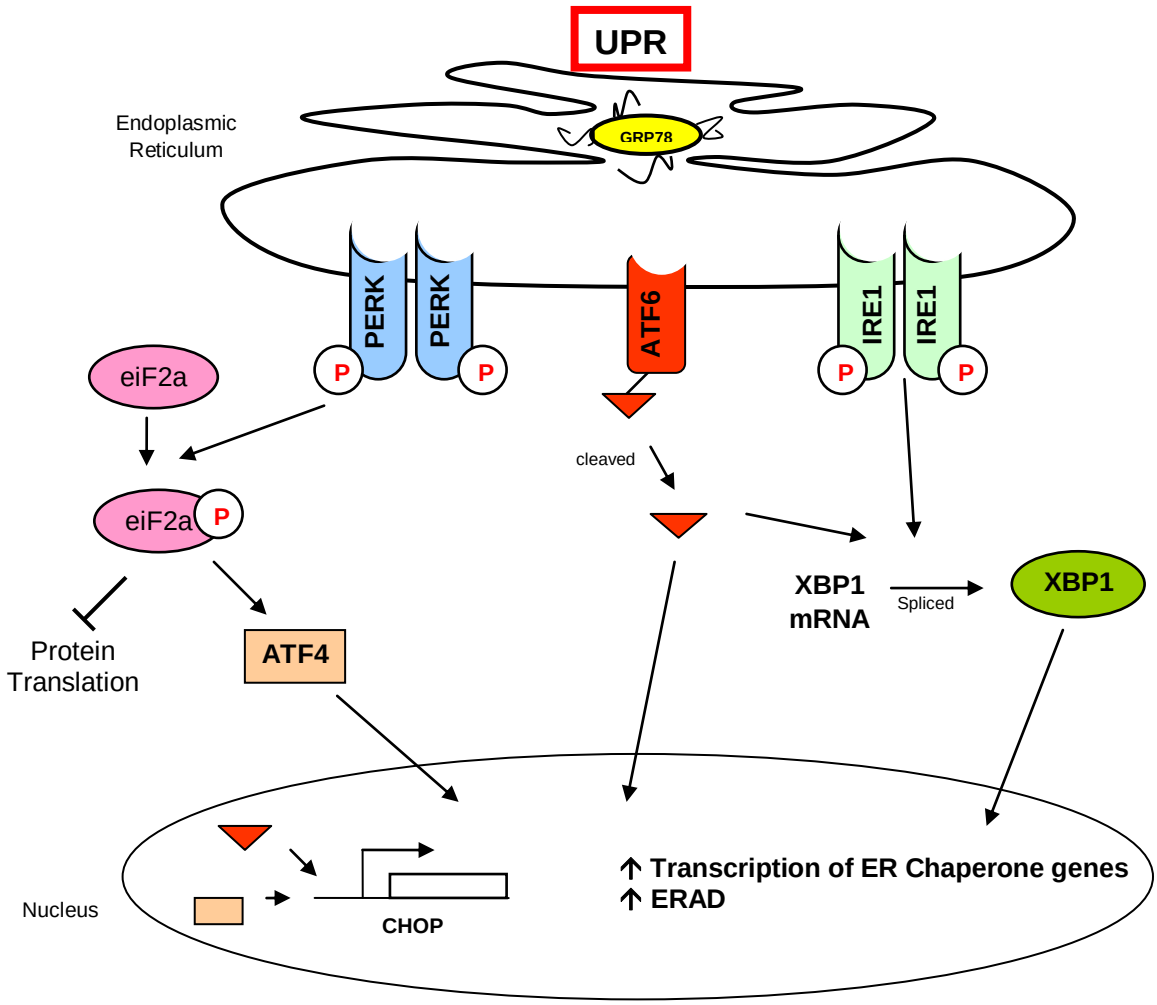

Figure 2: UPR signaling pathways.
Many factors such as the accumulation of unfolded proteins and disruption of ER function activate ER stress and results in the activation of the UPR. GRP78 dissociates into the ER lumen to correct the conformation of unfolded proteins. This releases the three ER transmembrane proteins, PERK, ATF6, and IRE1, which activate cytosolic and nuclear signaling pathways that upregulate transcription of ER chaperone genes and ERAD. XBP1, ATF4 and cleaved ATF6 translocate to the nucleus and both ATF4 and cleaved ATF6 regulate gene transcription of CHOP.

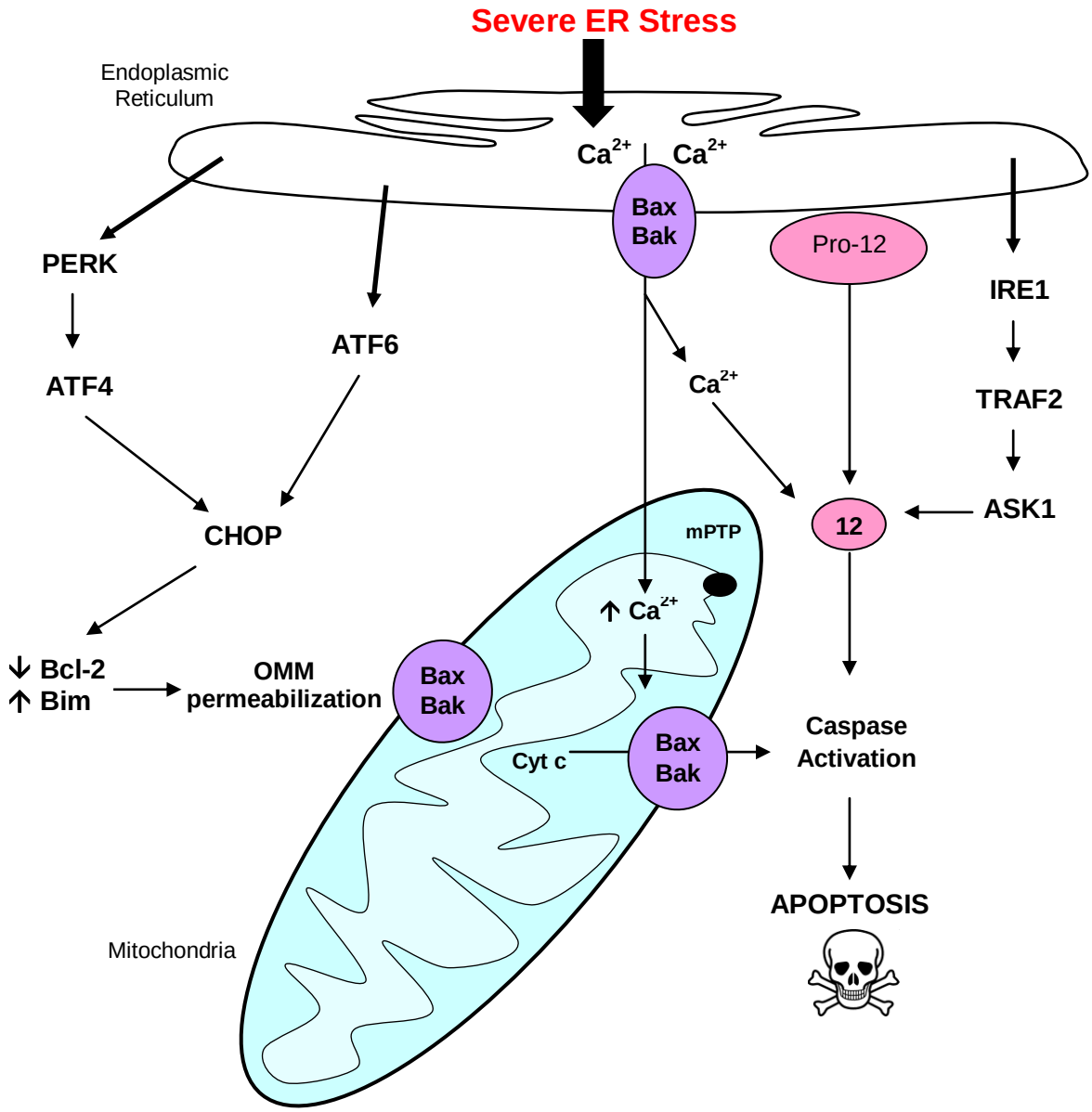

Figure 3: Severe ER stress induces apoptosis.
Activation of PERK and ATF6 both regulate CHOP expression that studies suggest to disrupt the balance between Bcl-2 and Bim, resulting in mitochondrial mediated apoptosis that can be amplified by the Bax/Bak channel. Calcium flux between ER and mitochondria also induces apoptosis. Activation of caspase-12 and IRE1 signaling through TRAF2 and ASK1 results in caspase mediated apoptosis.

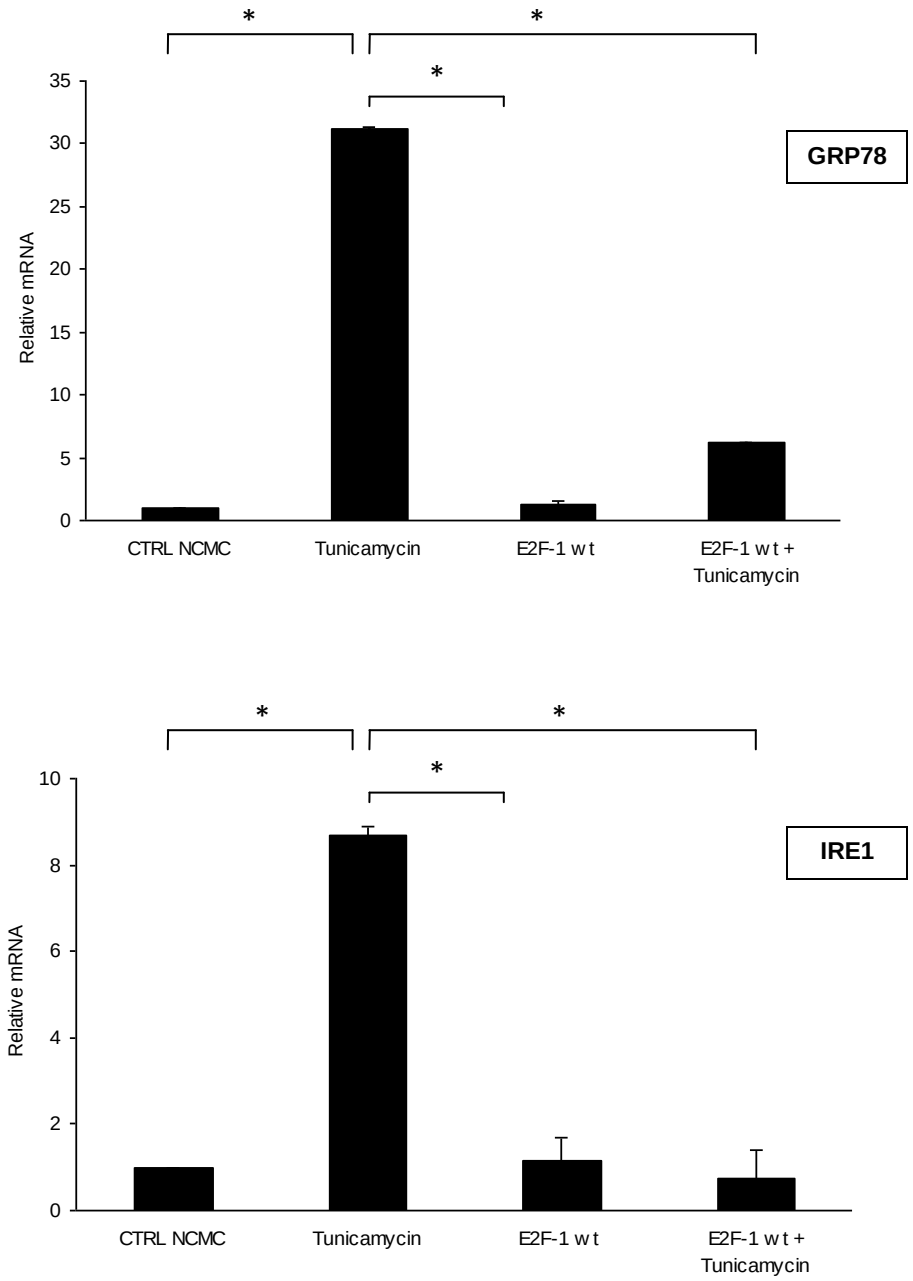

Figure 4: E2F-1 represses the UPR induced by tunicamycin in neonatal myocytes. Real-time qPCR analysis of relative mRNA expression levels of GRP78 and IRE1 during tunicamycin induced ER stress in cardiac myocytes. Data are expressed as mean±SE from n=3-4 independent experiments using triplicates for each condition tested. Statistical significance among the group is indicated * $p<0.05$.

E2F-1 repression of the UPR induced by tunicamycin in neonatal cardiac myocytes

To assess if E2F-1 could regulate biological processes occurring with the onset of ER stress, neonatal cardiac myocytes were infected with adenovirus encoding for E2F-1 wt to study the effects of E2F-1 on UPR genes. In Figure 4, tunicamycin-induced ER stress activated the UPR and resulted in an upregulation of gene transcription of GRP78 (30-fold increase, $p<0.05$) and IRE1 (8-fold increase, $p<0.05$). This effect was repressed by the presence of E2F-1 activity, thus down-regulating the UPR signals in cardiac myocytes.

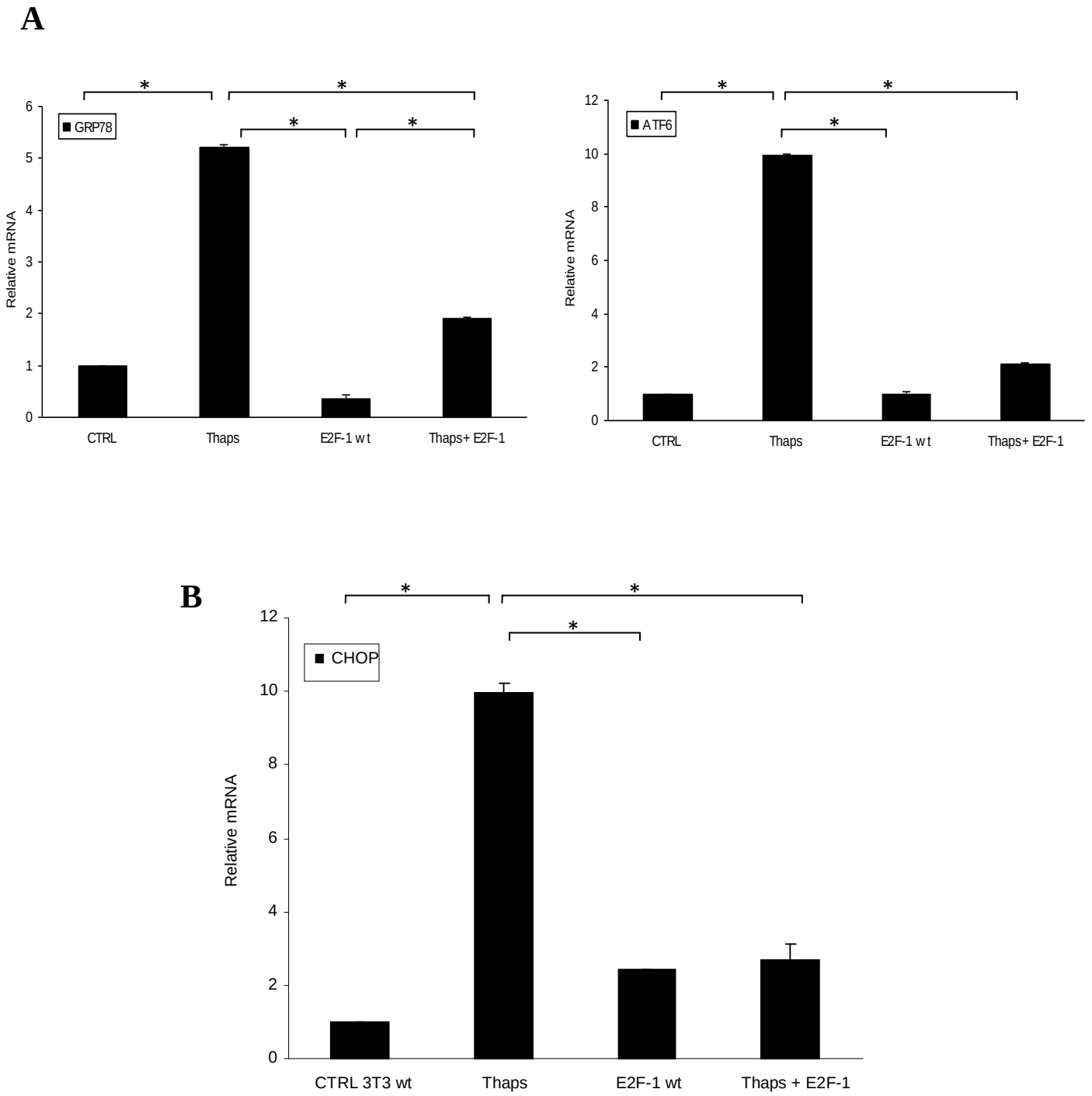

Figure 5: E2F-1 regulation of thapsigargin induced ER stress in neonatal myocytes and mouse embryonic fibroblasts. Panel A displays real-time qPCR analysis of relative mRNA expression levels of GRP78 and ATF6 in neonatal myocytes. Panel B displays gene transcription of CHOP in 3T3 wild-type MEFs. Data are expressed as mean±SE from n=3-4 independent experiments using triplicates for each condition tested. Statistical significance among the group is indicated * $p<0.05$.

Thapsigargin activation of the UPR is repressed by E2F-1.

In contrast to tunicamycin which increases accumulation of misfolded proteins in the ER lumen, another trigger of ER stress was utilized to study the effect of E2F-1 on the UPR. Thapsigargin induces ER stress by blocking the SERCA channel and inhibiting the uptake of Ca^{2+} into the ER lumen, thus increasing cytosolic $Ca^{2+.}$ Neonatal myocytes and 3T3 wild-type MEFs were infected with adenovirus encoding E2F-1 wt and followed by thapsigargin treatment. Real-time quantitative polymerase chain reaction (qPCR) analyzed mRNA expression levels. In Figure 5, thapsigargin treatment resulted in an increase of mRNA expression of the UPR markers. Panel A displays an upregulation in gene transcription of GRP78 (5-fold, $p<0.05$) and ATF6 (10-fold, $p<0.05$) and this effect was repressed by E2F-1 activity in cardiac myocytes. Repression of CHOP gene transcription by E2F-1 was displayed in wild-type MEFs during thapsigargin induced ER stress (panel B). The data suggests E2F-1 inhibition of the UPR signaling molecules is conserved among neonatal ventricular myocytes and wild-type MEFs.

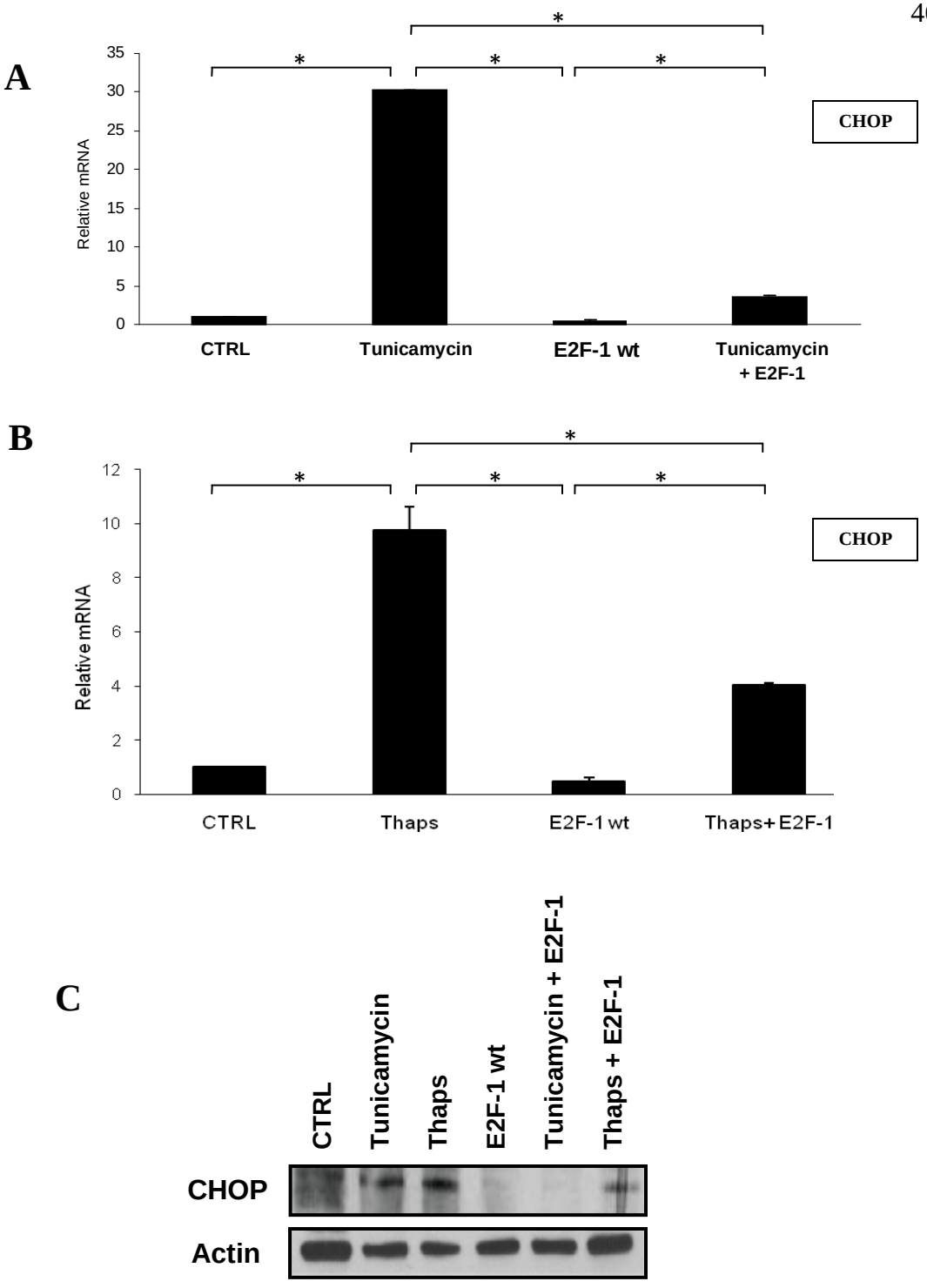

Figure 6: E2F-1 represses CHOP expression induced by ER stress in ventricular myocytes. Real-time qPCR analysis of relative mRNA levels of CHOP in neonatal myocytes during tunicamycin (Panel A) and thapsigargin (Panel B) induced ER stress. Data are expressed as mean±SE from n=3-4 independent experiments using triplicates for each condition tested. Statistical significance among the group is indicated * $p<0.05$, NS=non-significant. Panel C displays myocyte cell lysate subjected to western blot analysis with antibodies directed towards CHOP protein.

E2F-1 represses CHOP expression activated by ER stress in ventricular myocytes.

Real-time qPCR analysis of CHOP mRNA expression levels in myocytes infected with E2F-1wt were assessed during ER stress. Panel A (Figure 6) displays tunicamycin activation of CHOP gene transcription (30-fold, $p<0.05$) which was repressed by E2F-1 activity (3-fold, $p<0.05$). Panel B shows thapsigargin activation of CHOP gene transcription (10-fold, $p<0.05$) which was also in the presence of E2F-1. To verify if this effect on CHOP expression occurred at the protein level, western blot technique was analyzed. Protein expression of CHOP in neonatal myocytes (panel C, Figure 6) is increased during tunicamycin and thapsigargin induced ER stress. This effect was reversed with E2F-1 activity, resulting in repression of CHOP protein during tunicamycin and thapsigargin-induced ER stress. The data suggests that E2F-1 repressed CHOP expression at the gene transcription and protein expression level during ER stress, indicating repression of an UPR signal. Although tunicamycin and thapsigargin trigger ER stress by different mechanisms, both treatments induced expression of CHOP that was repressed by E2F-1 activity in ventricular myocytes.

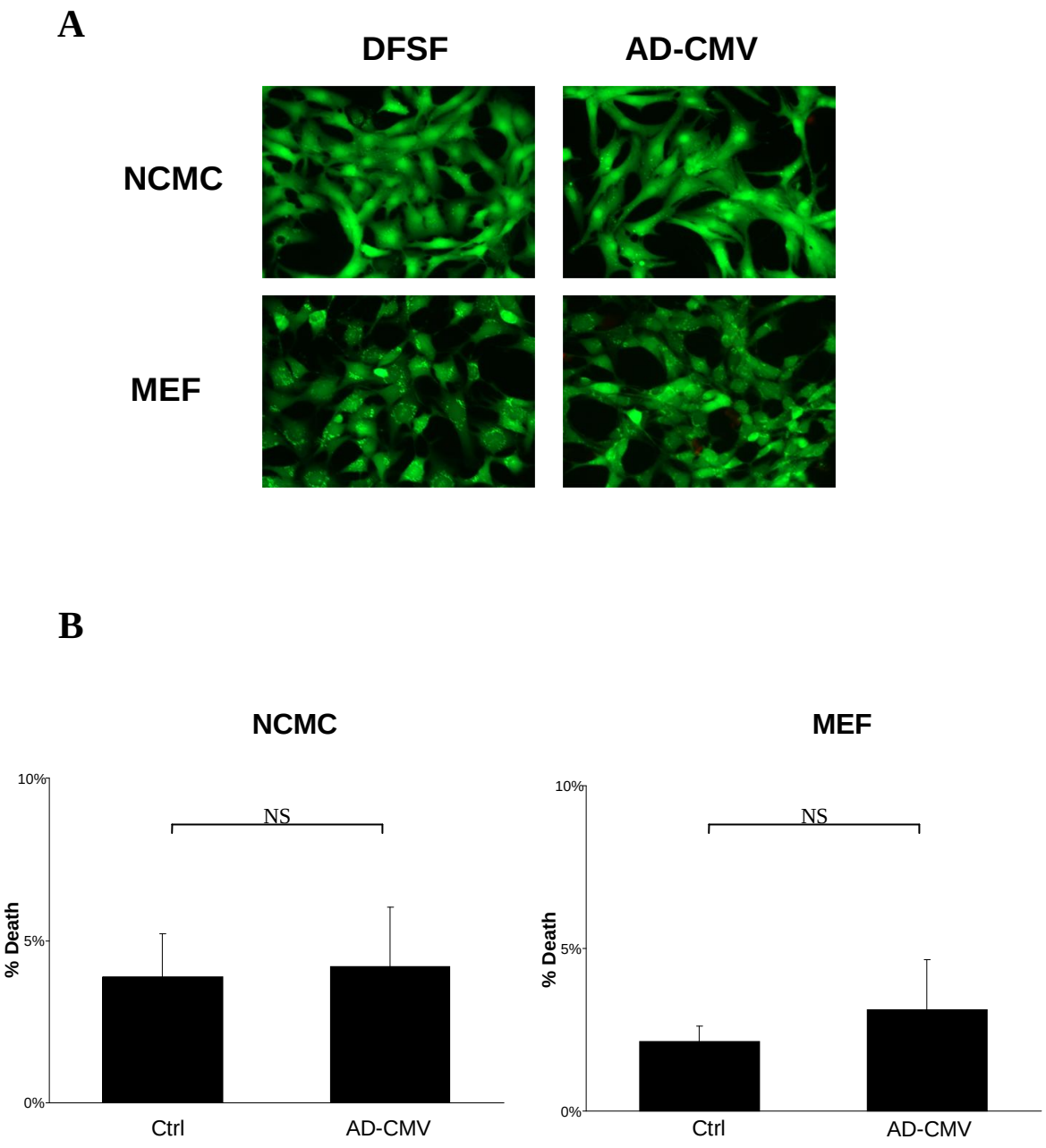

Figure 7: The effect of adenoviral infection in neonatal myocytes and wild-type MEFs on cell viability. Panel A displays representative images of ventricular myocytes and mouse embryonic fibroblasts stained with vital dyes calcein AM and ethidium homodimer to visualize live (green) and dead (red) cells to assess cell viability. Panel B displays quantification of data in panel A. Data were obtained from at least n=3 to n=4 independent experiments counting >200 cells from n=3 glass coverslips for each condition tested. Data are expressed as mean±SE. Statistical significance among the group is indicated * $p<0.05$, NS=non-significant.

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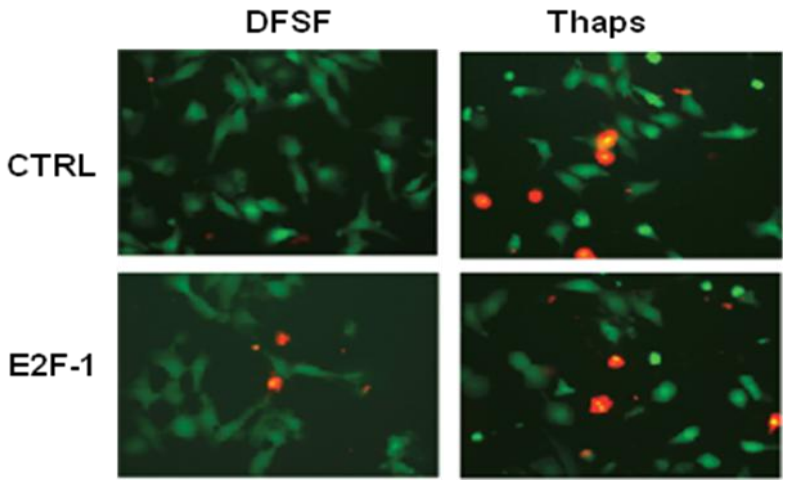

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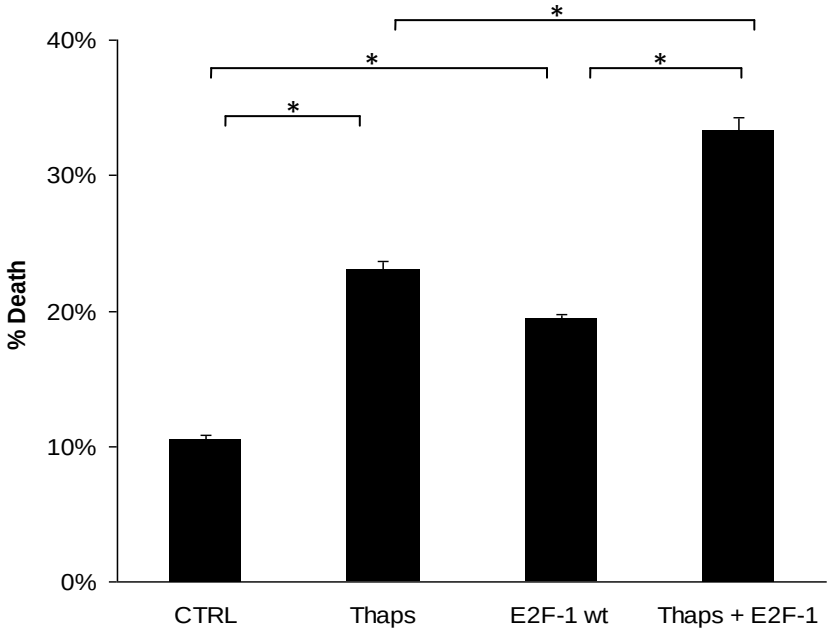

Figure 8: The effect of E2F-1 on cell viability during ER stress induced by thapsigargin in ventricular myocytes. Panel A displays representative images of ventricular myocytes stained with vital dyes calcein AM and ethidium homodimer to visualize live (green) and dead (red) cells to assess cell viability. Panel B displays quantification of data in panel A. Data were obtained from at least n=3 to n=4 independent myocyte isolations counting >200 cells from n=3 glass coverslips for each condition tested. Data are expressed as mean±SE. Statistical significance among the group is indicated * $p<0.05$, NS=non-significant.

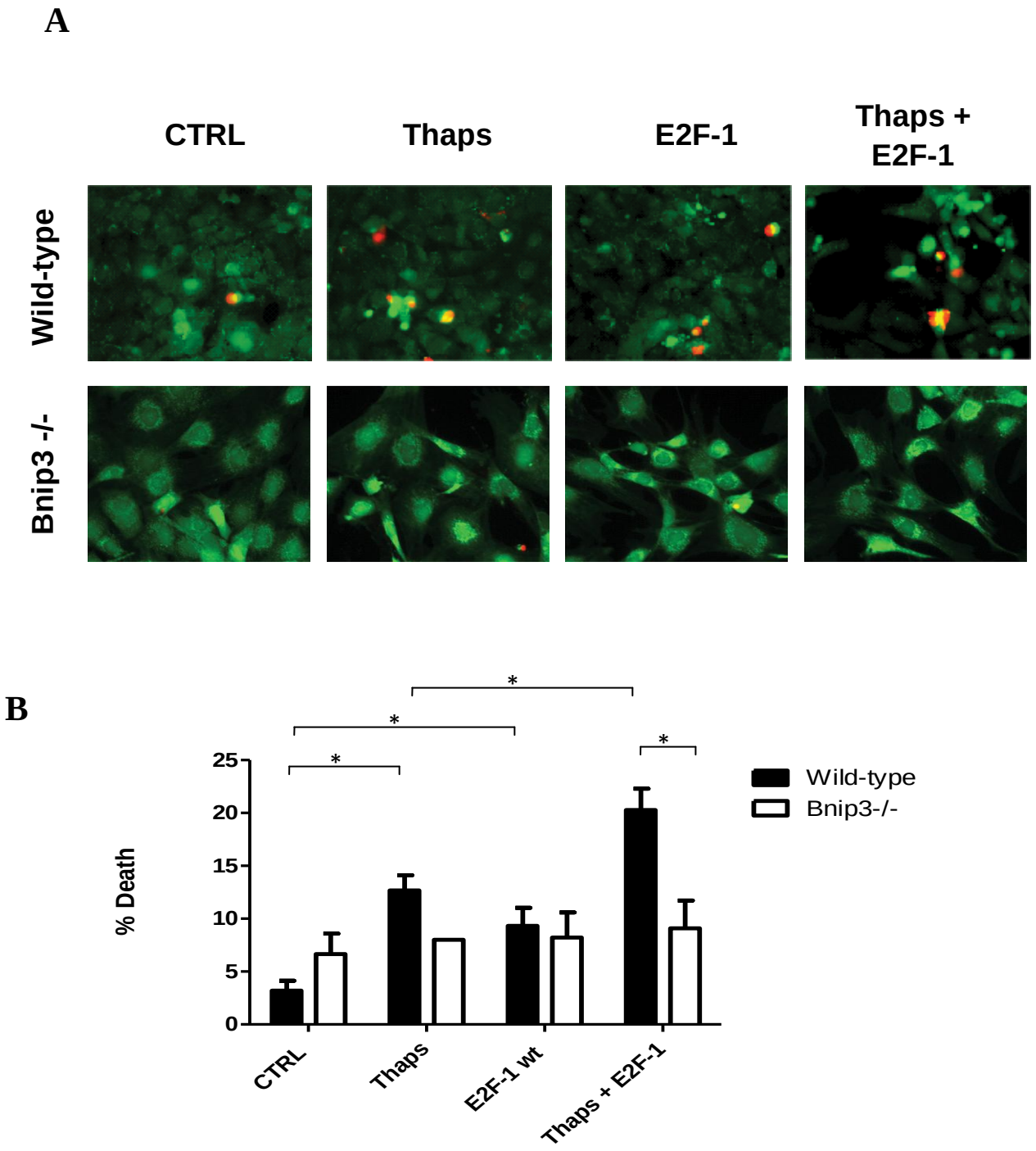

Figure 9: Thapsigargin induced cell death regulated by E2F-1 is abrogated in Bnip3-/- fibroblasts. Panel A displays fluorescent microscopy images of wild-type and Bnip3-/- MEFs stained with vital dyes to assess cell viability. Panel B displays quantification of data to measure cell death. Data were obtained from at least n=3 or n=4 independent experiments counting >200 cells from n=3 glass coverslips for each condition tested. Data are expressed as mean±SE. Statistical significance between groups is indicated * $p<0.05$.

Thapsigargin induced cell death regulated by E2F-1 is Bnip3 dependent.

To show the effect of adenoviral infection on cellular viability, AD-CMV, an adenovirus control which contains an empty expression cassette was used. Figure 7 displays the effect of AD-CMV infection of cardiac myocytes and mouse embryonic fibroblasts on cell viability in which quantification of the fluorescent images illustrates non-significant change from control. Therefore, the data shows adenoviral infection has no significant affect on cell viability.

Next, to study if E2F-1 played a role during ER stress mediated cell death, myocytes were stained with vital dyes, calcein-AM and ethidium homodimer to assess cell viability. Tunicamycin and thapsigargin trigger ER stress by independent pathways. However, the effect of E2F-1 inhibition of the UPR signaling molecules triggered by thapsigargin is conserved among cardiac myocytes and MEFs (Figure 5). Therefore, thapsigargin induction of ER stress is utilized for future experiments. ER stress induced by thapsigargin resulted in cell death and this effect was amplified with over expression of E2F-1 (Figure 8), indicating E2F-1 sensitizes cardiac cells during ER stress to promote cell death. E2F-1 activity increased cell death to 35% ($p<0.05$) in myocytes during thapsigargin treatment (Figure 8, panel B). Although E2F-1 is sufficient to induce cell death as a known activator of apoptosis, its functional role as a death-inducer is amplified during ER stress in ventricular myocytes.

The data reveals regulation of ER stress by E2F-1 provokes cell death in myocytes; however the molecular mechanisms of this pathway remain unknown. The Kirshenbaum lab has previously established that E2F-1 regulates Bnip3 during hypoxia. To assess if E2F-1 regulation of ER stress resulting in cell death was dependent on Bnip3

signaling, wild-type and Bnip3-/- MEFs were utilized. Figure 9 displays representative fluorescent microscopy images and quantification to assess cell viability in Bnip3-/- fibroblasts. Panel B indicates thapsigargin treatment and E2F-1 expression resulted in 12% ($p<0.05$) cell death in wild-type MEFs while Bnip3-/- cells did not display a significant change in cell death as compared to control. With the over expression of E2F-1 during ER stress in wild-type MEF, cell death is increased in comparison to control. However, under the same conditions, Bnip3-/- cells do not display the same pattern and show no significant change in relation to control. However, over-expression of E2F-1 during thapsigargin induced ER stress showed significant change ($p<0.05$) between wild-type and Bnip3-/- fibroblasts. In cells lacking Bnip3 gene function; the ability of E2F-1 to promote ER stress induced death is abolished. The data reveals that ER stress induced cell death is dependent on the function of Bnip3.

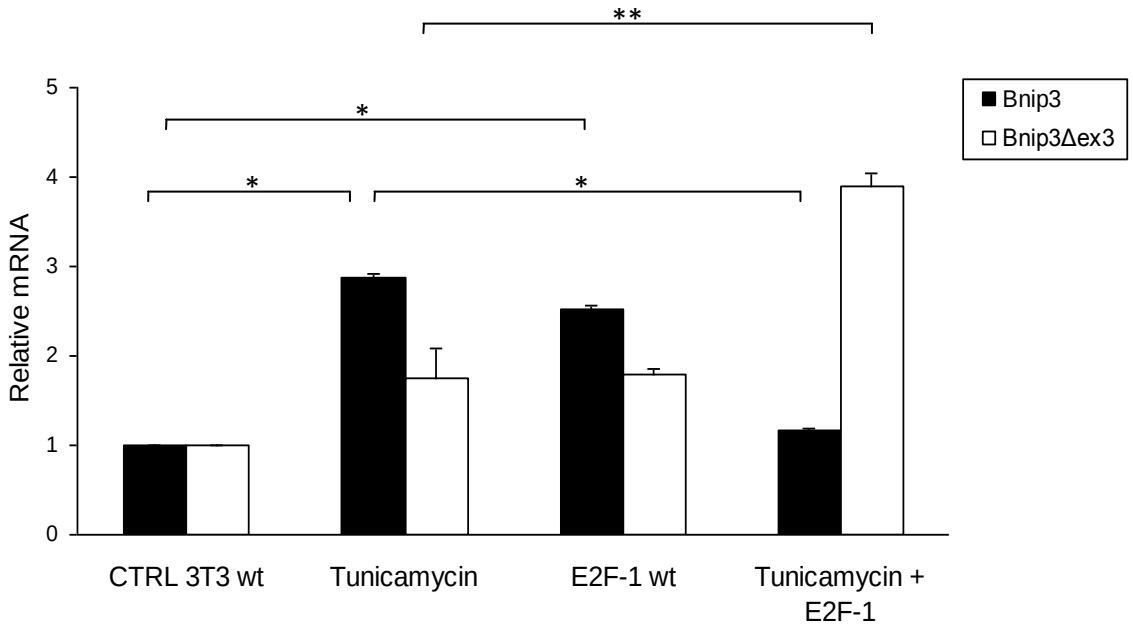

Figure 10: The effect of E2F-1 on cell death and cell survival gene transcription during ER stress in 3T3 wild-type MEFs. Real-time qPCR analysis of relative mRNA expression levels of Bnip3 and Bnip3Δex3 during thapsigargin treatment. Data are expressed as mean±SE from n=3-4 independent experiments. Statistical significance among the group is indicated * $p<0.05$ (Bnip3 relative mRNA); ** $p<0.05$ (Bnip3Δex3 relative mRNA).

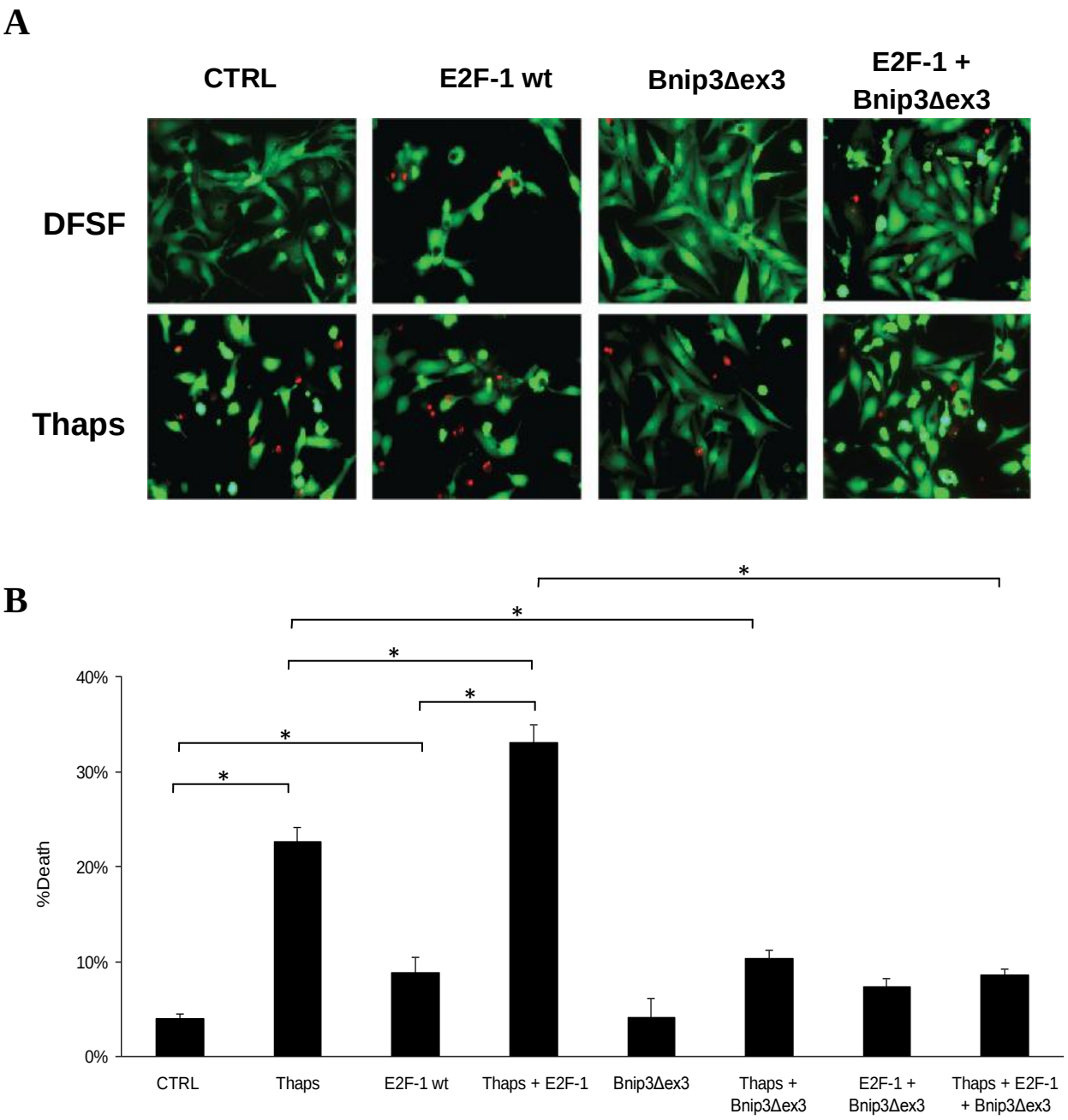

Figure 11: Bnip3Δex3 rescues cell death mediated by E2F-1 regulated ER stress. Panel A displays fluorescent microscopy images of ventricular myocytes stained with vital dyes to assess cell viability. Panel B displays quantification of data in panel A. Data were obtained from at least n=3 to n=4 independent myocyte isolations counting >200 cells from n=3 glass coverslips for each condition tested. Data are expressed as mean±SE. Statistical significance among the group is indicated * $p<0.05$, NS=non-significant.

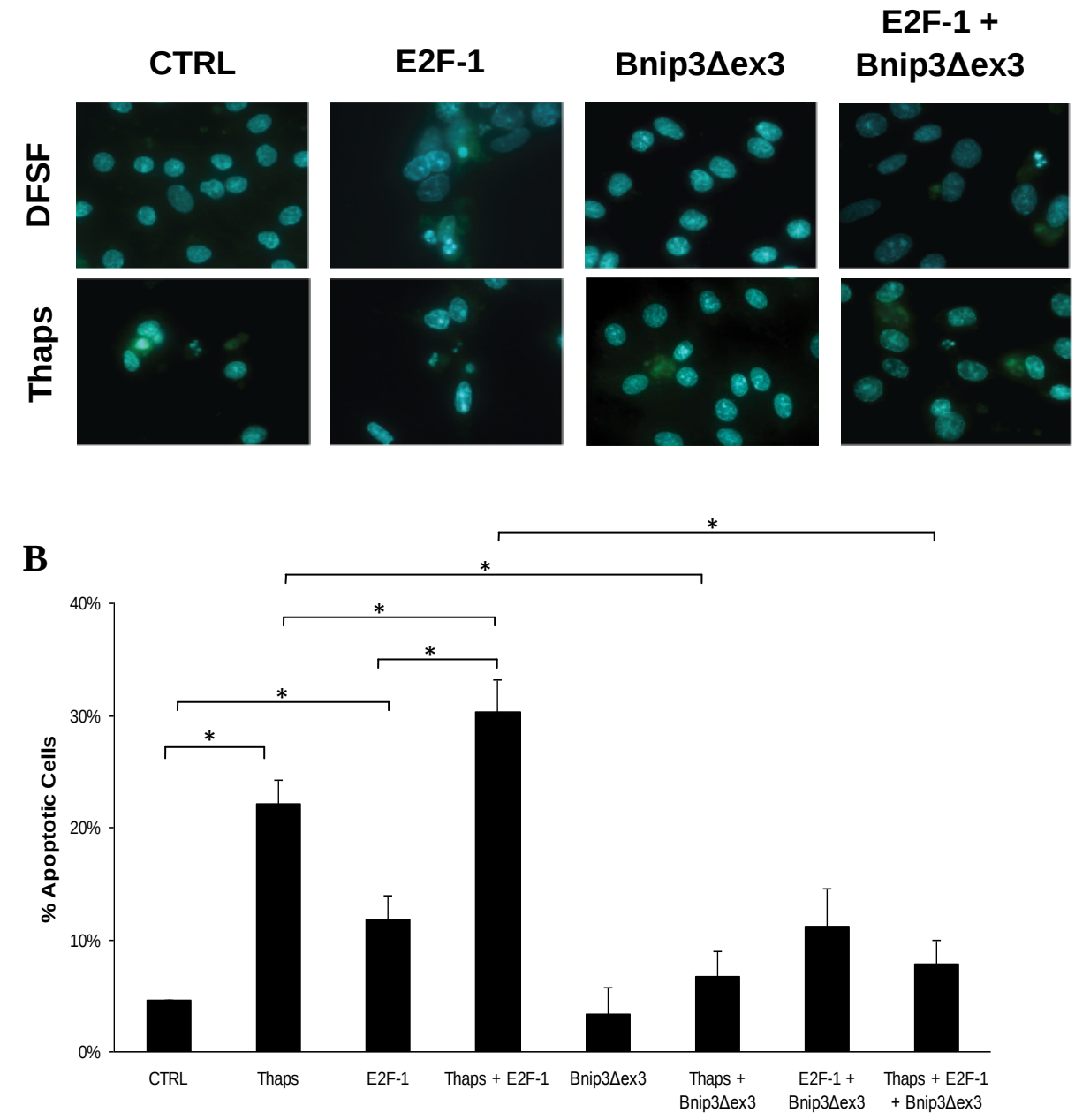

Figure 12: Bnip3Δex3 rescues E2F-1 regulated ER stress induced apoptosis. Panel A displays fluorescent microscopy images of myocytes stained with Hoechst 33258 to visualize apoptotic nuclei. Panel B displays quantification of data. Data were obtained from at least n=3 to n=4 independent myocyte isolations counting >200 cells from n=3 glass coverslips for each condition tested. Data are expressed as mean±SE. Statistical significance among the group is indicated * $p<0.05$.

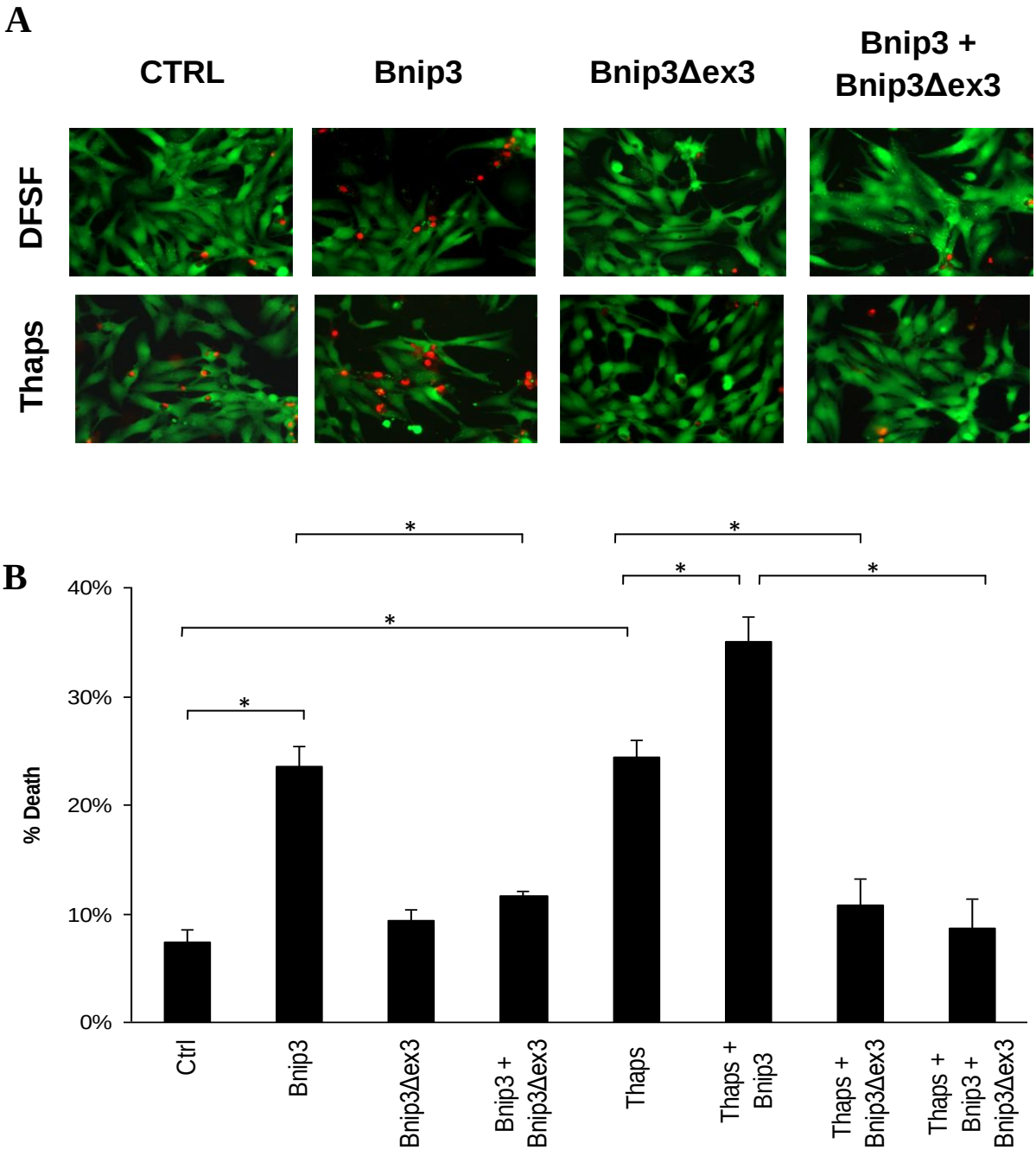

Figure 13: Bnip3Δex3 rescues Bnip3 and thapsigargin induced cardiac cell death. Panel A displays representative images of cell viability in neonatal myocytes during thapsigargin-induced ER stress. Panel B displays quantification of data. Data were obtained from at least n=3 to n=4 independent myocyte isolations counting >200 cells from n=3 glass coverslips for each condition tested. Data are expressed as mean±SE. Statistical significance among the group is indicated * $p<0.05$.

Bnip3Δex3 rescues E2F-1 regulated ER stress induced apoptosis in cardiac cells.

In a preliminary experiment involving tunicamycin treated wild-type mouse embryonic fibroblasts, mRNA expression levels of Bnip3 and Bnip3Δex3 were assessed by real-time qPCR analysis. Figure 10 displays a significant increase in Bnip3 gene transcription during ER stress and over expression of E2F-1 wt ($p<0.05$) in relation to control. Bnip3Δex3, a splice variant of Bnip3, mRNA expression is also present when Bnip3 is expressed as previously reported (136). However, E2F-1 regulation of ER stress induced by tunicamycin resulted in a decrease of Bnip3 mRNA expression while Bnip3Δex3 mRNA expression was increased (4-fold, $p<0.05$). Based on this observation regarding Bnip3Δex3 gene transcription, we wanted to investigate the role of Bnip3Δex3 during cardiac ER stress induced by thapsigargin in neonatal cardiac myocytes.

Bnip3Δex3 has been shown to rescue cardiac cells during hypoxic injury by blocking apoptotic cell death (136). To verify if E2F-1 regulation of ER stress induced cell death could be inhibited to promote cell survival; myocytes were infected with Bnip3Δex3 adenovirus and assessed for cell viability and Hoechst 33258 staining. The cell viability data in Figure 11 reveals that over expression of Bnip3Δex3 was successful in abrogating ER stress induced cell death as regulated by E2F-1 activity in thapsigargin treated cardiac myocytes. Figure 12 further reveals that regulation of ER stress by E2F-1 induced apoptotic cell death that was reversed by Bnip3Δex3, as indicated by Hoechst 33258 staining of apoptotic cells.

The Kirshenbaum lab has previously reported Bnip3 as a transcriptional target of E2F-1 in cardiac cells (11,13). We wanted to assess the effect of Bnip3 during cardiac ER stress and the role of Bnip3Δex3. In Figure 13, cell viability assessment of cardiac cells

indicate over expression of Bnip3 induced cell death in a similar manner to E2F-1 activity, and this effect of Bnip3 was amplified during ER stress induced by thapsigargin (35%-fold increase, $p<0.05$, panel B). These results suggest E2F-1 activity leads to ER stress induced apoptotic cell death by repressing UPR signals and regulating Bnip3.

To assess if Bnip3Δex3 could rescue ER stress-induced apoptotic cell death in the presence of Bnip3, cell viability of cardiac cells was analyzed (Figure 13). Panel B displays quantified data of the representative images, revealing Bnip3Δex3 rescued ER stress induced cardiac cell death, promoting cell survival under such conditions. The data suggest Bnip3 sensitizes cardiac myocytes to thapsigargin-induced ER stress and promotes cell death in a manner similar to E2F-1 regulated ER stress induced cell death which is inhibited by the presence of Bnip3Δex3.

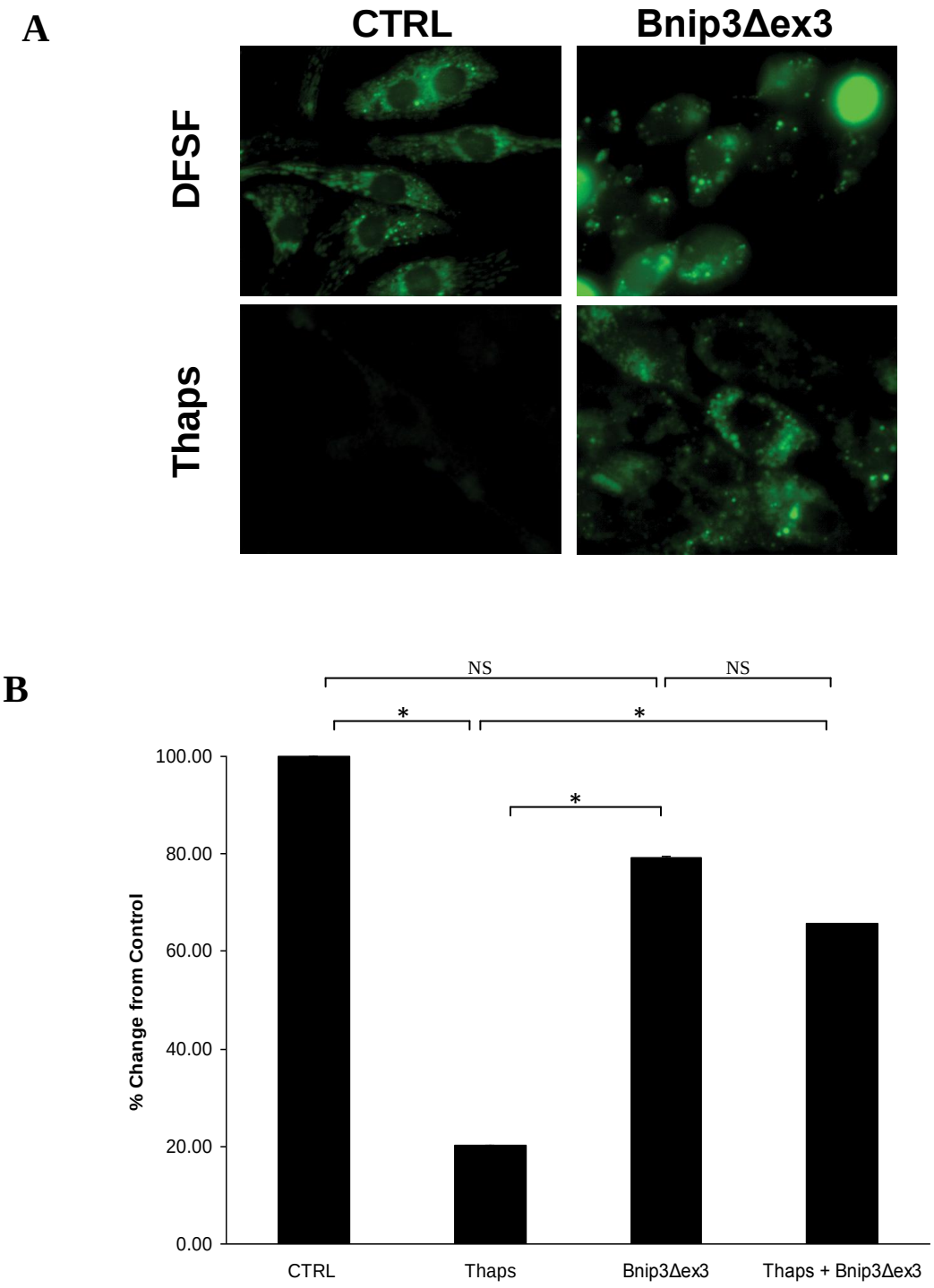

Figure 14: Thapsigargin induced cardiac cell death disrupts mitochondrial function by permeability transition pore opening which is reversed by Bnip3Δex3. Panel A displays fluorescent images of neonatal myocytes monitoring mitochondrial PTP opening. Myocytes treated with thapsigargin resulted in PTP opening. Panel B displays histogram of data shown in panel B relative to control. Data are expressed as mean±SE from n=3-4 independent experiments using triplicates for each condition tested. Statistical significance among the group is indicated * $p<0.05$, NS=non-significant.

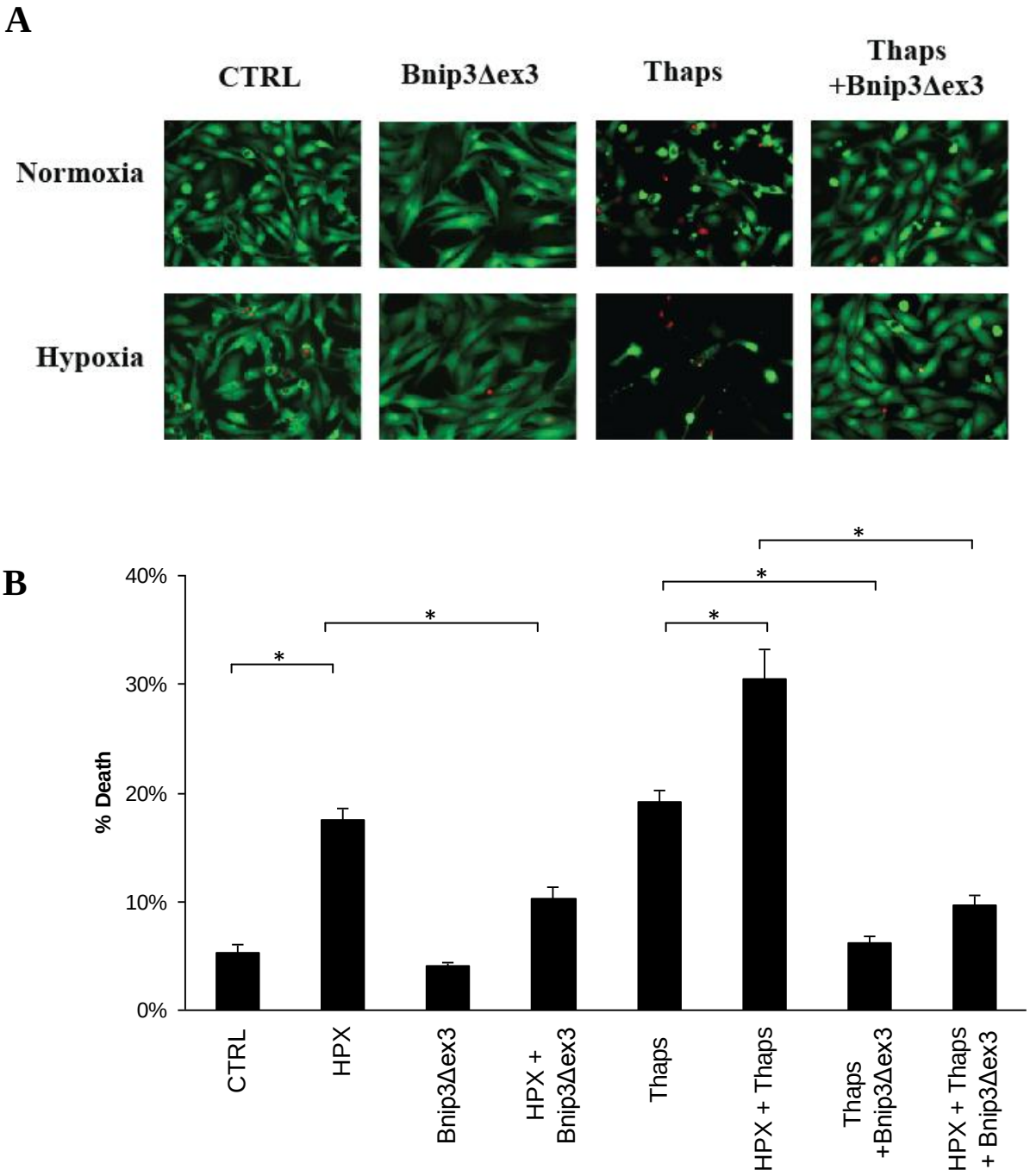

Figure 15: Bnip3Δex3 promotes cell survival in cardiac cells during hypoxia and ER stress. Panel A displays cell viability assessment of neonatal myocytes during hypoxia and ER stress induced by thapsigargin. Panel B displays quantification of data in panel A. Data were obtained from at least n=3 to n=4 independent myocyte isolations counting >200 cells from n=3 glass coverslips for each condition tested. Data are expressed as mean±SE. Statistical significance among the group is indicated * $p<0.05$, NS=non-significant.

Bnip3Δexon3 rescues thapsigargin induced disruption of mitochondrial PTP function and promotes cell survival during hypoxic ER stress.

Perturbations to mitochondria resulting from opening of the permeability transition pore are a known underlying feature of the intrinsic apoptotic death mechanism. Cardiac myocytes were analyzed to determine if ER stress-induced cell death by thapsigargin involved the role of mitochondrial function. Myocytes were loaded with calcein-AM in the presence of $CoCl_2$ and were assessed for mitochondrial PTP opening. This technique was analyzed by detecting changes in fluorescent intensity, in which opening of the pore resulted in the loss of fluorescent signal (178).

As shown in panel A (Figure 14), control myocytes displayed punctate green staining of mitochondria indicating the PTP in a closed configuration at basal function. However, a decrease in fluorescent signal is visualized during ER stress activated by thapsigargin, resulting in an opening of the PTP and mitochondrial dysfunction. This effect of thapsigargin on mitochondrial disruption was blocked in the presence of Bnip3Δex3, which restored mitochondrial PTP staining with an increase in fluorescent signal (Figure 14).

Panel B displays a histogram of the representative fluorescent images of the cardiac myocytes. The intensity of the fluorescent signal is corrected for area. The data indicates that Bnip3Δex3 rescues cell death and prevents mitochondrial PTP opening during ER stress induced by thapsigargin. This data indicates that ER stress provokes mitochondrial dysfunction through PTP opening, suggesting irregular interplay between ER and mitochondria during cardiac stress.

As mitochondrial dysfunction has been previously reported in cardiac cells from hypoxic injury (9,12), we wanted to investigate the role of Bnip3Δex3 during hypoxic

injury and ER stress in which both the mitochondria and ER are impaired. Figure 15 displays cell viability assessment of neonatal myocytes in which hypoxia and ER stress sensitized cardiac myocytes to an increase in cell death (30% death, $p<0.05$) which was abrogated in the presence of Bnip3Δex3. E2F-1 and Bnip3 were shown to increase cell death during ER stress. A similar pattern is displayed during cellular hypoxic conditions (Figure 15). Thus, the data reveals that Bnip3Δex3 can rescue cardiac cell death during physiological conditions of hypoxic injury and ER stress, highlighting the importance of the close relationship between mitochondria and ER in cardiac myocytes.

V. DISCUSSION

A healthy ER is important for regulating biological processes contributing to functional cardiac myocytes. The onset of ER stress activates the UPR to upregulate ER chaperone genes and signaling mechanisms to restore cellular ER homeostasis. However, irregular or excess ER stress has been shown to be detrimental to cells by activating apoptotic cell death mechanisms. As myocytes have a limited ability to regenerate with a fixed cell life cycle, cellular damage occurring from hypoxic injury results in a loss of functioning cardiomyocytes. As it is approximated that half a million people in Canada are living with heart failure (179), it is beneficial to study the biological processes implicated during cardiac failure. It is important to investigate the molecular factors and signaling pathways responsible for initiating cardiac apoptosis in hopes of developing new therapeutic treatments for cardiac disease and preventing heart failure.

The mode by which ER stress provokes cell death remains poorly defined. To study ER stress in cardiac myocytes and its effect on cell fate, the transcription factor E2F-1 is utilized as a tool to investigate the underlying pathways regulating cardiac cell death and cell survival during ER malfunction. E2F-1 transcriptional activity is known for regulating cell cycle machinery in proliferating cell types and triggering apoptotic cell death in cardiac myocytes (13,177). As E2F-1 has been shown to regulate thapsigargin induced ER stress with subsequent apoptosis (180,181) what remains to be defined is the role of E2F-1 during ER stress in cardiac myocytes. As E2F-1 has been reported to transcriptionally activate Bnip3 during hypoxia, the question to address is whether E2F-1 transcriptionally regulates other genes specifically involved in ER stress signaling

pathways. What also remains to be determined is the role of the UPR in cardiac cells and whether it promotes cell survival or cell death under cellular stress conditions.

To investigate ER stress, tunicamycin treatment was utilized as a tool to activate the UPR. The presence of E2F-1 repressed mRNA expression of UPR signaling molecules during tunicamycin-induced ER stress in ventricular cardiac myocytes (Figure 4). Cells were also treated with thapsigargin to assess ER stress induced by an alternative pharmacodynamic mechanism. E2F-1 repressed the UPR during thapsigargin induced ER stress in both cardiac myocytes and wild-type fibroblasts, suggesting that this form of ER stress is conserved amongst the two cell types utilized in these studies (Figure 5).

Consistent with another report that showed resistance to apoptotic death triggered by thapsigargin in E2F-1-/- fibroblasts (180), cardiac myocytes were also sensitive to ER stress-induced cell death triggered by thapsigargin. Gene transcription of GRP78 induced by thapsigargin was repressed by E2F-1 which sensitized myocytes to cell death (Figure 5). Based on previous reports, these findings suggest GRP78 as a pro-survival molecule. Our data is consistent with a study that reported GRP78 is protective during ischemic injury in astrocytes (182) and another study that reported E2F-1 repression of GRP78 increased sensitization of cancer cells to chemotherapy (183). In our findings, myocytes were sensitized to ER stress by E2F-1 repression of the pro-survival genes activated by the UPR (Figure 4-5).

CHOP is another molecule activated by the UPR that is known as a pro-apoptotic factor. In a recent study, CHOP-deficient mice displayed less apoptotic death and less cardiac dysfunction induced by pressure overload (165). In contrast, the present study reveals CHOP expression was repressed by E2F-1 activity during ER stress induced by

tunicamycin or thapsigargin in cardiac myocytes (Figure 6). Our data indicates E2F-1 represses CHOP activity which does not promote cell survival in ventricular myocytes (Figure 8). The data suggests repression of CHOP activity by E2F-1 may activate different apoptotic molecular pathways to promote cell death during ER stress.

Tunicamcycin and thapsigargin activate ER stress by increasing the accumulation of misfolded proteins in the ER lumen or inhibiting the SERCA channel and increasing cytosolic Ca^{2+} levels, respectively. Based on the conserved response of E2F-1 repression on the UPR in cardiac myocytes and fibroblasts, thapsigargin was further utilized to assess ER stress in ventricular myocytes in the next series of experiments. To study the effect of UPR inhibition by E2F-1 on myocyte cell fate, cell viability was analyzed. As a preliminary experiment, neonatal myocytes and MEFs were infected with an empty expression cassette adenovirus, AD-CMV, to show infection alone did not have an affect on cell viability (Figure 7). Cell viability analysis of thapsigargin induced ER stress revealed E2F-1 sensitized cardiac neonatal myocytes to ER-stress induced death (Figure 8).

A recent report revealed Noxa and Bim are necessary for ER stress induced apoptosis, emphasizing the role of BH3-only proteins of the Bcl-2 family in mediating different apoptotic pathways by the ER and mitochondria (187). Nickson and colleagues also reported PUMA, another pro-apoptotic BH3-only protein induced by many transcription factors including E2F-1 (188), is a critical factor in regulating ER stress induced apoptosis in cardiac myocytes (166). To study the mechanism by which E2F-1 regulates thapsigargin-induced ER stress cell death in myocytes (Figure 8), we determined the impact of ER stress in a knock out tissue culture model. The role of

Bnip3, a direct transcriptional target of E2F-1 and a BH3-only protein, was investigated during cardiac ER stress. Our data reveals that E2F-1 increased sensitivity to ER stress induced cell death which was abrogated in Bnip3-null fibroblasts (Figure 9), indicating E2F-1 represses the UPR and promotes cell death during ER stress dependent on Bnip3 function. As Bnip3 is reported to play a vital role during mitochondrial-mediated apoptosis (11,12,126), our findings indicate Bnip3 also plays a vital role in ER stress-mediated cell death which is consistent with a recent report by Zhang et al, illustrating the vital role of Bnip3 at the ER and mitochondria to mediate cell death (189).

Bnip3, a pro-apoptotic factor, was recently shown by the Kirshenbaum lab to be regulated by its hypoxia-inducible splice variant, Bnip3Δex3, to promote cell survival of neonatal myocytes (136). Preliminary experiments during E2F-1 regulated tunicamycin induced-ER stress displayed an increase in Bnip3Δex3 mRNA expression in wild-type cells (Figure 10). Due to the conserved response to thapsigargin, the functional role of Bnip3Δex3 during thapsigargin-induced ER stress was assessed as this has never been examined. The present study reveals that Bnip3Δex3 was able to rescue ventricular myocytes from E2F-1 regulated thapsigargin induced apoptosis (Figure 11-12). The activity of Bnip3Δex3 suppressed thapsigargin induced cardiac cell death in the presence of Bnip3 to promote cardiac myocyte survival (Figure 13).

Yet the mechanism by which Bnip3Δex3 blocks thapsigargin induced cell death and promotes cell survival is unknown. Consistent with a recent report (190), we showed thapsigargin induced myocyte death by mitochondrial PTP opening. The disruption of mitochondrial function was repressed by the presence of Bnip3Δex3 and restored mitochondrial PTP function (Figure 14). Therefore, our data reveals thapsigargin induced

cardiac cell death is mediated by mitochondrial dysfunction which is inhibited by Bnip3Δex3 to promote cardiac cell survival. As ER stress has been documented during cardiac dysfunction, our data reveals Bnip3Δex3 was able to rescue ER stress and hypoxia induced cell death in neonatal myocytes (Figure 15).

In the context of ventricular myocytes, defining the line between ER and SR are debatable. Thapsigargin inhibits the pumping function of SERCA which raises cytoplasmic Ca^{2+} levels while simultaneously depleting ER/SR Ca^{2+}, thus altering Ca^{2+} regulation and promoting Ca^{2+}-mediated cell death. The mitochondria are important organelles for sequestering cytosolic Ca^{2+}, which subsequently stimulates oxidative metabolism. However, mitochondrial Ca^{2+} overload sensitizes mitochondrial PTP opening and triggers intrinsic programmed cell death. Therefore, our findings reveal a critical interaction between ER and mitochondria in mediating cellular death pathways that is suggestive of a mechanism dependent on Ca^{2+} signal transmission in cardiac myocytes which is consistent with previous reports (190,191,192,193,194).

The present study has identified the mechanism by which E2F-1 provokes ER stress-induced apoptotic death in cardiac myocytes which involves interruption of Ca^{2+} signaling that may be mediated between the ER-mitochondria. This death trigger was inhibited by the pro-survival factor Bnip3Δex3 that suppressed mitochondrial dysfunction. As myocytes have a limited ability to actively divide and enter the cell cycle, investigating the regulatory mechanisms of ER-mitochondria Ca^{2+} signal transmission in heart failure models may further help us understand cell death in cardiac cells. The site for ER-mitochondrial communication of detrimental signaling molecules serves as a potential regulatory site for cell death signals and targets for therapeutic

treatments for heart failure patients. The development of a pharmacological agent that could increase levels of Bnip3Δex3 in cardiac cells could be a potential mechanism for treating cardiac ischemia and preventing heart failure.

VI. CONCLUSION

The transcription factor, E2F-1 triggers apoptotic signaling mechanisms in different cell types. Using neonatal ventricular myocytes, we examined the mode by which E2F-1 provokes ER stress-induced apoptosis. The present study verified that E2F-1 sensitized cardiac myocytes to ER stress-induced cell death, and determined the mechanism involved in inhibiting thapsigargin induced apoptosis as regulated by E2F-1. In summary, the conclusions from this study are:

1. E2F-1 represses UPR signaling triggered by ER stress in ventricular myocytes.
2. E2F-1 sensitizes neonatal myocytes to ER stress induced cell death.
3. E2F-1 regulated ER stress induced cell death is Bnip3 dependent
4. Over-expression of Bnip3Δex3 rescued E2F-1 regulated ER stress-induced apoptosis
5. Thapsigargin induces ER stress and mitochondrial defects including opening of mitochondrial permeability transition pore
6. Bnip3Δex3 inhibits E2F-1 sensitization of myocytes to ER stress induced apoptosis and rescues mitochondrial defects induced by ER stress to promote cell survival.

E2F-1 has been evident in mediating hypoxic injury; this study indicates that it also serves a role in regulating ER stress in ventricular myocytes. Therefore, based on the findings of the present study, we visualize a model in which the activation of the UPR and synthesis of Bnip3Δex3 are provoked to stimulate cell survival to oppose ER stress induced apoptotic death and mitochondrial defects. As myocyte function is greatly

affected during ischemic injury and infarction, contractility is also compromised which contributes to the progression of heart disease. This study emphasizes the importance of understanding the underlying molecular pathways of ER-mitochondria interplay during Ca^{2+} regulation in mediating cell death and cardiac dysfunction which requires further investigation. Hence, Bnip3Δex3 may provide an adaptive mechanism by limiting Ca^{2+} signaling between ER and mitochondria during cardiac injury which can alleviate cellular damage and confer a survival role during ER stress.

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