

**TRANSCRIPTIONAL REGULATION OF THE PRO-APOPTOTIC GENE BNIP3
BY P65 NF- κ B, HISTONE DEACETYLASE 1, AND E2F-1
IN POSTNATAL VENTRICULAR MYOCYTES**

BY

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ABSTRACT

Apoptotic cell death of cardiac myocytes plays an important pathological role after a myocardial infarction and during heart failure. Apoptotic myocytes are not regenerated because of the restricted ability of terminally differentiated cardiac myocytes to undergo cell division. Because ventricular function is directly related to the number of active muscle cells, the inappropriate loss or premature death of cardiac myocytes results in reduced cardiac performance. Bnip3 was previously identified by Dr. Lorrie Kirshenbaum's laboratory as a critical mediator of hypoxia-induced apoptosis in the heart. Importantly, his lab established that the cytoprotective actions of NF- κ B during hypoxia included the transcriptional repression of Bnip3. However, the mechanism by which NF- κ B acted as a transcriptional repressor was undefined. The present work strongly supports the hypothesis that NF- κ B-mediated inhibition of Bnip3 transcription is dependent on the recruitment of the corepressor protein HDAC1. Immunoprecipitation experiments revealed that HDAC1 and p65 NF- κ B formed protein-protein interactions. ChIP assays demonstrated that HDAC1 and p65 NF- κ B associated with the Bnip3 promoter. HDAC1-mediated repression of Bnip3 was lost in cells deficient for p65 NF- κ B, and restored upon repletion of p65. A second avenue of investigation described in this work demonstrated that the cell cycle factor E2F-1 directly activated Bnip3 transcription. Earlier work by Dr. Kirshenbaum found that adenovirus-mediated overexpression of E2F-1 in ventricular myocytes induced apoptosis. Herein, it is shown that E2F-1-mediated cell death is largely Bnip3-dependent because functional loss of Bnip3 inhibited E2F-1-induced cell death. Concerning hypoxia, Bnip3 expression is

dependent upon the loss of p65/HDAC1-mediated repression, and on the presence of transcriptionally active E2F-1. During hypoxia, overexpression of p65, HDAC1, or Rb, an endogenous inhibitor of E2F-1-dependent transcription, attenuated hypoxia-induced Bnip3 transcription. Based on these findings, future therapies may be designed to repress Bnip3 gene expression after a myocardial infarction, thereby averting cardiac cell death and preserving cardiac function post-infarction.

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LIST OF ABBREVIATIONS

Abeta – amyloid beta
ACE – angiotensin converting enzyme
AIDS – acquired immunodeficiency syndrome
AIF – apoptosis inducing factor
ANT – adenine nucleotide translocator
Apaf – apoptotic protease activating factor
ARB – angiotensin receptor blocker
ARC – apoptosis repressor with caspase recruitment domain
ASA – acetylsalicylic acid
ATP – adenosine triphosphate
Atg5 – autophagy 5
BAFF – B-cell activating factor
Bad – Bcl-2 antagonist of cell death
Bak – Bcl-2 antagonist killer
Bax – Bcl-2 associated protein X
Bcl-2 – B-cell lymphoma 2
Bcl-X_L – Bcl-2 related protein, long isoform
Bcl-X_S – Bcl-2 related protein, short isoform
BH – Bcl-2 homology
Bid – BH3-interacting domain death agonist
Bik – Bcl-2 interacting killer
Bim – Bcl-2 interacting mediator of cell death
Bnip3 – Bcl-2/adenovirus E1B 19 kDa interacting protein 3
Bnip3L – Bcl-2/adenovirus E1B 19 kDa interacting protein 3-like
CABG – coronary artery bypass graft
CAD – caspase-activated DNAase
Calcein-AM – calcein-acetoxymethylester
CamKIV – calcium/calmodulin-dependent protein kinase IV
CARD – caspase recruitment domain
CBP – CREB binding protein
C. elegans – *Caenorhabditis elegans*
CD – conserved domain (within the Bnip3 protein)
Cdc5 – cell division cycle 5
CDK – cyclin dependent kinase
Ced – cell death
ChIP – chromatin immunoprecipitation
CK – creatine kinase
CKII – casein kinase II
CMV – cytomegalovirus
CNTL – control
CoREST – RE1-silencing transcription factor corepressor
DB – DNA binding
DD – death domain
DED – death effector domain

DIABLO – direct IAP-binding protein with low pI
DISC – death inducing signaling complex
DMEM – Dulbecco's modified Eagle's medium
DNA – deoxyribonucleic acid
DP – differentiation regulated transcription factor protein
DR – death receptor
DSG – disuccinamidyl glutarate
ECL – enhanced chemiluminescence
EGF – epidermal growth factor
EGR2 – early growth response 2
Endo G – endonuclease G
ETC – electron transport chain
FADD – Fas associated via death domain
FasL – Fas ligand
FBS – Fetal Bovine Serum
FLICE – FADD-like ICE
FLIP – FLICE inhibitory protein
FoxO3a – Forkhead Box O3a
GSK-3 β – glycogen synthase kinase-3 β
HAT – histone acetyltransferase
HAX-1 – HS-1 associated protein 1
HDAC – histone deacetylase
HF – heart failure
HIF – hypoxia inducible factor
Hsp – heat shock protein
Hop – homeodomain-only protein
HYPX – hypoxia
IAP – inhibitor of apoptosis
ICAD – inhibitor of caspase-activated DNase
ICE – interleukin 1-beta converting enzyme
IGF – insulin-like growth factor
I κ B α – inhibitor of kappa light chain gene enhancer in B cells
IKK – inhibitor of kappa light chain gene enhancer in B cells, kinase of
IL – interleukin
iNOS – inducible nitric oxide synthase
I.P. – immunoprecipitation
I/R – ischemia followed by reperfusion
ISOL – in situ oligoligation
Jab1 – Jun activation domain binding protein 1
LAD – left anterior descending
LDH – lactate dehydrogenase
LDL – low density lipoprotein
LPS – lipopolysaccharide
Mcl-1 – myeloid cell leukemia 1
MEF – myocyte enhancer factor; mouse embryonic fibroblast
MI – myocardial infarction

MOI – multiplicity of infection
MnSOD – manganese superoxide dismutase
MOMP – mitochondrial outer membrane permeabilization
MSK – mitogen and stress activated protein kinase
mTOR – mammalian target of rapamycin
NEMO – NF- κ B essential modulator
NFAT – nuclear factor of activated T-cells
NF- κ B – nuclear factor- κ B, nuclear factor of kappa light chain gene enhancer in B cells
NIK – NF- κ B inducing kinase
Nix – Nip3-like protein X
NLS – nuclear localizing signal
NS – not significant
NuRD – nucleosome remodelling and histone deacetylation
PARP – poly(ADP-ribose) polymerase
PBS – phosphate buffered saline
PCD – programmed cell death
PCR – polymerase chain reaction
PDTC – pyrrolidine dithiocarbamate
PKA – protein kinase A
PKC – protein kinase C
RSK1 – ribosomal S6 kinase
PCNA – proliferating cell nuclear antigen
PTCA – percutaneous transluminal coronary angioplasty
PTP – permeability transition pore
Puma – p53-upregulated modulator of apoptosis
PVDF – polyvinylidene fluoride
qPCR – quantitative PCR
RANKL – receptor activator of NF- κ B ligand
Rb – retinoblastoma
RIP – receptor interacting protein
RNA – ribonucleic acid
ROS – reactive oxygen species
RT-PCR – reverse transcriptase PCR
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA – short interfering RNA
shRNA – short hairpin RNA
Smac – second mitochondrial activator of caspases
SMRT – silencing mediator for retinoid and thyroid hormone receptors
sNix – alternatively spliced Nix
SRF – serum response factor
TAD – transcription activation domain
TAK1 – transforming growth factor beta activated kinase 1
t-Bid – truncated Bid
TBK1 – TANK-binding kinase 1
TBS – tris buffered saline
TM – transmembrane

TNF α – tumor necrosis factor alpha
TNFR1 – TNF receptor 1
TNFRSF – TNF receptor superfamily
TORC1 – target of rapamycin complex 1
TRAF – TNF receptor associated factor
TRADD – TNFR1 associated via death domain
TRAIL – TNF-related apoptosis-inducing ligand
TSA – trichostatin A (an HDAC inhibitor)
TUNEL – terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate
nick end labeling
UV – ultraviolet
VDAC – voltage-dependent anion channel
XIAP – X-linked inhibitor of apoptosis
YY1 – yin yang 1

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I. INTRODUCTION

As multicellular organisms, many of our cell populations are constantly renewed. This regeneration occurs regularly for hematopoietic, skin, and liver cells; however, neurons and cardiac myocytes are examples of terminally differentiated cell populations that are resistant to cell division (Alberts et al., 2002). As the complex process of cell division is highly regulated, so is the mechanism for controlled cell death. The body can remove damaged or unwanted cells by a mechanism called apoptosis, which is an active and regulated process also known as programmed cell death (Kerr et al., 1972). Cells undergoing apoptosis exhibit a characteristic apoptotic phenotype that results in the methodical breakdown of the cell while leaving the surrounding cells and tissue relatively undisturbed (Kerr et al., 1972; Wyllie, 1997).

Apoptosis is an important functional process during development. For example, apoptosis of surplus neurons ensures proper nervous system function (Hamburger and Levi-Montalcini, 1949; Garcia et al., 1992; Gagliardini et al., 1994). In the developing heart, apoptosis is observed in specific loci including the outflow tract and the atrioventricular cushions (Keyes and Sanders, 2002; Poelmann and Gittenberger-de Groot, 1999), and has been implicated as a key process for outflow tract remodelling (Watanabe et al., 1998; Rothenberg et al., 2002; Poelmann and Gittenberger-de Groot, 2005). In mammalian hands and feet, apoptosis is involved in the removal of soft tissue between the digits during development (Mori et al., 1995). Apoptosis is the process by which the Müllerian duct is regressed in a developing male during sex determination (Price et al., 1977; Roberts et al., 1999). Thus, developmental apoptosis can generally be

thought of as a sculpting tool that the body uses to arrive at its functional gross morphology.

It is clear that apoptosis plays critical physiological roles in the body. Accordingly, deregulation of apoptosis is often associated with abnormal development or disease. For example, inhibition of apoptosis in the interdigital spaces leads to webbed digits (Macias et al., 1996). More seriously, excessive apoptosis can lead to degenerative conditions, whereas defects in apoptosis may lead to hyperproliferative disorders such as cancer (Fischer and Schulze-Osthoff, 2005).

In context of the heart, apoptosis is known to play a detrimental role in many cardiovascular disease conditions including MI, particularly in the infarct border zone post-MI, and to a lesser extent in the remote myocardium (Gottlieb et al., 1994; Kajstura et al., 1996; Olivetti et al., 1997; Olivetti et al., 1996; Saraste et al., 1997; Narula et al., 1996; Fliss and Gattinger, 1996; Piro et al., 2000; Schwarz et al., 2006). Because cardiac myocytes have a limited capacity for regeneration after injury (Grove et al., 1969; Okazaki and Holtzer, 1966), apoptosis contributes to myocyte loss, ventricular remodelling, and ultimately to heart failure (Wencker et al., 2003; Hayakawa et al., 2003). Thus, the inhibition of cardiomyocyte apoptosis by future therapies may help preserve cardiac function in patients after an injury such as MI.

Dr. Lorrie Kirshenbaum's laboratory at the Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, University of Manitoba has been studying the mechanisms and signaling pathways involved in cardiac cell survival and cell death for a number of years. As a first step towards understanding cardiac apoptosis, his lab

has focused on the death factor Bnip3. Dr. Kirshenbaum and others have established that transcription of Bnip3 is upregulated during ischemic HF and by hypoxia in isolated ventricular myocytes (Regula et al., 2002b; Kubasiak et al., 2002). It has been hypothesized that the death promoting actions of Bnip3 may underlie hypoxia-induced apoptosis of ventricular myocytes (Regula et al., 2002b). Recently, the role of cardiac Bnip3 expression was examined *in vivo* in mice in the context of MI (Diwan et al., 2007). Transgenic mice with cardiac-specific overexpression of Bnip3 fared worse after MI compared to controls, whereas Bnip3 knock-out mice fared better (Diwan et al., 2007). Thus, Bnip3 is a candidate gene that may be targeted by future therapeutic interventions to reduce cardiomyocyte cell death after injury. Presently, it is important to understand how Bnip3 expression is regulated in the heart under normal and hypoxic conditions.

Although the regulation of Bnip3 is still not completely understood, evidence from Dr. Kirshenbaum's laboratory has revealed NF- κ B as a potential inhibitor of Bnip3 gene expression in the heart (Baetz et al., 2005). The NF- κ B signaling pathway is conserved throughout many organisms and cell types, and has been previously shown to affect many cellular processes including immunity and inflammation, reviewed by (Courtois and Gilmore, 2006). With respect to cell death, NF- κ B is typically characterized as anti-apoptotic, although it should be noted that this is not universally agreed upon (Radhakrishnan and Kamalakaran, 2006). Earlier work from Dr. Kirshenbaum's laboratory demonstrated a cytoprotective role for NF- κ B signaling in the heart by suppressing hypoxia-induced cell death and mitochondrial defects such as the loss of mitochondrial membrane potential ($\Delta\psi_m$) and the opening of the permeability transition

pore in ventricular myocytes (Regula et al., 2004) (see chapter III, section 2.2.3 for further description of the PTP). It was subsequently established that NF- κ B conferred its anti-apoptotic effects, at least in part, by inhibiting the hypoxia-induced transcription of the death gene Bnip3 by associating with the NF- κ B binding element within the Bnip3 promoter (Baetz et al., 2005). NF- κ B was further determined to be important for the basal silencing of Bnip3 because deregulated expression would otherwise inappropriately induce apoptosis.

The p65 subunit of NF- κ B was established as the critical NF- κ B subunit for the inhibition of Bnip3 expression; however, many of the described NF- κ B target genes are transcriptionally activated by NF- κ B, for example, Bcl-2 (Catz and Johnson, 2001), IL-6 (Libermann and Baltimore, 1990), and the IAPs (Stehlik et al., 1998). Because NF- κ B classically acts as an activator of transcription, one objective of the present work was to understand how NF- κ B functioned as a transcriptional repressor of the death gene Bnip3. Based on earlier work from other laboratories, which characterized a relationship between p65 and HDAC1 (Ashburner et al., 2001; Lee et al., 2000b; Zhong et al., 2002), as well as with other HDACs (Zhang and Kone, 2002; Ito et al., 2000; Chen et al., 2001a; Yu et al., 2002b) for the regulation of NF- κ B target genes, we hypothesized that HDACs may be involved in the p65-mediated repression of Bnip3 transcription. The present work explores the relationship between p65 and HDAC1 for the transcriptional repression of Bnip3 in postnatal ventricular myocytes.

In addition to understanding the mechanisms that inhibit Bnip3 expression, our laboratory has also investigated the cellular mechanisms that underlie the activation of Bnip3

transcription. Further analysis of the Bnip3 promoter revealed the presence of a consensus E2F-1 binding site, in addition to the NF- κ B element. E2F-1 is a transcription factor important in G1 to S phase transition and DNA synthesis during the cell cycle (Chen et al., 1989; DeCaprio et al., 1989; Goodrich et al., 1991; Dalton, 1992; Blake and Azizkhan, 1989), and in this regard E2F-1 activity is directly controlled by Rb, a tumour suppressor protein that can bind E2F-1 and inhibit E2F-1-dependent transcription (Hiebert et al., 1992; Hamel et al., 1992; Helin et al., 1993). Proliferative signals from growth factors or cytokines activate Cyclin-Cdk complexes that phosphorylate Rb (Lees et al., 1991; Lin et al., 1991), thereby signaling the release of E2F-1 for the activation of cell cycle genes. Overexpression of E2F-1 has previously been shown by Kirshenbaum et al. (1996) to induce DNA synthesis in ventricular myocytes. However, instead of proceeding to mitosis, the myocytes underwent apoptotic cell death (Kirshenbaum et al., 1996). The underlying mechanism was undetermined (Kirshenbaum et al., 1996; Kirshenbaum and Schneider, 1995). Therefore, the second objective of the present work was to ascertain the relationship between E2F-1 and Bnip3 in the heart. The work described in chapter IV, section 3 supports a model in which Bnip3 is a downstream transcriptional target of E2F-1.

Herein, this report demonstrates how HDAC1 and p65 NF- κ B cooperate to repress Bnip3 gene expression. Further, the activation of Bnip3 transcription is shown to underlie E2F-1-mediated cell death. Finally, hypoxia-induced Bnip3 transcription is driven by a loss of NF- κ B/HDAC1 inhibitory signals and a gain of E2F-1 activation signals.

II. RATIONALE AND HYPOTHESIS

Despite years of work, the regulation of cardiac apoptosis is still not completely understood. Deregulated myocyte apoptosis in the heart is a serious concern because cardiomyocytes are not able to regenerate after an injury in the damaged area (Grove et al., 1969; Okazaki and Holtzer, 1966). Because excessive myocyte apoptosis has been observed in the heart after ischemic injury, and concurrently identified as a contributor to ventricular remodelling and HF (Wencker et al., 2003; Hayakawa et al., 2003), it is likely that therapeutic inhibition of cardiomyocyte apoptosis may be of significant benefit to patients post-MI. Therefore, a better understanding of hypoxia and ischemia-induced myocyte apoptosis may allow for the design of new therapies to intervene in these pathways.

To this end, the mitochondrial death gene Bnip3 is an excellent candidate for therapeutic targeting because it induces apoptosis, and possibly other forms of cell death, and is upregulated in the heart by ischemic HF and hypoxia. Previous work from Dr. Kirshenbaum's laboratory identified p65 NF- κ B as a transcriptional repressor of Bnip3; however, the underlying mechanism was unknown (Baetz et al., 2005). In the present work our goal was to ascertain the mechanism by which p65 NF- κ B transcriptionally repressed Bnip3 in ventricular myocytes. Based on earlier work describing a relationship between p65 and HDAC1 (Ashburner et al., 2001; Lee et al., 2000b; Zhong et al., 2002), **we tested the hypothesis that p65 was mechanistically acting through a co-repressor protein such as HDAC1 to inhibit Bnip3 transcription in ventricular myocytes.**

Specific aims were to determine whether HDAC1 repressed Bnip3 gene transcription, to

determine whether p65 NF- κ B established protein-protein interactions with HDAC1, to determine the requirement of p65 NF- κ B for HDAC1-mediated repression of Bnip3, and to determine whether HDAC1 could prevent hypoxia-induced Bnip3 expression.

Our lab is also interested in the molecular mediators of Bnip3 gene activation. Earlier work from Dr. Kirshenbaum established that over-expression of the cell cycle factor E2F-1 induced apoptosis in ventricular myocytes; however, the pathway for E2F-1 dependent apoptosis was not defined (Kirshenbaum et al., 1996; Kirshenbaum and Schneider, 1995). The presence of an E2F-1 binding site in the Bnip3 promoter region suggested to us that Bnip3 may be a downstream target of E2F-1. **We tested the hypothesis that E2F-1 transcriptionally activated Bnip3, and that E2F-1-mediated apoptosis was dependent on the presence of Bnip3 in ventricular myocytes.** Specific aims were to determine whether overexpression of E2F-1 upregulated Bnip3 transcription, to determine whether functional removal of Bnip3 protein activity attenuated E2F-1-induced cell death, and to determine whether genetic knockdown or inhibition of E2F-1 reduced hypoxia-induced Bnip3 transcription.

III. LITERATURE REVIEW

1.0 Cardiovascular Disease in North America

1.1 Mortality, Hospitalization, and Cost

Even after many years of diligent research by the scientific and medical communities, cardiovascular disease is still a major burden on Canadians and is a leading cause of death in Canada and North America (Heart and Stroke Foundation of Canada, 2003; Rosamond et al., 2008; Pilote et al., 2007; Chow et al., 2005; Manuel et al., 2003).

Although the number of deaths per year in Canada due to all cardiovascular diseases has remained approximately constant since 1990, over a third of all Canadian deaths in 1999 were due to cardiovascular disease, and nearly half of those were due to ischemic heart disease (Heart and Stroke Foundation of Canada, 2003). Looking specifically at ischemic heart disease and MI, the number of deaths in Canada has been declining since the 1980s and this trend is expected to continue. However, the observed decline in ischemic cardiovascular mortality is not the whole story. The advances in medicine over the last twenty five years have likely allowed for better management of cardiovascular disease, thus eliminating many patients from the mortality statistics that they otherwise would have contributed to. The decline in mortality, therefore, does not necessarily indicate that cardiovascular disease is disappearing in Canada.

The prevalence of heart disease is defined as the total number of people with heart disease divided by the total number of people in the population, over a period of time.

Prevalence, in general, is a useful indicator of the extent to which a particular disease is present in a population. Unfortunately, reliable prevalence data is not available in Canada, and instead federal agencies use hospitalization data to indicate the number of people with cardiovascular disease (Heart and Stroke Foundation of Canada, 2003). Because hospitalization does not include the number of patients seen on an emergency or outpatient basis, it likely underestimates the true number of patients living with cardiovascular disease.

Cardiovascular disease was the leading cause of hospitalization in Canada in 2000 and 2001 (Heart and Stroke Foundation of Canada, 2003; Tsuyuki et al., 2003). The raw number of hospitalizations for all types of cardiovascular disease increased between 1985 and 2001, and is expected to continue to increase over the next twenty years due to the aging Canadian population (Heart and Stroke Foundation of Canada, 2003). In the USA, mortality due to cardiovascular disease is declining; however, the prevalence in America, as estimated by survey and interview data, has increased from approximately 34.2% in 2003 (Thom et al., 2006) to 37.1% in 2005 (Rosamond et al., 2008). Despite the decline in mortality, these trends indicate that cardiovascular disease in Canada and the United States is a growing problem and not a shrinking one (Johansen et al., 2003).

In addition to the significant emotional hardship and loss involved with cardiovascular disease, it also draws the most financial resources from the Canadian health care system, costing \$18.5 billion in 1998 (Health Canada, 2002). This amount translated into 11.6 % of total direct and indirect illness cost that year (Health Canada, 2002). In the United States, the estimated direct and indirect cost for all cardiovascular disease in 2008 is

\$448.5 billion, although this figure is derived from a broader definition of cardiovascular disease than the Canadian estimate (Rosamond et al., 2008). Although hospital care, drugs, and disability are major direct costs, especially for the elderly population, indirect costs reflect the lost economic contributions of young and middle-aged Canadians who may die prematurely (Heart and Stroke Foundation of Canada, 2003). Clearly, cardiovascular disease is a worthy focus for research to improve the lives of Canadians and reduce the cost to the health care system.

1.2 Epidemiology

Preventative medicine has become an active area of research given the seemingly gloomy scenario our society is facing in terms of rising health care costs. As a first step towards preventing cardiovascular disease, studies have identified a number of behavioural and biological parameters that may elevate an individual's risk for cardiovascular disease. In this context, a risk factor is said to increase one's risk for a disease if it has been shown in large studies that there is an association between exposure to the risk factor and the subsequent development of disease.

Identified modifiable behavioural risk factors for cardiovascular disease include tobacco smoking, physical inactivity, obesity, and a diet low in fruits and vegetables (Heart Failure Society of America, 2006; Heart and Stroke Foundation of Canada, 2003).

Biological risk factors such as dyslipidemia and hypertension may be modified in the long term by controlling diet, exercise, and stress, but often pharmacological interventions are required (Heart and Stroke Foundation of Canada, 2003). Additionally, the presence of

diabetes also increases a patient's risk for cardiovascular disease (Heart and Stroke Foundation of Canada, 2003). A family history of heart disease also puts an individual at an increased risk, and suggests that there are genetic factors at play that may make some individuals more or less susceptible to heart disease. Many of these risk factors were identified during the Framingham Heart Study, a research initiative that began in 1948 and is still ongoing (Anderson et al., 1991). Interestingly, social determinants of health such as income and socioeconomic status may also play an indirect role in an individual's overall risk for cardiovascular disease since, for example, a lower income may limit access to healthy food and exercise facilities.

Although it is much easier said than done, by reducing exposure to these risk factors there is good evidence that we may also reduce the burden our society faces with respect to cardiovascular disease.

1.3 Heart Failure and Myocardial Ischemia

1.3.1 Heart Failure

Simply stated, HF is the failure of the heart as a pump. The Frank-Starling law describes the intrinsic regulation of cardiac function and states that the amount of blood entering the heart is proportional to the force of the contraction. More formally, as the left ventricular end-diastolic pressure and volume increase, there is an intrinsic increase in contractility and cardiac output (Guyton and Hall, 2006). This response occurs because the increased pressure causes increased stretch of the myocardial wall during diastole, and results in a greater overlap of actin and myosin filaments within a sarcomere, thereby

increasing the number of cross-bridges available for contraction (Guyton and Hall, 2006). Compared to a healthy heart, a failing heart will have a weaker contraction at the same left ventricular end diastolic pressure or volume.

There are two phases of HF, the compensated phase and the decompensated phase. During compensated HF, reduced cardiac output activates the sympathetic nervous system through the baroreceptors, resulting in increased heart rate and contractility (Kumar et al., 2005). In addition to this, the renin-angiotensin system is activated by reduced renal perfusion. Angiotensin II activates aldosterone and results in sodium and water retention in the collecting duct of the kidney, while angiotensin II itself induces vasoconstriction and also activates sympathetic autonomic activity (Guyton and Hall, 2006; Kumar et al., 2005). The end result of these reflex measures is increases in: force and rate of contraction, preload, afterload, blood volume, cardiac output; and the resulting maintenance of blood pressure and tissue perfusion. Ventricular remodelling, including cellular hypertrophy, ventricular dilation, and changes in cytokine levels are also associated with compensated HF (Kumar et al., 2005).

Unfortunately, over a prolonged period of time, these mechanisms lead to fibrosis, extracellular matrix remodelling via collagen deposition, wall thinning, defects in calcium handling, and an elevated level of apoptotic cell death that each impair cardiac function during the decompensated phase. In some cases, diastolic dysfunction, which is an impairment in the ability of the heart to relax, may also occur, for example, during extreme fibrosis or hypertrophy. Needless to say, prognosis is poor for patients in late-stage HF (Kumar et al., 2005).

Left-sided HF is the most common form of HF. Impaired left ventricular contraction results in incomplete ejection during systole, resulting in increased left atrial pressure, increased pulmonary venous pressure, and increased capillary pressure in alveoli. With sufficient pressure, pulmonary edema may occur, resulting in congestive heart failure (Kumar et al., 2005; Lilly, 2007). Right sided HF may occur independently or in conjunction with left-sided failure. Because the right side of the heart receives blood from the peripheral venous circulation, right ventricular systolic dysfunction causes increased venous pressure, peripheral edema, and congestion of internal organs such as the liver and spleen (Kumar et al., 2005). During left-sided failure, the increased pressure in the lungs will force the right side to work harder and may lead to right-sided failure.

Four classes of drugs that may be used to manage HF are diuretics, beta blockers, ACE inhibitors (or the ARBs), and inotropes. Loop diuretics such as furosemide reduce blood volume and are primarily used to “dry up” patients with pulmonary edema (Weinstein and Solis-Gil, 1966; Arnold et al., 2006; Hunt, 2005). Importantly, before the onset of diuresis, furosemide has a therapeutic effect via venodilation (Dikshit et al., 1973; Pickkers et al., 1997), thereby reducing pulmonary venous pressure and favouring capillary fluid resorption (Lilly, 2007). Another diuretic, spironolactone, is an aldosterone inhibitor that has been shown to reduce mortality in patients with HF (Pitt et al., 1999). Beta-1 selective blockers such as metoprolol or bisoprolol, or non-selective beta-blockers such as carvedilol are useful post-MI and during HF starting at a low dose to control heart rate, and ultimately to improve survival, systolic and diastolic function, as well as response to exercise (Metra et al., 2000; MERIT-HF Study Group, 1999; CIBIS-II

Investigators and Committees, 1999; Packer et al., 1996; Packer et al., 2001). The mechanism for these effects is thought to be that the beta-blockers prevent and reverse the HF-induced downregulation of beta-1 adrenergic receptors, as well as antagonizing the cytotoxic effects of chronic adrenergic stimulation on cardiac myocytes (Bristow, 1997; Lilly, 2007). ACE inhibitors such as enalapril have been shown to improve symptoms and reduce mortality in patients with HF (The SOLVD Investigattors, 1992; The SOLVD Investigators, 1991; The CONSENSUS Trial Study Group, 1987). These agents prevent the synthesis of angiotensin II and its resulting effects. They decrease preload and afterload by inhibiting angiotensin II-mediated aldosterone activity, vasoconstriction, and increases in sympathetic tone (Lilly, 2007). ARBs such as valsartan are an alternative to ACE inhibitors, but are typically reserved for those patients who can not tolerate the ACE inhibitor-induced cough. Finally, the cardiac glycoside digoxin is an inotrope that increases contractility without increasing myocardial oxygen demand. Although digoxin improves quality of life, it does not prolong it (The Digitalis Investigation Group, 1997). It functions on a molecular level by inhibiting the $\text{Na}^+\text{-K}^+$ ATPase, thereby increasing intracellular sodium. With a weaker sodium gradient, the $\text{Na}^+\text{-Ca}^{2+}$ exchanger subsequently removes less calcium from the cell, leading to an increase in intracellular calcium that increases the force of contraction (Howland and Mycek, 2006). Digoxin also mediates therapeutic effects through modulation of the autonomic nervous system (Howland and Mycek, 2006). Other inotropes such as dobutamine are used in late-stage decompensated HF, or in emergency, to preserve contractility (Arnold et al., 2006).

It is important to note that HF is a condition that can result from a variety of different cardiovascular diseases. Ischemic heart disease is the most common and is discussed in detail below, but other types of cardiac defects and injuries may also lead to HF. These include congenital defects, valvular stenosis or regurgitation, myocarditis, and other cardiomyopathies (Kumar et al., 2005).

1.3.2 Ischemic Heart Disease

Although the heart is responsible for pumping blood throughout the body, the blood in the cardiac chambers does not supply the heart muscle itself with blood or nutrients. Instead, the heart receives its oxygen and nutrients from blood pumped into the coronary circulation, which forms a branching structure and perfuses the cardiac tissue (Guyton and Hall, 2006).

The term ischemia refers to a decrease in the blood flow supplying an organ or tissue. Myocardial ischemia is a reduction in blood flow in the coronary circulation. This may occur during disease conditions when a complete or partial block of a coronary artery results in decreased perfusion of the downstream myocardium. It is important to note that hypoxia is an important component of ischemia because blood flow is the regular mechanism for oxygen delivery. The process of tissue damage following ischemia is known as infarction, and in the heart, a myocardial infarction (MI). In layman's terms, an MI is often referred to as a heart attack.

The build up of an atherosclerotic plaque within a coronary artery is the most common mechanism of coronary occlusion. A plaque generally forms over several years or

decades, and may be comprised of a variety of cell types, including endothelial, arterial smooth muscle, and immune cells, as well as deposits of cholesterol in the form of LDL (Kumar et al., 2005). A partial blockage of coronary blood flow by an atherosclerotic plaque may lead to an imbalance in myocardial oxygen supply and oxygen demand, leading to angina, and past a certain threshold, the blockage may lead to an infarction (Lilly, 2007). The surface of some atherosclerotic plaques has been found to be pro-coagulant (Lilly, 2007; Kumar et al., 2005) and furthermore, the rupture of a plaque may expose thromboembolic triggers. Because a thrombus would completely block coronary blood flow, an MI may suddenly occur in individuals with atherosclerosis due to the activation of platelets and other clotting factors.

Patients with an MI typically experience a crushing chest pain; however, some MIs are asymptomatic and may only be discovered to have occurred later by electrocardiography, analysis of serum LDH or CK levels, or future autopsy (Lilly, 2007). Sudden death from MI is typically due to ventricular arrhythmias such as ventricular fibrillation, in which the asynchronous ventricular contractions are incapable of pumping blood (Kumar et al., 2005). On arrival to the emergency room, individuals having a heart attack are treated with the goal of restoring blood flow to the ischemic area. This is typically achieved either pharmacologically with thrombolytics such as streptokinase or tissue-plasminogen activator to dissolve the blood clot (Howland and Mycek, 2006), or with surgical interventions. Common surgical treatments to restore blood flow include coronary artery bypass grafting (CABG) and percutaneous transluminal coronary angioplasty (PTCA) (Lilly, 2007). Treatment must be administered as fast as possible, since the amount of

myocardial damage is proportional to the length of time spent ischemic. In surviving patients, the wound heals over time, ultimately forming a fibrous scar.

In response to hypoxia or ischemia, cardiac myocytes are unable to continue aerobic respiration and oxidative phosphorylation to produce ATP. Using glycogen stores, anaerobic glycolysis produces ATP, but this process can not maintain sufficient energy levels for normal cardiac function (Lilly, 2007). Within the ischemia area, ATP depletion inhibits contraction and also the activity of ATP-dependent ion pumps, leading to elevated intracellular levels of Na^+ and Ca^{2+} , and elevated extracellular K^+ (Kumar et al., 2005; Lilly, 2007). Together these contribute to cellular swelling and membrane depolarization, the latter of which may give rise to arrhythmias (Lilly, 2007). If prolonged for more than 20-30 minutes, ischemia will lead to irreversible injury and cardiac cell death (Lilly, 2007). Supposing that therapeutic reperfusion takes place, the infarct size will be substantially smaller than if no intervention were performed, but paradoxically, reperfusion itself induces damage. There is further injury, in part, because of generation of ROS from the restarted ETC in cardiac myocytes (Yellon and Hausenloy, 2007). ROS are oxygen radicals, meaning that they have a highly reactive unpaired electron in their outer orbit. They are cytotoxic due to their ability to induce DNA damage, mitochondrial perturbations, and may affect enzyme activity (Yellon and Hausenloy, 2007). Under basal conditions, ROS are removed by superoxide dismutase, catalase, and glutathione peroxidase (Venardos et al., 2007). The elevated intracellular Ca^{2+} also contributes to reperfusion injury by promoting mitochondrial perturbations involving PTP opening, hypercontraction upon repletion of ATP, and by activating

cellular enzymes such as calpain, which contribute to cell death (Yellon and Hausenloy, 2007; Logue et al., 2005). A special form of cell death called apoptosis has been observed following ischemia alone, and after ischemia followed by reperfusion (Borutaite and Brown, 2003). This death pathway has been shown to contribute to the loss of cardiac function post-MI (Yaoita et al., 1998). Apoptotic cell death will be discussed further in the next section.

2.0 Pathways of Cell Death

Classically speaking, there are two cellular pathways for cell death: necrosis and apoptosis. A process called autophagy has been recently implicated as a third, potentially unique, mode of cell death. These will be discussed below.

2.1 Necrosis and Autophagy

Historically, the term necrosis was used to characterize more generally dying or dead tissue. At the cellular level it refers to death that occurs by accidental means. In this regard, classical necrosis usually begins with cellular swelling, loss of regulation of cellular processes, and culminates with membrane rupture and cell lysis. Consequently, the cellular contents, including degradative lysosomal enzymes, are released into the surrounding area and result in local inflammation and damage to the neighbouring cells. Necrosis has been traditionally considered to occur as a passive process, not requiring ATP, but recent evidence suggests that there may be a genetic element involved in the control of the necrosis pathway (Tavernarakis, 2007; Golstein and Kroemer, 2007). In

the context of the heart, necrosis is one way in which cells die in the infarct region.

Autophagy is a cellular process involving the breakdown of proteins and organelles via a lysosomal pathway either to eliminate damaged cellular components or to generate free amino acids and sugars during low-nutrient conditions (Levine, 2005). The role of autophagy in the heart is debated at present. Some evidence suggests that autophagy may be a form of cell death (Valentim et al., 2006; Zhu et al., 2007; Miyata et al., 2006; Shimomura et al., 2001), while other researchers have reported that it is cardioprotective (Hamacher-Brady et al., 2006; Nakai et al., 2007; Yan et al., 2005). Interestingly, it has been suggested that there may be some cross talk between the apoptotic and the autophagic pathways involving proteins such as Beclin 1, Atg5, Bcl-2, and Bcl-X_L, although there is controversy surrounding the pro- or anti-autophagic properties of Bcl-2 (Shimizu et al., 2004; Pattingre et al., 2005; Criollo et al., 2007; Brady et al., 2007; Yousefi et al., 2006; Pyo et al., 2005). One interpretation of the current literature is that autophagy is, fundamentally, a protective process, but deregulated or excessive autophagy may be detrimental to a cell (Shaw and Kirshenbaum, 2008). Further research is required in order to fully understand autophagy so that it may be therapeutically targeted in the future.

2.2 Apoptosis

2.2.1 Introduction to Apoptotic Pathways

Apoptosis is a highly regulated and ordered cell death process that requires the expenditure of cellular ATP. Also known as programmed cell death (PCD),

characteristic morphology of apoptosis includes nuclear and cytoplasmic condensation, membrane blebbing, and finally, cellular fragmentation. Because this occurs without the loss of membrane integrity, this process avoids the resulting inflammation caused by necrosis (Kerr et al., 1972; Wyllie, 1974). Seminal work in *C. elegans* by Horvitz and others have identified the specificity of the apoptotic process by determining that the selective removal of specific cells at certain times is crucial for normal worm development. This led to the discovery of the ced gene family and mammalian caspase counterparts, reviewed in ref. (Metzstein et al., 1998; Alnemri et al., 1996).

Caspase activation and DNA laddering are two biochemical hallmarks of apoptosis. Caspases will be discussed in detail in section 3, but generally are a family of cysteine proteases responsible for apoptotic signal transduction. Initiator caspases such as caspase 8, 9, and 10 are upstream of the effector caspases 3, 6, and 7, reviewed in ref. (Fuentes-Prior and Salvesen, 2004; Shi, 2004). DNA laddering occurs due to the activation of nucleases that cleave DNA at the unprotected DNA strands between nucleosomes. This results in the creation of DNA fragments with approximate sizes that are multiples of the approximate 146 bp coiled around a nucleosome (Shi et al., 1990). In some instances, caspase independent apoptosis has been reported to occur via the liberation of endonuclease G (EndoG), Apoptosis Inducing Factor (AIF), and Smac (Second Mitochondrial Activator of Caspases) from the mitochondria, reviewed in (Kim et al., 2005; van Gurp et al., 2003; Lockshin and Zakeri, 2002). As shown in figure 1, there are two major pathways for apoptosis activation: the extrinsic and intrinsic pathways, as discussed in sections 2.2.2 and 2.2.3, respectively.

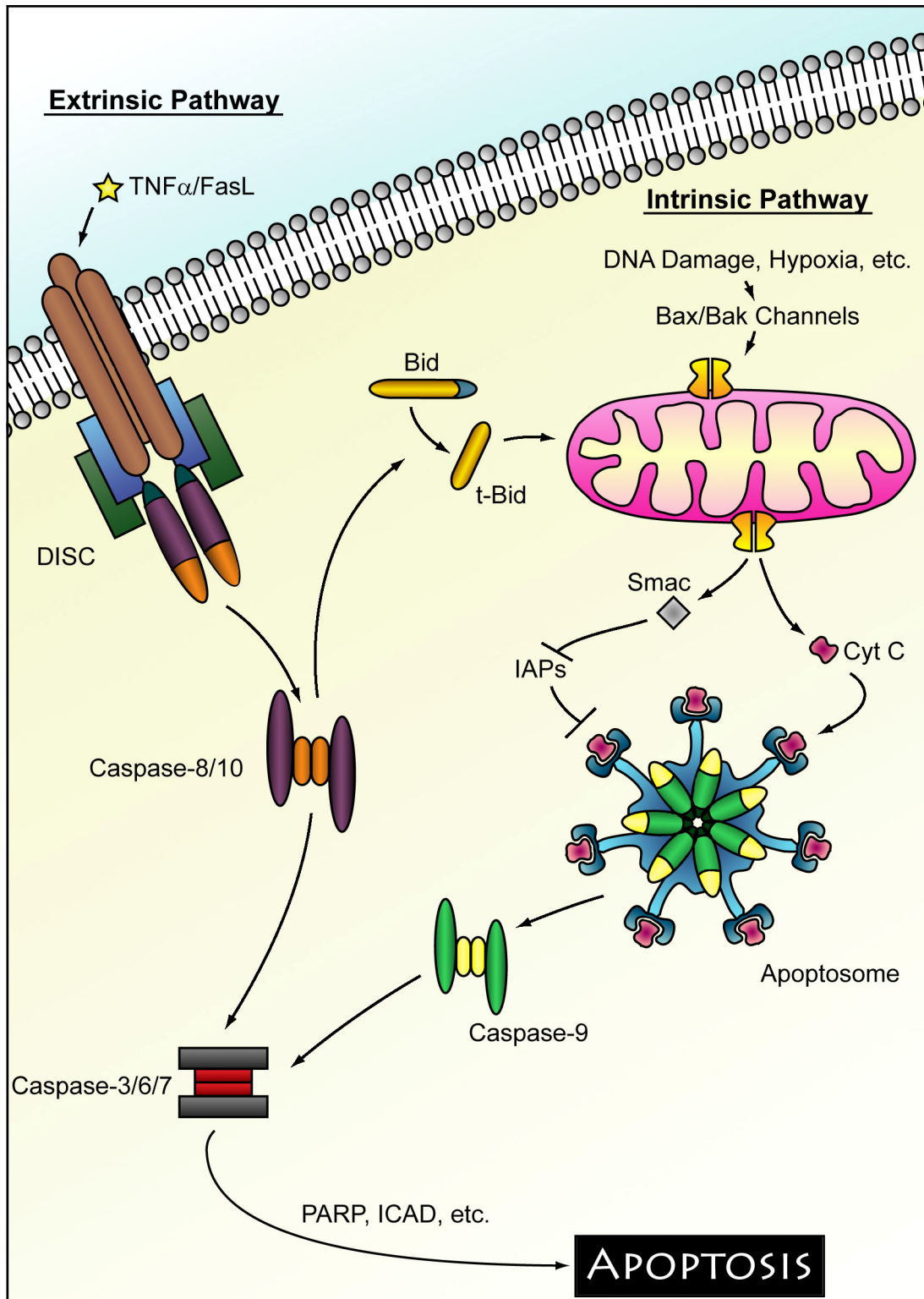


Figure 1. Extrinsic and intrinsic pathways of apoptosis. Receptor-mediated (left) and mitochondrial (right) signals culminate in the activation of the caspase cascade and apoptotic cell death. Cross-talk is mediated via caspase-8-dependent cleavage of Bid.

Since apoptosis is a regulated process, there is a potential for pharmacological or genetic intervention to prevent unwanted or untimely cell death. Accordingly, there has been considerable interest toward elucidating the underlying genetic and signal transduction pathways that govern apoptosis with the ultimate goal of identifying therapeutic targets for not only heart disease, but also for the modulation of inappropriate cell death in neurodegenerative diseases such as Alzheimer's and Parkinson's, and proliferative conditions such as cancer and tumor growth.

2.2.2 Death Receptor "Extrinsic" Pathway

The extrinsic apoptotic pathway is initiated at the cell surface by the ligand-dependent activation of death receptors (DR). In response to the association of ligands in the tumor necrosis factor (TNF) family such as Fas ligand (FasL) (Itoh et al., 1991), TNF α (Robaye et al., 1991), and TRAIL (TNF-Related Apoptosis-Inducing Ligand) (Pan et al., 1997), the receptors (Fas (TNF Receptor Superfamily 6), TNF-R1 (TNF Receptor Superfamily 1a), and DR4 (TNF Receptor Superfamily 10a), respectively) trimerize and recruit a series of cytoplasmic adapter proteins containing death domains (DD) such as FADD (Fas Associated via Death Domain) (Chinnaiyan et al., 1995) and TRADD (TNFR1 Associated via Death Domain) (Hsu et al., 1995) to form the Death Inducing Signaling Complex (DISC). An initiator caspase such as caspase 8 is recruited to the DISC via the interaction of its death effector domain (DED) with the DED of FADD, and undergoes proteolytic cleavage and biological activation (Muzio et al., 1996; Muzio et al., 1998), reviewed in (Curtin and Cotter, 2003). Caspases are believed to function in a hierarchal manner such that up-stream caspases subsequently activate down-stream death effector

caspace 3, and commit the cell to an apoptotic fate by biochemical destruction of the cell.

2.2.3 Mitochondrial “Intrinsic” Pathway

Whereas extrinsic pathway activation relies on ligand-receptor interaction at the cell surface, the intrinsic pathway can be activated by both external and internal signals such as DNA damage, drugs/toxins, UV radiation, and hypoxia. These signals are sensed by the cell and induce a variety of effects including changes in protein half life, phosphorylation, subcellular localization, as well as the activation of death genes and the repression of survival genes. One important family of proteins in the intrinsic pathway is the Bcl-2 family, which has both pro-apoptotic and cytoprotective members, but all share one or more Bcl-2 homology (BH) domain. The anti-apoptotic Bcl-2 family members include Bcl-2 itself, Bcl-XL, and Bcl-w; and the pro-apoptotic members include Bax, Bak, Bid, Puma, Noxa, and Bnip3, reviewed in the context of the heart in (Gustafsson and Gottlieb, 2007), and discussed further in section 7. It is believed that the “decision” a cell makes regarding whether it will undergo apoptosis is, in part, determined by the relative ratio of pro- to anti-apoptotic Bcl-2 proteins present in the cell. One theory purports that an abundance of pro-apoptotic proteins, such as Bax or Bak, will titrate the life-promoting Bcl-2 family members, resulting in mitochondrial defects, and leading to the release of cytochrome c, Smac/DIABLO (Direct IAP-Binding Protein with Low pI), AIF, and endo G that each promote apoptosis. Following a death signal, basal levels of cytoplasmic Bax and Bak undergo conformational change and mitochondrial translocation, acting to disrupt the mitochondrial membrane potential and inducing the

release cytochrome c and other factors (Wolter et al., 1997; Narita et al., 1998), reviewed in (Lalier et al., 2007). Smac/DIABLO inhibits the IAPs (Inhibitors of Apoptosis)(Du et al., 2000; Verhagen et al., 2000); endo G translocates to the nucleus to fragment DNA(Li et al., 2001); AIF activates downstream targets such as the nuclear protein PARP-1 (poly(ADP-ribose) polymerase-1) (Yu et al., 2002a; Susin et al., 1999); and cytochrome c combines in the cytoplasm with APAF-1 (Apoptotic Protease Activating Factor), procaspase 9, and dATP to form the apoptosome. This multiprotein complex is the activating platform for caspase 9 (Zou et al., 1999), which will be discussed further in section 3.

The opening of the mitochondrial PTP is a recognized event in the intrinsic apoptotic pathway that occurs downstream of apoptosis activation signals, but upstream of many biochemical apoptotic markers such as caspase-3 activation (Bradham et al., 1998; Marchetti et al., 1996). The PTP has a channel-like structure, allowing passage for molecules up to 1.5 kDa without directional specificity. It is comprised of several protein subunits including VDAC, ANT, and cyclophilin D, among others (Marzo et al., 1998b; Beutner et al., 1998; Krauskopf et al., 2006). Because the PTP spans both mitochondrial membranes, an open conformation depletes the proton gradient across the inner mitochondrial membrane, and depolarizes mitochondrial membrane potential ($\Delta\psi_m$) (Bernardi et al., 1998). It is believed that the swelling of the inner mitochondrial membrane following PTP opening breaches the outer membrane and causes the release of pro-apoptotic factors such as cytochrome c that otherwise would be sequestered in the intermembrane space, reviewed in (Crompton, 1999; Javadov and Karmazyn, 2007).

In the heart, Ca^{2+} overload during reperfusion is thought to induce apoptosis and mitochondrial perturbations, such the opening of the PTP (Yellon and Hausenloy, 2007). Additionally, the pro-death protein Bnip3 has been characterized as an inducer of the mitochondrial apoptotic pathway in cardiac myocytes by a mechanism involving the PTP (Regula et al., 2002b). A detailed discussion of Bnip3 is available in section 8.

2.2.4 “Cross Talk” between the Extrinsic and Intrinsic Pathway

There is some degree of cross talk between the intrinsic and the extrinsic apoptotic pathways. The classic example is the cleavage of the pro-apoptotic Bcl-2 family member protein Bid to t-Bid by caspase 8 (Li et al., 1998). The c-terminal fragment of t-Bid subsequently translocates to the mitochondrial outer membrane where it presumably activates the intrinsic pathway. Whether t-Bid acts alone or in concert with other pro-apoptotic Bcl-2 family member proteins is debatable and remains a point of controversy (Wei et al., 2001; Willis et al., 2007). Besides caspase 8, another protein implicated in both the intrinsic and extrinsic pathways is the cytoprotective protein ARC (Apoptosis Repressor with Caspase recruitment domain). ARC is unique in its cytoprotective effects in that it can inhibit both the extrinsic and intrinsic pathway. ARC has been reported to inhibit the extrinsic pathway by interacting with caspase 8 and components of the DISC such as FADD. The inhibition of Bax activation and mitochondria translocation has been suggested to underlie its inhibition of the intrinsic pathway. Thus, ARC can prevent apoptosis induced by both pathways (Koseki et al., 1998; Gustafsson et al., 2004; Nam et al., 2004).

2.2.5 The Role of Apoptosis in Heart Disease

Because several research groups have independently implicated apoptosis in the pathology of heart disease, this notion is now fairly well accepted. Although necrosis is the major death mechanism in the core of a myocardial infarct, apoptosis has been observed in the border zone around the infarct and in the remote myocardium away from the infarct (Gottlieb et al., 1994; Kajstura et al., 1996; Olivetti et al., 1997; Olivetti et al., 1996; Saraste et al., 1997; Narula et al., 1996; Fliss and Gattinger, 1996; Piro et al., 2000; Schwarz et al., 2006). Interestingly, there are conflicting reports regarding the timing of apoptosis with respect to ischemia and reperfusion. Some evidence has suggested that apoptosis is minimal during ischemia alone, but that reperfusion induces an increase in the rate of apoptosis (Gottlieb et al., 1994; Zhao et al., 2000). Other evidence has suggested that ischemia or hypoxia alone is sufficient to induce apoptosis in whole hearts and in isolated cardiac myocytes, and that reperfusion may or may not further increase apoptosis (Veinot et al., 1997; Fliss and Gattinger, 1996; Chakrabarti et al., 1997; Black et al., 1998; De Moissac et al., 2000). A safe interpretation is that some level of apoptosis is occurring during ischemia and reperfusion in the heart.

Before the landmark study by Wencker et al (Wencker et al., 2003), cell death was known to occur during heart failure, but it was unknown to what extent the loss of myocytes was causally contributing to the cardiac pathology and decline in hemodynamic parameters. By generating a transgenic mouse with inducible caspase 8 activity in the heart, they demonstrated that an extremely low level of apoptosis induced a lethal dilated cardiomyopathy, implicating apoptosis in the disease process. The proportion of

apoptotic myocytes sufficient to induce heart failure in mice in this study was well under the previously reported proportion of apoptotic myocytes in human dilated cardiomyopathies, further suggesting that a very low, albeit increased, rate of apoptosis is an important component of heart failure. In another study, a caspase inhibitor was used to reduce apoptosis and improve survival in transgenic mice that overexpress *Gαq*, an intracellular molecule that transduces signals from α -adrenoreceptors and angiotensin II receptors, mediating hypertrophy and impaired contractility (Hayakawa et al., 2003). This evidence, along with other studies that have observed apoptosis in failing human hearts (Narula et al., 1996; Olivetti et al., 1997) suggests that apoptosis is a contributor to heart failure.

One previous criticism of some studies in which apoptosis was implicated during heart failure was the potential for false positives from the TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) assay, a popular technique for detecting apoptotic cells in tissue. As shown by Kanoh et al (Kanoh et al., 1999) the TUNEL assay will give a false-positive result when the cell has a high rate of DNA repair. While this has significant implications for investigations, the integrity of the work described above is not affected because of the many experimental approaches employed such as DNA laddering and caspase activity assays to verify the relationship between apoptosis and cardiomyopathy. Lesauskaite et al (Lesauskaite et al., 2004) suggested that in situ oligoligation (ISOL) may be a more suitable alternative to TUNEL. Proteins involved in the regulation of apoptosis include caspases and Bcl-2 family members. Through experimental manipulation of these proteins, investigators have

further demonstrated a role for apoptosis in the heart. This evidence is discussed in sections 3.2 and 7.3.

3.0 Caspases

3.1 Introduction to Caspases

In 2002, Robert Horvitz shared the Nobel Prize in Medicine with Sydney Brenner and John Sulston for their contributions to our understanding of apoptotic cell death.

Horvitz's investigations with the nematode *Caenorhabditis elegans* lead to his discovery and characterization of the genes *ced-3*, *ced-4*, and *ced-9* as critical regulators of programmed cell death in the worm (Ellis and Horvitz, 1986; Hengartner et al., 1992).

Ced-9 was characterized as a Bcl-2 homolog (Hengartner and Horvitz, 1994), Ced-4 as an Apaf-1 homolog (Zou et al., 1997), and Ced-3 as a cysteinyl-aspartate specific protease, similar to the mammalian protein ICE (Yuan et al., 1993), which was the first mammalian caspase identified (Cerretti et al., 1994; Miura et al., 1993).

There have been 13 other caspases subsequently discovered; however, many were initially labelled with unique names, creating confusion in the literature. In 1996 a committee of researchers coined the term "caspase", based on this protein family's ability to cleave its targets after an aspartic acid residue in a cysteine-dependent manner. ICE was renamed caspase-1 because it was the first caspase discovered, and the others were named in order of discovery (Alnemri et al., 1996).

Before addressing the apoptotic actions of caspases, it should be noted that the primary

role of some family members lies outside the classical apoptotic caspase cascade. For example, caspase-14 is implicated in terminal differentiation of keratinocytes by a non-apoptotic cell death mechanism called cornification (Eckhart et al., 2000; Lippens et al., 2000). Caspase-14 knock-out mice exhibited defects in skin water retention and corneocyte barrier formation (Denecker et al., 2007). Caspase-1, -4, -5, -11, and -12 appear to be involved in inflammation, reviewed in (Martinon and Tschopp, 2004).

Regarding apoptosis, caspases are broadly classified into two groups according to their place in the caspase activation cascade. These caspases are translated as inactive zymogens called procaspases, and consist of a pro-domain, a small subunit, and a large subunit, connected by interchain linkers.

The apical/initiator caspases are caspase-2, -8, -9, and -10, and their role is to activate the downstream executioner/effector caspases. Structurally, caspase-2 and -9 both contain a CARD domain (Li et al., 1997; Duan et al., 1996; Hofmann et al., 1997), and caspase-8 and -10 both contain a DED domain (Muzio et al., 1996). These caspases exist basally as inactive monomers (Boatright et al., 2003). Upon death receptor activation in the extrinsic apoptotic pathway, caspase-8 or -10 bind to FADD at the DISC via their DED domain (Muzio et al., 1996). Multiple caspase monomers are brought in close proximity and dimerize, thereby forming an active conformation. Importantly, cleavage of pro-caspase-8 may occur, but this step is not required for its activation (Muzio et al., 1998; Boatright et al., 2003).

In the intrinsic pathway, caspase activation occurs according to a similar paradigm of recruitment to an activation platform. The release of cytochrome c from the

mitochondria following mitochondrial perturbation signals the formation of the apoptosome. This specialized multiprotein structure serves as an activation platform for caspase-9 in a manner similar to DISC-mediated caspase-8 activation. This structure is comprised of cytochrome c, Apaf-1, procaspase-9, and dATP arranged in a symmetrical wheel-like formation (Acehan et al., 2002; Li et al., 1997). Pro-caspase-9 is recruited to the apoptosome via CARD-CARD interactions between pro-caspase-9 and Apaf-1. Again, in this model the induced proximity of the caspase monomers is sufficient to induce dimerization and catalytic activation (Salvesen and Dixit, 1999; Boatright et al., 2003).

The activated initiator caspases cleave and activate the effector caspases: caspase-3, -6, and -7. These molecules exist basally as inactive dimers, and are also known as executioner caspases because they act directly on nuclear and cytoskeletal components, and facilitate the apoptotic disassembly of the cell. The activation of effector caspases involves a linker region cleavage-dependent conformation change that generates the active site and stabilizes the substrate binding region (Stennicke et al., 1998; Zhou and Salvesen, 1997; Boatright et al., 2003). Downstream caspase targets include PARP (Lazebnik et al., 1994; Tewari et al., 1995; Nicholson et al., 1995), nuclear proteins such as lamin A (Orth et al., 1996; Takahashi et al., 1996), cytoskeletal proteins such as alpha fodrin (Cryns et al., 1996; Janicke et al., 1998; Zheng et al., 1998) and actin (Mashima et al., 1997), as well as ICAD, an inhibitor of CAD (Sakahira et al., 1998; Enari et al., 1998). Activation of these target proteins results in the biochemical destruction of the cell including dismantling the cytoskeleton and DNA laddering.

There are many layers of control over the apoptotic cascade, allowing the cell precise control over the death machinery. In the intrinsic arm of the pathway, caspase-9 activation is inhibited by the IAPs (c-IAP1, c-IAP2, and XIAP), and this process is inhibited by Smac/DIABLO (Srinivasula et al., 2001; Du et al., 2000; Verhagen et al., 2000). Similarly, HAX-1 has been identified as an inhibitor of caspase-9 activation (Han et al., 2006), as have the heat shock proteins Hsp70 and Hsp90 (Saleh et al., 2000; Beere et al., 2000; Pandey et al., 2000). Intriguingly, recent evidence suggests that caspase-3-mediated cleavage of caspase-9 abrogates the inhibitory actions of XIAP on caspase-9 activity (Denault et al., 2007), thereby amplifying the apoptotic signal transduction in a cell. Regarding the extrinsic pathway, caspase-8 and -10 activity is inhibited by FLIP, which competes with those caspases for binding to FADD (Irmeler et al., 1997; Srinivasula et al., 1997; Ekert et al., 1999); however, some reports have proposed that a FLIP-procaspase-8 dimer may become catalytically active (Chang et al., 2002; Micheau et al., 2002; Boatright et al., 2004). Finally, viral proteins such as p35 (Bump et al., 1995) and crmA (Zhou et al., 1997) are also known to inhibit caspase activity.

3.2 The Role of Caspases in the Heart

Because caspases are integral to the classical apoptotic pathways, investigations that implicate caspase activity as a contributor to the pathology of heart failure equally implicate apoptosis. Researchers have used caspase inhibitors to examine the effects of cardiac ischemia and reperfusion in the absence of caspase activity. One such agent is the synthetic peptide Z-VAD-fmk, a commonly used nonselective caspase inhibitor (Slee et al., 1996); however, more specific agents also exist. DEVD-fmk is an inhibitor of the

effector caspases, caspase-3 and -7, and Z-IETD-fmk and Z-LEHD-fmk inhibits the initiator caspases, caspase-8 and caspase-9, respectively. These agents are competitive inhibitors of caspase activity because they act as decoy substrates. Conjugating the peptides to fluoro- or chloromethylketone (fmk, cmk) generates an irreversible competitive inhibitor, whereas the aldehyde (-cho) conjugates are reversible inhibitors (Ekert et al., 1999).

Experimental results of caspase inhibitors used in the context of cardiac ischemia-reperfusion have been encouraging with regard to therapeutic potential. Typical effects include reduction of infarct size, attenuation of left ventricular dysfunction, and reduction in apoptosis. For example, a study by Mocanu et al. (2000) tested specific and broad spectrum caspase inhibitors in isolated rat hearts perfused by a Langendorff system. A caspase inhibitor was added to the perfusate after 30 minutes of regional ischemia, induced by left coronary artery ligation, and 5 minutes prior to reperfusion. They found that both the selective and the non-selective caspase inhibitors equivalently reduced infarct size, as measured by tissue staining with triphenyltetrazolium chloride. Because apoptosis may be accelerated by reperfusion, they proposed that caspase inhibitors might be beneficial adjuncts to thrombolytic treatment for patients presenting with MI (Mocanu et al., 2000). A similar study only found that a non-selective caspase inhibitor improved cardiac function, reduced infarct size, and apoptosis after I/R, compared to a selective inhibitor. Although these results point to the same general conclusion, the second study cited differences in dose and experimental timing of drug delivery for the discrepant results (Kovacs et al., 2001). In vivo studies have shown that the pan-caspase inhibitor

ZVAD-fmk, delivered before ischemia and periodically during reperfusion, reduced infarct size and TUNEL-positive cells in rats subjected to 30 minutes of coronary occlusion followed by 24 hours of reperfusion (Yaoita et al., 1998). Another in vivo study, looking only at ischemia without reperfusion, found that the caspase inhibitor DEVD-cho reduced apoptosis in the border zone at 24 hours post infarction. In the long term (8 weeks), they found that the mice on the inhibitor exhibited better hemodynamics, left ventricular geometry, and a trend toward increased survival compared to controls. This study did not observe a change in infarct size (Balsam et al., 2005). Lastly, adenovirus-mediated delivery of the viral protein p35 by direct injection into the heart attenuated apoptosis, reduced infarct size, and improved left ventricular function in rats subjected to LAD ligation compared to controls (Bott-Flugel et al., 2005). The beneficial effects of the caspase inhibitors support a detrimental role for apoptosis in ischemic heart disease, and suggest that such agents may be of use to human patients.

In spite of these studies, there is no evidence available showing that caspase inhibitors are useful drugs for humans during cardiac ischemia/reperfusion. Interestingly, the caspase inhibitor IDN-6556 has been used in the context of human liver transplant to attenuate apoptosis following cold ischemia/warm reperfusion (Baskin-Bey et al., 2007).

Many studies have focused on the role of cardiomyocyte apoptosis, but it remains to be determined whether apoptosis of non-myocyte cells such as cardiac fibroblasts plays a pathological role post-MI. Along this line, it has been shown that cardiac fibroblasts may have an innate resistance to apoptosis, mediated by Bcl-2 (Mayorga et al., 2004). Future studies may therefore make important distinctions between cardiac myocyte and cardiac

fibroblast apoptosis post-MI.

4.0 Nuclear Factor – κ B

4.1 Characterization of NF- κ B

Nuclear Factor- κ B (NF- κ B) was originally discovered by David Baltimore's laboratory in 1986 (Sen and Baltimore, 1986). Using B lymphocytes, the newly named NF- κ B was characterized as a factor that bound to the immunoglobulin kappa light chain enhancer DNA sequence, coincident with increased kappa chain gene expression (Sen and Baltimore, 1986). Since then, NF- κ B activity has been implicated in a large array of biological processes including immunity, inflammation, proliferation, and cell survival (Beg and Baltimore, 1996; Beg et al., 1995; Van Antwerp et al., 1996; Hayden et al., 2006; Van Waes, 2007; Uwe, 2008). Likewise, defects in NF- κ B signaling have been associated with a multitude of human diseases including cancers of the lung (Tang et al., 2006; Zhang et al., 2007a), prostate (Huang et al., 2001; Lessard et al., 2006), breast (Nakshatri et al., 1997; Sovak et al., 1997), pancreas (Holcomb et al., 2008), as well as in lymphomas (Jost and Ruland, 2007), collectively reviewed in (Karin, 2006). Other diseases include those with an inflammatory component such as rheumatoid arthritis (Roman-Blas and Jimenez, 2006), inflammatory bowel disease (Atreya et al., 2008), and asthma (Catley et al., 2005; Newton et al., 2007), as well as AIDS (Puca et al., 2007; Pande and Ramos, 2003; Griffin et al., 1989), Alzheimer's Disease (Collister and Albeni, 2005), and many others (Courtois and Gilmore, 2006; Pande and Ramos, 2005). The role of NF- κ B in the heart will be discussed in section 4.3.

NF- κ B itself is comprised of two subunits in either a homo- or heterodimeric complex. In the NF- κ B family there are five protein subunits: p50 (precursor p105), p52 (precursor p100), p65 (aka RelA), RelB, and c-Rel, which are encoded by the following genes, respectively: *NFKB1*, *NFKB2*, *RELA*, *RELB*, and *REL*. Each of these proteins contains a Rel homology domain (RHD), which is used for dimerization and DNA binding. The most well characterized NF- κ B dimer is comprised of the p50 and p65 subunits (Hayden and Ghosh, 2008).

Our laboratory has focused our studies on the p65 subunit of NF- κ B because many of the biological actions attributed to NF- κ B are linked to p65. Structurally, p65 is a 551 amino acid protein comprised of an N-terminal RHD, an NLS, and a C-terminal TAD, as shown in the schematic diagram in figure 2 (Hayden and Ghosh, 2008). Because it can bind DNA and activate transcription, p65-containing NF- κ B dimers are transcription factors, and indeed, NF- κ B is generally thought to exert its influence on the cell through activation of target genes. However, in some situations, NF- κ B has been shown to repress transcription (Ashburner et al., 2001; Baetz et al., 2005; Zhang and Kone, 2002; Zhang et al., 2005; Chen et al., 2003). This notion will be developed further in sections 4.2 and 5.4.

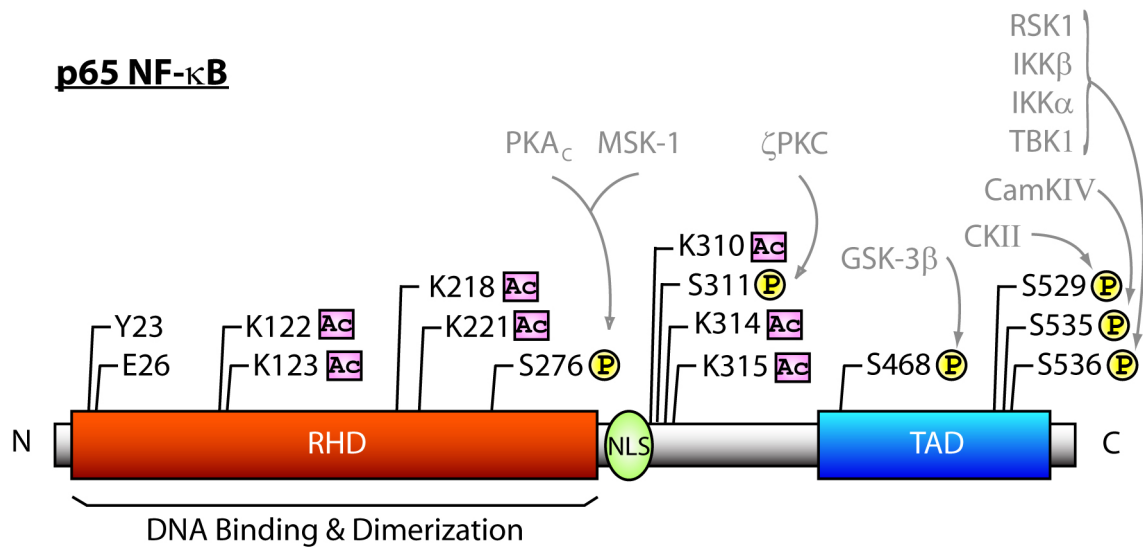


Figure 2. Schematic diagram of the p65 NF- κ B protein. Shown is the Rel homology domain (RHD, red), nuclear localization signal (NLS, green), and transcription activation domain (TAD, blue). Also indicated are residues for phosphorylation (indicated by yellow circles) and acetylation (indicated by purple squares). Kinases for phosphorylation of p65 at designated residues are shown in grey.

Post-translational modifications of p65 have been described that impart functional changes to p65 and add extra layers of regulation over a given NF- κ B response beyond the classical NF- κ B signaling described in the next section. Phosphorylation on serine residues S276, S311, S468, S529, S535, and S536 have each been shown to increase the transcriptional activity of p65. The kinases are reported to be PKA_C and MSK-1 for S276 (Zhong et al., 1997; Zhong et al., 1998; Okazaki et al., 2003; Vermeulen et al., 2003), ζ PKC for S311 (Duran et al., 2003), CKII for S529 (Wang and Baldwin, Jr., 1998; Wang et al., 2000b), CamKIV for S535 (Jang et al., 2001; Bae et al., 2003), and IKK β , IKK α , TBK1, and RSK1 for S536 (Sakurai et al., 1999; Buss et al., 2004b; Bohuslav et al., 2004). One of the most well characterized phosphorylation sites for the transcription potential of p65 is arguably S536. Phosphorylation at this residue occurs after stimulation with TNF α and LPS and contributes to the activation of NF- κ B target genes (Sakurai et al., 2003; Yang et al., 2003). Phosphorylation at S529 has also been shown to be induced by TNF α , and the loss of this phosphorylation site was associated with less NF- κ B-dependent transcription after stimulation (Wang and Baldwin, Jr., 1998). However, because of the many kinases involved in p65 phosphorylation, the phosphorylation status and resultant capability of p65 for transcriptional activation likely depends on a balance of multiple inputs. Interestingly, basal phosphorylation of p65 on S468 is maintained by GSK-3 β , and inhibits its transcriptional activity (Buss et al., 2004a).

Acetylation of p65 also affects the biological function of p65. Acetylation on lysine residue 221 is important for DNA binding and for inhibiting the association between p65

and the inhibitor protein I κ B α (Chen et al., 2002a). Accordingly, deacetylation of this residue promotes p65- I κ B α binding, cytoplasmic localization of p65, and the termination of NF- κ B activity. Furthermore, acetylation at lysine 310 was shown to increase the transcription potential of p65 (Chen et al., 2002a; Chen et al., 2001a). Most interestingly, it appears that the phosphorylation of p65 at S276 and S536 may “prime” p65 for acetylation at K310 due to increased association with the acetyl transferase p300 (Chen et al., 2005). This sequential modification of p65 is required for its full transcriptional activity. It is also suspected that phosphorylation of S311 may have a similar role in the induction of acetylation (Chen et al., 2005). Another group has proposed a slightly different model, in which p65 acetylation at K122 and K123 promotes dissociation from DNA and termination of NF- κ B activity (Kiernan et al., 2003). Finally, two other putative p65 acetylation sites were recently identified: K314 and K315 (Buerki et al., 2008). The study by Buerki et al. (2008) found that neither acetylation of K314, K315, nor of the previously identified K310, had any general effect on subcellular localization, DNA binding, or overall transcriptional activity of p65. However, microarray analysis suggested that expression of specific NF- κ B target genes may be selectively modulated by acetylation of p65 (Buerki et al., 2008).

There is strong evidence that these post translational modifications of p65 have biological significance. Regarding the discrepancies in the literature about the functional significance of certain residues, they may only apply to the experimental conditions tested, such as a particular NF- κ B stimulus on a particular cell or tissue type. Excitingly, it is because of these different reports that researchers have realized that post-translational

modification of p65 may provide the cell with a mechanism to “flavour” a given NF- κ B response in a context-dependent manner.

4.2 NF- κ B Signaling

Under normal conditions, NF- κ B is predominantly localized to the cytoplasm. Research over the last number of years has identified two major signaling pathways for NF- κ B activation and translocation to the nucleus, where it may exert its influence over cellular gene expression. The most well described of these pathways is known as the classical or canonical pathway and the other is known as the non-canonical or alternative pathway. Below, these pathways will be described in the reverse direction, starting with NF- κ B nuclear translocation and moving backwards to the initial activation signal such as surface receptor-ligand binding.

In the canonical pathway, as diagrammed in figure 3, the inhibitor protein I κ B α maintains the cytoplasmic localization of the p50/p65 NF- κ B heterodimer under basal conditions by mutually masking the NLSs of p65 and I κ B α (Beg et al., 1992; Zabel et al., 1993; Sachdev et al., 1998). However, it is important to note that the p65 subunit has been observed in the nucleus of unstimulated cells due to basal shuttling to and from the nucleus, incomplete masking of the p50 NLS by I κ B α , and the fact that a certain fraction of cellular NF- κ B is simply not associated with I κ B α at a given time (Carlotti et al., 2000; Birbach et al., 2002; Huang et al., 2000; Harhaj and Sun, 1999; Johnson et al., 1999; Malek et al., 2001).

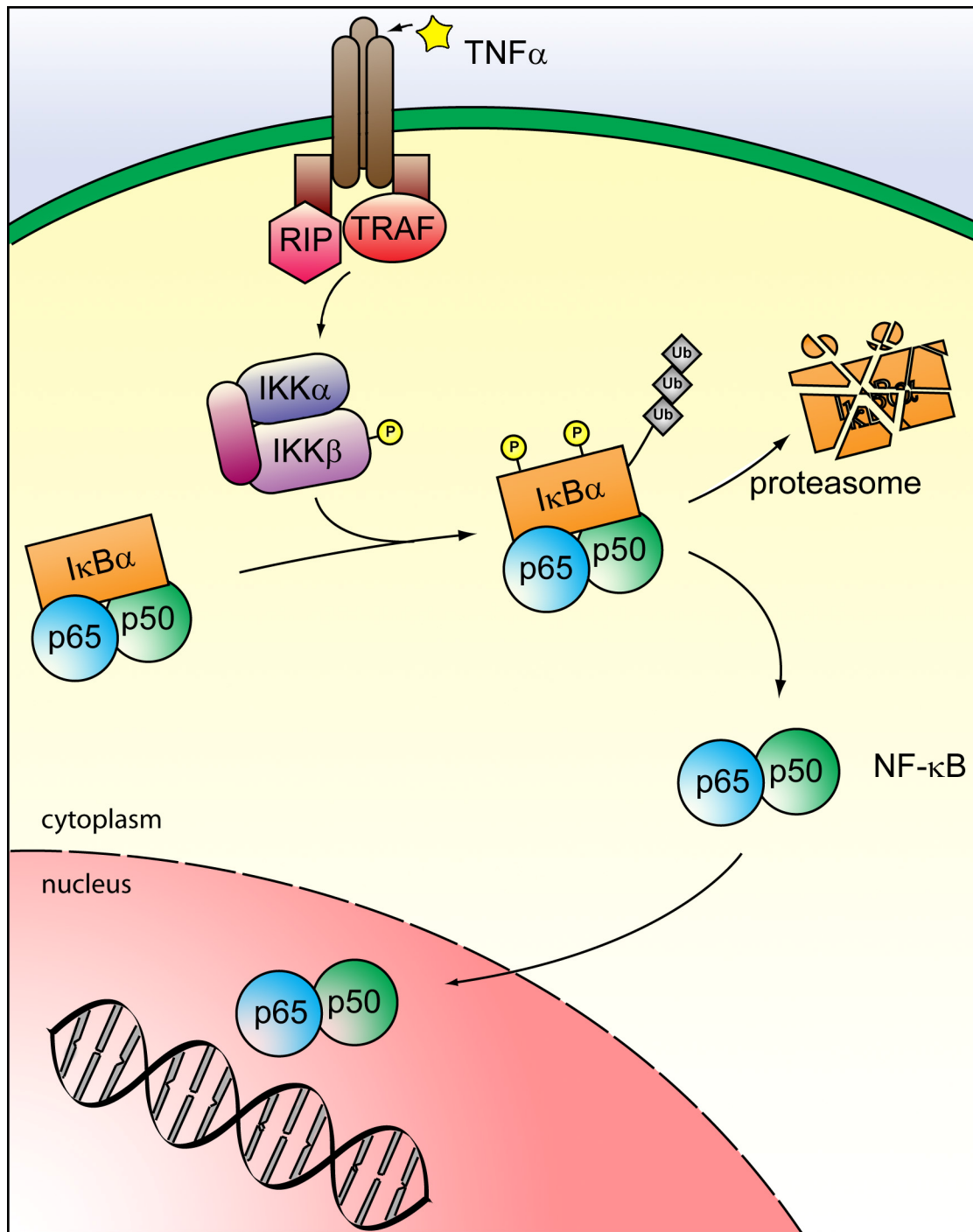


Figure 3. Activation of canonical NF- κ B signaling. Ligands such as TNF α bind TNF receptors and recruit TRAF and RIP proteins, which mediates activation of the IKK complex. Phosphorylation-dependent degradation of I κ B α by IKK β allows NF- κ B nuclear translocation and subsequent regulation of downstream target genes.

The phosphorylation-dependent ubiquitination of I κ B α at serines 32 and 36 signal its degradation by the 26S proteasome (Chen et al., 1995), thereby unmasking the p65 NLS, and shifting the localization equilibrium of NF- κ B to the nucleus (Beg et al., 1992). Accordingly, serine to alanine substitution mutations at these residues on I κ B α results in a “super repressor” protein that cannot be degraded and prevents NF- κ B activation by holding it in the cytoplasm (Brown et al., 1995).

Phosphorylation of I κ B α is primarily achieved by the kinase activity of the IKK complex, which has three core subunits. IKK α and IKK β subunits both have kinase activity, and IKK γ , aka NEMO, is characterized as an essential regulatory subunit (Chen et al., 1996b; Zandi et al., 1997; Rothwarf et al., 1998; Yamaoka et al., 1998). IKK activation involves the phosphorylation of IKK β in its kinase domain on two serine residues, S177 and S181 (Carter et al., 2001), and phosphorylation of IKK α on S176 and S180 (Ling et al., 1998; Mercurio et al., 1997). Importantly, knock-out and loss-of-function experiments have implicated IKK β as the major kinase in the canonical pathway. IKK β ^{-/-} mice die at embryonic day ~14.5 from apoptotic liver degeneration, similar to p65^{-/-} mice that die at approximately the same time with similar liver apoptosis. Furthermore, embryonic cells derived from both of these lines were less resistant to TNF α -induced cell death and displayed reduced NF- κ B target gene activation, implying a critical role for the beta subunit that cannot be complemented by IKK α (Tanaka et al., 1999; Li et al., 1999d; Beg et al., 1995). IKK α ^{-/-} mice exhibit skeletal and skin developmental abnormalities and do not survive after birth. However, in IKK α ^{-/-} MEFs, there was no change in I κ B α phosphorylation after TNF α stimulation, and a considerable amount of IKK activity was

retained in these cells, revealing that IKK α activity is dispensable with regard to the cytokine-induced NF- κ B response (Li et al., 1999c; Takeda et al., 1999).

The activation of the IKK complex is a complicated process partially dependent on the initial stimulus. Ligands such as TNF α , LPS, and IL-1 have each been shown to stimulate IKK-NF- κ B activity, but the array of adapter proteins recruited to the receptor in each case slightly differs (Hayden and Ghosh, 2008). In the case of TNF α , ligand binding induces receptor trimerization and the recruitment of TRAF2, TRAF5, and RIP1 via TRADD (Micheau and Tschopp, 2003). The IKK complex may also be recruited to the receptor via RIP1-NEMO interactions (Poyet et al., 2000; Inohara et al., 2000). It has been suggested that this may induce a conformation change in the IKK complex, leading to autophosphorylation and activation; however, some evidence also supports the role of TAK1 as an IKK-kinase in the canonical pathway (Hayden and Ghosh, 2008). Aside from the typical NF- κ B activators TNF α , LPS, and IL-1, there are a wide array of other activators, reviewed in (Pahl, 1999). Thus, the major events and participating factors have been identified in the canonical NF- κ B pathway, but the fine details, especially the role of ubiquitination in IKK activation, are still areas of active research.

Termination of NF- κ B activation is classically mediated by a negative feedback loop in which NF- κ B induces the expression of I κ B α , which enters the nucleus, binds NF- κ B, and subsequently exports it to the cytoplasm (Sun et al., 1993; Chiao et al., 1994; Sachdev et al., 1998; Arenzana-Seisdedos et al., 1997). Furthermore, activation of the deubiquitinases A20 and CYLD by NF- κ B has been shown to contribute to the attenuation of the signal, possibly by inhibiting IKK activation (Wertz et al., 2004;

Brummelkamp et al., 2003). As previously mentioned, post-translational modifications of p65 may also be involved in signal termination.

One fundamental difference between the canonical and non-canonical pathways is that the non-canonical pathway is regulated by proteolytic cleavage of precursor proteins, rather than by I κ B proteins. Aside from the absence of I κ B-mediated regulation, the non-canonical pathway features p52-containing dimers, such as p52-RelB, as opposed to p50-containing dimers in the canonical pathway (Bours et al., 1994). Briefly, the phosphorylation-dependent cleavage of the precursor protein p100 to p52 allows p52-containing NF- κ B dimers to translocate the nucleus and affect gene transcription (Heusch et al., 1999; Betts and Nabel, 1996). The processing of p100 is mediated by IKK α and the upstream kinase NIK, which may, in fact, target both IKK α and p100 (Senftleben et al., 2001; Ling et al., 1998; Xiao et al., 2004). IKK α is activated by a similar collection of proteins as IKK β is (Hayden and Ghosh, 2008). A subgroup of TNFRSF members becomes activated on the cell membrane when bound by ligands in the TNF superfamily such as BAFF (Claudio et al., 2002) and RANKL (Novack et al., 2003). Because the subsequently generated NF- κ B dimers contain p52 and not p50, the non-canonical NF- κ B target genes are distinct from the canonical target genes (Dejardin, 2006).

Example target genes that are activated by canonical NF- κ B signaling include Bcl-2 (Catz and Johnson, 2001), IL-6 (Libermann and Baltimore, 1990), FLIP (Micheau et al., 2001), MnSOD (Das et al., 1995) and the IAPs (Stehlik et al., 1998; Wang et al., 1998). In this regard, NF- κ B activity is usually characterized as anti-apoptotic or pro-inflammatory. Interestingly, TNF α is associated with the activation of both the extrinsic

apoptotic pathway and with the activation of NF- κ B signaling. It is the activation of NF- κ B-dependent antiapoptotic genes such as FLIP, Bcl-2, the IAPs, and A20 that antagonize TNF α -induced apoptosis (Micheau et al., 2001). Accordingly, the loss of NF- κ B signaling is associated with increased susceptibility to TNF α -induced apoptosis (Beg and Baltimore, 1996; Mustapha et al., 2000).

In addition to its well described role as transcriptional activator, there are reports in the literature indicating that NF- κ B activity in some cellular contexts can repress transcription (Ashburner et al., 2001; Zhang and Kone, 2002; Zhang et al., 2005; Grimm et al., 2005). In MDA-MB-231 and MCF-7 cells, p65 repressed expression of DR4 and caspase-8 following stimulation with TRAIL, a ligand in the TNF α family that induces apoptosis (Chen et al., 2003). Using cardiac myocytes, our laboratory showed that p65 directly repressed the transcription of Bnip3, an apoptotic factor that will be discussed in detail in section 8 (Baetz et al., 2005). Furthermore, evidence has suggested a role for HDACs in the repression of NF- κ B target genes and in this regard the relationship between NF- κ B and HDACs will be discussed in section 5.4.

Another way that NF- κ B activity deviates from its classic and established role as a cytoprotective transcription factor is that it has been shown in some cases to activate apoptosis-inducing genes, and has been directly implicated in the apoptotic process. This is supported by the fact that NF- κ B upregulates the death inducing ligands TNF α (Shakhov et al., 1990), FasL (Kasibhatla et al., 1998; Matsui et al., 1998), and TRAIL (Baetu et al., 2001), some death receptor genes (Ravi et al., 2001; Kasof et al., 2001; Chan et al., 1999), and other pro-apoptotic proteins such as p53 (Wu and Lozano, 1994;

Kim et al., 2002) and Bcl-Xs (Shou et al., 2002). Some reports show that NF- κ B activation is pro-apoptotic in cardiomyocytes and endothelial cells under oxidative stress (Wang et al., 2002; Aoki et al., 2001). Because NF- κ B appears to have both anti- and pro-apoptotic capabilities, its role in cell death is still a source of debate; however, the majority of the evidence points to an anti-apoptotic role for NF- κ B. In light of this, perhaps the detrimental effects of NF- κ B are activated under more extreme circumstances such as excess NF- κ B activation, as in the case of A20 knockout mice (Lee et al., 2000a), or chronic absence of NF- κ B, in the case of p65 knockout mice (Beg et al., 1995). Future studies will further characterize the contexts in which NF- κ B signaling is pro- or anti-apoptotic.

4.3 Role of NF- κ B in the Heart

Not only is there debate over the nature of NF- κ B signaling in a cell, there is also controversy over the role of NF- κ B in heart disease. Interestingly, NF- κ B activation has been observed in many cardiovascular disease states including ischemic and dilated heart failure, ischemic preconditioning, and myocarditis (Frantz et al., 2003; Wong et al., 1998; Morgan et al., 1999; Xuan et al., 1999; Yokoseki et al., 2001). Furthermore, stimuli such as ischemia-reperfusion and ROS (Shimizu et al., 1998; Li et al., 1999a; Peng et al., 1995), TNF α (De Moissac et al., 1998), as well as β -adrenergic stimulation (Takemoto et al., 1999; Chandrasekar et al., 2004) have been shown to activate NF- κ B in the heart. Because of these observations, it is believed that NF- κ B may be somehow involved in heart disease. However, despite a sizable body of research, the exact role of NF- κ B in the heart has yet to be fully elucidated. The critical question is whether NF- κ B signaling

is an important cardioprotective pathway, or alternatively, if NF- κ B contributes to cardiac pathology post-MI. The answer may be both, and dependent upon the timing of NF- κ B activation.

Because the heart's response to ischemia-reperfusion is dynamic and complex, time is an important variable in experiments examining NF- κ B activity in the heart following ischemia-reperfusion. For example, Chandrasekar and Freeman (1997) observed two waves of NF- κ B activity in the heart after ischemia-reperfusion. In their experiments they observed NF- κ B activity following 15 minutes of ischemia and 15 minutes of reperfusion, which lasted until 1 hour of reperfusion. The second wave began at 3 hours reperfusion and lasted until 6 hours (Chandrasekar and Freeman, 1997). In another study using an MI model of ischemia only, NF- κ B activation was found to be maximum at 3 days post-MI in the infarcted area, and had declined by day 7 (Shimizu et al., 1998). In Langendorff-perfused rat hearts subjected to global ischemia and reperfusion it was found that NF- κ B activity was induced as early as after 5 minutes of ischemia with I κ B α degradation observed at 4 minutes of ischemia and resynthesis at 30 minutes. In this study, NF- κ B DNA binding was only slightly increased by reperfusion. Interestingly, the antioxidant PDTC (pyrrolidine dithiocarbamate) prevented ischemia-induced NF- κ B activity, further implicating ROS as an NF- κ B activator (Li et al., 1999a). Finally, in primary cultures of cardiac myocytes, NF- κ B is activated by 1 hour of hypoxia (Matsui et al., 1999), but inhibited by 24 hours of hypoxia (Baetz et al., 2005). Clearly, the timing of NF- κ B activation in the heart following ischemia and reperfusion must be carefully considered if NF- κ B signaling is to be therapeutically exploited. It is important to keep

in mind, however, that NF- κ B signaling is generally cyclical in nature, in large part due to the continuous loop of I κ B α degradation and resynthesis (Hoffmann et al., 2006).

Despite this, the point is that when considering how to therapeutically exploit NF- κ B to treat heart disease, there may be an optimal window for therapeutic NF- κ B activation, and an equally therapeutic window for NF- κ B inhibition that needs to be determined experimentally.

As previously stated, the role of NF- κ B in the heart is not completely understood at present. On the basis that cardiac ischemia and subsequent reperfusion injury induces NF- κ B activity (Shimizu et al., 1998; Li et al., 1999a) and inflammatory cytokine expression (Herskowitz et al., 1995), several groups have tested a variety of NF- κ B inhibitors in the context of ischemia reperfusion. There are a variety of compounds known to inhibit the NF- κ B signaling pathway, including proteasome inhibitors, IKK inhibitors, and NF- κ B decoy oligonucleotides. In a pig model of ischemic heart disease, the proteasome inhibitor PS-519, administered just prior to ischemia/reperfusion, attenuated NF- κ B activation and reduced infarct size compared to controls (Pye et al., 2003). Although this type of compound has consistently been cardioprotective in these studies, it is conceivable that the effects of proteasome inhibitors in the heart extend beyond NF- κ B, and it is unlikely that this cardioprotective effect is mediated solely through NF- κ B inhibition (Luss et al., 2002; Meiners et al., 2004).

To more definitively ascertain the role of NF- κ B in the heart a more specific compound is required. The selective inhibitor Bay 65-1942 has been characterized as a competitive inhibitor of IKK β . It competes with ATP for IKK β binding and prevents IKK-mediated

I κ B α phosphorylation and degradation (Ziegelbauer et al., 2005). This compound, when delivered either prior to the onset of ischemia, or at the time of reperfusion, resulted in smaller infarcts, improved myocardial function, and a reduction in TNF α and IL-6 release (Moss et al., 2007). Another type of NF- κ B inhibitor, NF- κ B decoys, which are oligonucleotides that compete with NF- κ B target genes for NF- κ B binding, have also been characterized as cardioprotective during MI (Morishita et al., 1997). Finally, mice with cardiac-specific overexpression of a non-phosphorylatable I κ B α “super repressor” protein demonstrated significant reduction in infarct size after ischemia-reperfusion, again suggesting a pathological role for NF- κ B (Brown et al., 2005). Further evidence in support of this idea is that TNF α , an inducer and target of NF- κ B, is increased in the heart post MI, and contributes to infarct size and the decline in cardiac function, reviewed in (Schulz et al., 2004). This statement is based on the fact that antibodies directed against TNF α reduced infarct size (Belosjorow et al., 2003; Li et al., 1999b), as did genetic knock out of TNF α (Maekawa et al., 2002). However, the role of TNF α is also unclear, and like NF- κ B, some studies support an alternate, protective role for them both, as described in the next paragraph.

Despite the above evidence supporting the notion that NF- κ B inhibition is potentially therapeutic, the following strongly suggests an alternate point of view, that NF- κ B is cardioprotective. In this context, NF- κ B signaling has been shown to not only avert apoptotic cell death in the heart, but also to attenuate infarct size and, importantly, to play a role in cardiac preconditioning, which is a reduced susceptibility to ischemic damage after previous short exposures to ischemia. Indeed, NF- κ B inhibition has been shown to

abrogate the protective effects of myocardial preconditioning, and thus, NF- κ B may have a protective role in the heart under certain circumstances (Morgan et al., 1999; Xuan et al., 1999).

Because of the established anti-apoptotic properties of NF- κ B signaling, researchers have asked whether these effects are seen in the heart. In a transgenic mouse, cardiac specific overexpression of a Δ N I κ B α mutant that retains NF- κ B in the cytoplasm and attenuates NF- κ B signaling demonstrated larger infarcts 24 hours post LAD ligation, and substantially increased myocardial apoptosis at 3 and 6 hours, suggesting a protective role for NF- κ B (Misra et al., 2003). A study involving the knock-out of both TNFR1 and TNFR2 revealed that these mice had larger infarcts and increased apoptosis compared to wild types, suggesting that TNF α may activate a protective pathway, such as NF- κ B (Kurrelmeyer et al., 2000). In support of this, pre-treatment of hearts or cardiac myocytes with TNF α conferred protection from ischemia and hypoxia as shown by reduced LDH release compared to controls (Eddy et al., 1992; Nakano et al., 1998). Moreover, another study showed that cardiac-specific overexpression of the anti-apoptotic protein Bcl-2 reduced infarct size and apoptosis following ischemia reperfusion. Because Bcl-2 has been shown to activate NF- κ B in the heart (De Moissac et al., 1998), this is consistent with the notion that NF- κ B is cytoprotective, although the many other anti-apoptotic mechanisms of Bcl-2, including the inhibition of pro-apoptotic Bcl-2 family members likely played a strong role in this result (Chen et al., 2001c).

Work from Dr. Kirshenbaum's laboratory has revealed a cytoprotective role for NF- κ B by averting hypoxia-induced cell death of isolated ventricular myocytes. In this study,

adenovirus-mediated delivery of IKK β to rat ventricular myocytes attenuated hypoxia-induced mitochondrial perturbations, caspase activity, and cell death (Regula et al., 2004). Interestingly, NF- κ B was detected in both the cytoplasm and at the mitochondria in these cells, suggesting that NF- κ B may directly inhibit cell death at the mitochondria in addition to its described role as a transcriptional regulator. Furthermore, loss of NF- κ B signaling was shown to be sufficient for increased susceptibility to TNF α -induced cell death (Mustapha et al., 2000). Interestingly, NF- κ B was shown to inhibit the transcription of the hypoxia-inducible death factor Bnip3 in cardiac myocytes, thereby providing another mechanism for NF- κ B cytoprotection (Baetz et al., 2005). Collectively, these results strongly support a cytoprotective role for NF- κ B in the heart.

How can these apparent discrepancies in the literature be resolved? The differences in the literature are: differences in experimental models, such as ischemia/reperfusion vs. ischemia alone, degree of coronary artery banding, localized ischemia vs. global ischemia, isolated hearts vs. whole animal experiments, as well as species differences including mouse strains, and experimental time points. Because of the dynamic nature of the NF- κ B response in the heart as previously discussed, it may be that the different time points used in the experiments may generate apparently different results if examined in isolation by independent groups. Further to this point, researchers often constitutively express transgenes and base their findings on the subsequent results. However, because of the dynamic nature of NF- κ B signaling in the heart, this approach may be inadequate to fully address the role of NF- κ B. On the premise that NF- κ B activity may be protective or detrimental, the notion of an inducible transgene may allow researchers to dissect out

when NF- κ B is protective, and when it is not, in relation to the onset of ischemia or reperfusion.

Insight into the discrepancies involving the role of TNF α in the heart may be ascertained from a study investigating the different NF- κ B responses from acute vs. chronic TNF α exposure. In response to acute TNF α exposure, a normal NF- κ B response was observed and comprised largely of p65/p50 NF- κ B heterodimers entering the nucleus. In the context of prolonged, chronic TNF α elevation, as in TNF α overexpressing transgenic animals or in heart failure, p50/p50 NF- κ B homodimers were observed in addition to p65/p50 NF- κ B heterodimers, suggesting a different pattern of NF- κ B gene expression (Haudek et al., 2001).

Thus, the role of NF- κ B is still not fully understood in the heart, and NF- κ B may be both “good” and “bad” in the heart, depending at least on the timing of its activation post-MI. Early activation of NF- κ B mediates preconditioning, and IKK β overexpression strongly inhibits hypoxia induced death of isolated cardiac myocytes. Furthermore, pre-treatment with TNF α confers protection against ischemia. Taken together, these results suggest a protective role for early NF- κ B activation (Morgan et al., 1999; Xuan et al., 1999; Eddy et al., 1992; Nakano et al., 1998). However, the protective effect of NF- κ B chemical inhibitors or NF- κ B decoys, delivered prior to ischemia/reperfusion, argue against this (Moss et al., 2007; Morishita et al., 1997). Late NF- κ B activation by the elevated levels of TNF α in congestive heart failure, coupled with the altered NF- κ B gene expression from chronic TNF α exposure and the cardiodepressant effects of TNF α in the myocardium suggests that chronic NF- κ B activation may be detrimental in the long run

(Schulz et al., 2004).

Future experiments will further characterize NF- κ B in the heart, and may discover an optimal protocol for NF- κ B modulation that minimizes damage post-MI. Post-translational modifications of p65 may also be investigated for their role in heart disease. However, because the regulation of NF- κ B is complex, and because NF- κ B itself regulates so many downstream targets that are involved in different cellular processes, therapeutic interventions directly targeted towards NF- κ B may be difficult to successfully implement without unwanted side effects. Instead, it may be more effective to identify particular NF- κ B target genes that are relevant to improving patient outcomes post-MI, and subsequently target that specific subset of genes with a cocktail of therapeutic factors. Accordingly, the work presented in chapter IV, sections 1 and 2 is focused on understanding the NF- κ B-mediated regulation of the death factor Bnip3, an NF- κ B target gene implicated in hypoxia-induced cardiomyocyte apoptosis. Bnip3 itself is discussed further in chapter III, section 8.

5.0 Histone Deacetylases

5.1 Chromatin Structure

One of the many amazing things about the cell is the nuclear DNA packaging system.

The nucleus of a human cell typically contains 22 pairs of autosomal chromosomes, plus two sex chromosomes for a total of 46. Chromosomes are made up of chromatin, which is a general term used to describe all the DNA and proteins within a chromosome. In a human cell, the nucleus is approximately 6 μ m in diameter and contains about 2 m of

DNA (Griffiths et al., 2000). Clearly, the DNA must be packaged very efficiently to fit in such a small space. In order to package DNA into the nucleus, DNA is first coiled around support proteins called histones. A nucleosome is a protein octamer comprised of two of each of the histone proteins H2A, H2B, H3, and H4, with 146 base pairs of DNA wrapped around it (Kornberg and Lorch, 1999; Luger et al., 1997). Nucleosomes are present all along a single strand of DNA, and they are packaged with other histones and scaffolding proteins into higher order structures that ultimately compact the nuclear DNA into the nucleus (Griffiths et al., 2000).

5.2 Histone Deacetylation Inhibits Transcription

In the genome, local histone acetylation status is an important determinant of chromatin conformation and a qualitative predictor of the extent of local gene transcription. In a nucleosome, lysine residues of histone proteins, particularly in the N-terminal tails of histones H3 and H4, are targets for acetylation, which has been shown to increase transcriptional activity (Jenuwein and Allis, 2001). Because lysine residues impart a positive charge on histone proteins, they attract negatively charged DNA (Luger et al., 1997). Thus, the acetylation of histone proteins by histone acetyltransferases (HATs) eliminates the positive charge and results in a weakened association between the acetylated histones and DNA (Grunstein, 1997). The conformation of the histones themselves may also be altered, and the net effect is a more relaxed chromatin structure more suitable for interactions between DNA and transcription factors, polymerases, and other regulatory molecules, thereby favouring transcription (Lee et al., 1993; Kuo et al., 1998; Martinez-Balbas et al., 1998; Lu et al., 2002). Similarly, when the histones are

deacetylated by histone deacetylases (HDACs), the positively charged lysine side chain is restored and attracts DNA (Grunstein, 1997). This locally compacts the chromatin and restricts access to the DNA, thereby downregulating local transcription (Rundlett et al., 1996; Lee et al., 1993). The opposing actions of HATs and HDACs regulate chromatin structure and are intimately linked to gene transcription.

5.3 Classification of HDACs

There are four classes of HDACs, and many HDACs are evolutionarily conserved (Yang and Seto, 2008). The class I HDACs share homology with the yeast protein Rpd3 (Taunton et al., 1996; Furukawa et al., 1996; Yang et al., 1996; Yang et al., 1997; Hu et al., 2000; Van, I et al., 2000). This group, comprised of HDAC1, 2, 3, and 8 are primarily involved in transcriptional repression of target genes in the nucleus (Grozingler and Schreiber, 2002). The class I HDACs share an N-terminal deacetylase domain, and histidine 141 of HDAC1 has been shown to be critical for deacetylase activity (Hassig et al., 1998). Since HDACs themselves do not bind to DNA, it is thought that they are recruited to promoters by other factors (Mehnert and Kelly, 2007). To this end, HDAC1 and HDAC2 are components of multiprotein complexes such as the Sin3, Mi-2-NuRD, and CoREST histone deacetylase complexes that have been shown to interact with DNA binding proteins and mediate chromatin remodelling and transcriptional repression (Heinzel et al., 1997; Johnstone, 2002; You et al., 2001; Grozingler and Schreiber, 2002; Yang and Seto, 2008). HDAC3 associates with the SMRT and N-CoR corepressor proteins (Li et al., 2000). Another mechanism for the regulation of transcription by the class I HDACs is the direct deacetylation of non-histone targets such as the

transcriptional regulator YY1 (Yao et al., 2001). Finally, it is important to note that HDACs may also repress transcription through interaction with sequence-specific transcriptional regulators (Ashburner et al., 2001; Zhang and Kone, 2002).

The class II HDACs share homology with the yeast protein Hda1, and are comprised of HDAC 4, 5, 6, 7, 9, and 10 (Yang and Seto, 2008). One well characterized function of HDAC 4, 5, 7, and 9, the so-called class IIa proteins, is the repression of MEF(myocyte enhancer factor)2-dependent transcription (McKinsey et al., 2001). Unlike the class I HDACs that are primarily localized in the nucleus, the subcellular localization of the class II HDACs is determined by their phosphorylation-dependent interaction with 14-3-3 proteins (Grozinger and Schreiber, 2000; Wang et al., 2000a). The class III HDACs are a group comprised of the sirtuin family of proteins (Sirt1-7), which impact upon a wide variety of biological processes, reviewed in (Haigis and Guarente, 2006). HDAC11 is the only member of class IV HDACs and it has homology to both Rpd3 and Hda1 (Gao et al., 2002; Gregoretta et al., 2004).

5.4 Relationship between NF- κ B and HDACs

There are several reports in the literature implicating HDAC proteins as key regulators of NF- κ B signaling. As mentioned in section 4.1, the p65 subunit of NF- κ B is subject to reversible acetylation/deacetylation, and deacetylation of p65 has been shown to inhibit its transactivation potential and DNA binding, and promote a cytoplasmic subcellular localization through the establishment of p65-I κ B α interactions (Chen et al., 2001a; Chen et al., 2002a). These effects were mediated through direct interaction with HDAC3, and

the deacetylation by HDAC3 was important for the termination of NF- κ B activity.

Another mechanism by which HDACs affect NF- κ B dependent gene transcription is through the p65-mediated recruitment of HDAC corepressor proteins to NF- κ B target genes for transcriptional repression (Ashburner et al., 2001). In this study, a non-specific HDAC inhibitor de-repressed a NF- κ B luciferase reporter construct, and increased TNF α -induced IL-8 expression. HDAC1 and HDAC2 interacted with p65, and Gal4 fusion experiments in this study demonstrated that HDAC1 specifically inhibited the transactivation potential of p65 (Ashburner et al., 2001). One interesting implication of this is that TNF α -dependent NF- κ B target gene activation involves a reduction in HDAC1 protein levels to allow for full activation of the transactivation properties of p65 (Vashisht Gopal et al., 2006). It has been reported that repression of NF- κ B target genes by p50 is thought to depend on the recruitment of HDAC1 (Zhong et al., 2002; Williams et al., 2006a). Importantly, p65 NF- κ B has been characterized as a transcriptional repressor of some genes, and the mechanism was reportedly the recruitment of HDACs (Aarenstrup et al., 2008; Zhang and Kone, 2002; Shetty et al., 2005; Campbell et al., 2004).

Thus, the role of HDACs must now be considered for future therapies that attempt to intervene in NF- κ B signaling. For example, in inflammatory disorders such as COPD, HDAC2 has been implicated in glucocorticoid receptor-mediated inhibition of inflammatory NF- κ B target gene expression (Ito et al., 2000; Ito et al., 2006; Mroz et al., 2007). Interestingly, low doses of theophylline have been shown to stimulate HDAC activity and may potentiate the anti-inflammatory effect of on-board glucocorticoids (Ito

et al., 2002; Cosio et al., 2004).

HDAC inhibitors are being tested for their efficacy against certain types of cancer (Imre et al., 2006; Carey and La Thangue, 2006). Potential mechanisms for their anti-tumour effects include upregulation of pro-apoptotic NF- κ B target genes such as DR5 (Shetty et al., 2005) as well as reduction in TNF α -mediated NF- κ B activity (Imre et al., 2006) and the downregulation of the NF- κ B dependent proliferative gene cyclin D1 (Hu and Colburn, 2005). Activation of the cell cycle arrest protein p21 (Archer et al., 1998) and even caspase activation (Medina et al., 1997) may also underlie the anti-cancer properties of HDAC inhibitors. These responses vary by cell type, and resistance of cells to the death-inducing properties of HDAC inhibitors is reportedly due to drug-induced activation of cytoprotective NF- κ B signaling (Mayo et al., 2003; Kim et al., 2006a; Kim et al., 2006b; Dai et al., 2005a). This view is not universally agreed upon, however, and more work needs to be done to fully address the impact of HDAC inhibitors on NF- κ B signaling (Imre et al., 2006). Nevertheless, it is apparent that there is a strong precedent in the literature for an intimate relationship between NF- κ B activity and HDACs.

5.5 The Role of HDACs in the Heart

Insight into the role of HDACs in the heart has been gained from the use of chemical inhibitors, and from transgenic and knockout studies. Regarding the class I HDACs, cardiac specific knockout of either HDAC1 or HDAC2 alone was not associated with a phenotype, but double knockout of both genes resulted in heart failure (Montgomery et al., 2007). Interestingly, mice completely deficient for HDAC1 were embryonic lethal

(Lagger et al., 2002; Montgomery et al., 2007), but universal knock-out of HDAC2 was not. However, there are varying reports of the subsequent phenotype of HDAC2^{-/-} mice, ranging from reduced overall size (Zimmermann et al., 2007; Trivedi et al., 2007) to cardiac deformations with and without apoptosis (Montgomery et al., 2007; Trivedi et al., 2007). Together, this suggests that some functional aspects of HDAC1 and HDAC2 can not be complemented by other class I HDACs.

Overexpression of HDAC2 induced cardiac hypertrophy, implicating it as a mediator of the hypertrophic process (Trivedi et al., 2007). Similarly, hearts overexpressing HDAC3 were also enlarged, although it was suggested that this occurred through hyperplastic growth and not hypertrophic means (Montgomery et al., 2007). Moreover, TSA and a class I specific HDAC inhibitor SK-7041, have both been shown to blunt cardiac hypertrophy in response to stimuli such as phenylephrine and angiotensin II (Antos et al., 2003; Kee et al., 2006). Thus, it has been posited that the class I HDACs are mediators of cardiac hypertrophy, perhaps via repression of anti-hypertrophic genes (Kook et al., 2003). One mechanism for this may be through association with Hop, an atypical homeodomain transcription factor, because HDAC2 activity was important for Hop-induced hypertrophy (Kook et al., 2003). Another potential mechanism is the derepression of p21, which has been shown to be anti-hypertrophic (Nozato et al., 2000). The effects of HDAC inhibitors are discussed further below.

The work of Dr. Eric Olson and others has uncovered a role for the class II HDACs for the repression of cardiac growth (Backs and Olson, 2006). HDAC5^{-/-} and HDAC9^{-/-} mice each spontaneously develop cardiac hypertrophy (Zhang et al., 2002; Chang et al., 2004).

Furthermore, HDAC4 is reportedly involved with the differentiation of cardiac stem cells into cardiac myocytes (Karamboulas et al., 2006) and HDAC7 is crucial for endothelial cell adhesion and the blood vessel function (Chang et al., 2006). Indeed, treatment of cardiac myocytes with phenylephrine induced phosphorylation-dependent cytoplasmic localization of class II HDACs, thereby derepressing the activity of the transcription factor MEF2, which has been shown to be a mediator of cardiac hypertrophy (Lu et al., 2000; Miska et al., 1999; Passier et al., 2000). Other hypertrophic mediators are also basally repressed by class II HDACs, including SRF and NFAT (Davis et al., 2003; Dai et al., 2005b; Wilkins et al., 2004).

Thus, the class II HDACs appear to antagonize hypertrophy, but this raises a question of how HDAC inhibition by TSA (a class I and II inhibitor) reportedly blocks hypertrophy (Antos et al., 2003). It was predicted, based on the class II HDACs alone, that TSA would exacerbate hypertrophy (Antos et al., 2003). Antos et al. (2003) suggested that the derepression of class I HDAC-regulated anti-hypertrophic genes was dominant over the derepression of class II HDAC-regulated pro-hypertrophic genes (Antos et al., 2003). Another possible explanation is centred around the fact that in the presence of a hypertrophic signal, the class II HDACs are compartmentalized to the cytoplasm, and that inhibition of their deacetylase activity may not further de-repress the genes under their control such as MEF2 (Kong et al., 2006). Conversely, TSA alone in the absence of a hypertrophic signal, has been shown to induce hypertrophy (Alcendor et al., 2004). This apparent discrepancy may be explained by differences in the basal regulation of hypertrophic genes compared to stimulus-induced hypertrophy. In the basal situation,

TSA-dependent inhibition of the class II HDACs may be primarily responsible for the observed hypertrophy (Alcendor et al., 2004). More work is required to fully understand the cellular effects of HDAC inhibition.

The class III HDAC Sir2 α has been characterized as anti-apoptotic in cardiac myocytes (Alcendor et al., 2004). The proposed mechanism for this effect was through deacetylation-dependent inhibition of the transcriptional potential of p53, an apoptosis-inducing cellular factor. Chemical or genetic interventions that inhibited Sir2 α resulted in apoptosis, suggesting a necessary role for Sir2 α in the regulation of basal cell survival (Alcendor et al., 2004).

Based in part on the above observations, the use of HDAC inhibitors has been suggested for the treatment of heart disease. TSA has been shown to reduce infarct size after ischemia-reperfusion (Granger et al., 2008), and inhibit ischemia- and pressure-overload-induced hypertrophy (Lee et al., 2007; Kong et al., 2006; Kee et al., 2006). Further beneficial cardiac effects of HDAC inhibitors extend to their use for pharmacological preconditioning (Zhao et al., 2007). Considering that HDAC inhibitors are undergoing clinical trials for cancer therapy (Carey and La Thangue, 2006), the effects of these drugs on the heart may need to be monitored.

In summary, the class I and II HDACs divergently regulate cardiac hypertrophy. Generally speaking, the class II HDACs repress pro-hypertrophic genes, and the class I members repress anti-hypertrophic genes (Bucks and Olson, 2006). HDAC inhibitors appear to block hypertrophy, and the resultant cellular phenotype likely depends on the balance between the activation of hypertrophic signaling and the derepression of anti-

hypertrophic genes. There appears to be a gap in the current literature regarding the effect of class I HDACs on the regulation of pro-apoptotic genes in the heart.

6.0 E2F Family

6.1 The E2F Family and the Cell Cycle

E2F was so named because of its initial characterization as a necessary transcription factor for the activation of the adenoviral E2 promoter by the adenovirus protein E1A (Kovesdi et al., 1986; Kovesdi et al., 1987; Yee et al., 1989). To date, at least eight E2F family members have been identified and designated E2F-1 to -8 (Iaquinta and Lees, 2007). With the exception of E2F-7 and E2F-8, each of the E2F proteins dimerizes with DRFT-protein-1 and -2 (DP-1 and DP-2) for the transcriptional regulation of target genes (Trimarchi and Lees, 2002).

The E2F proteins are best known for their role in the cell cycle during the G1 to S phase transition where they regulate genes required for DNA synthesis and S phase entry (Johnson et al., 1993; Xu et al., 1995; Singh et al., 1994). E2F-1, -2, and -3a are activators of transcription and are functionally regulated by interaction with the pocket protein Rb via its L-X-C-X-E domain (Knudsen and Wang, 1997; Sun et al., 2007). E2F-4 and -5 are best characterized as transcriptional repressors, and have been shown to interact with the pocket proteins p107 and p130, in addition to Rb (Hijmans et al., 1995; Beijersbergen et al., 1994; Ginsberg et al., 1994; Trimarchi and Lees, 2002). E2F-6, -7, and -8 also repress transcription, but do not interact with pocket proteins (Iaquinta and

Lees, 2007).

During G1 or G0, E2F target genes are transcriptionally repressed because their promoters are bound by E2F-4 and E2F-5 in complex with p107, p130, and Rb (Verona et al., 1997; Takahashi et al., 2000; Trimarchi and Lees, 2002). Additionally, E2F-1, -2, and -3a are associated with Rb proteins, and by this virtue are unable to activate transcription (Takahashi et al., 2000; Trimarchi and Lees, 2002). In late G1, hyperphosphorylation of Rb by cyclin D/E-Cdk complexes results in the liberation of associated E2F proteins and activation of E2F target genes (Yam et al., 2002). Because E2F-4 and E2F-5 do not have an NLS, loss of pocket protein binding results in their nuclear export (Muller et al., 1997; Verona et al., 1997; Takahashi et al., 2000; Trimarchi and Lees, 2002). Importantly, overexpression of E2F-1 alone is sufficient to induce DNA synthesis and S phase entry in quiescent, serum starved cells (Johnson et al., 1993), implicating it as a crucial factor in this process. With respect to the cell cycle and DNA synthesis, E2F target genes include CDK2, cyclin A, dihydrofolate reductase, thymidylate synthetase, PCNA, and many others (DeGregori et al., 1995; Bracken et al., 2004; Ishida et al., 2001; Ren et al., 2002).

Inhibition of E2F-dependent transcription by Rb is thought to occur through two possible mechanisms. One is that Rb blocks the transcription activation domain of E2F (Ross et al., 1999), and the second is that Rb recruits co-repressor proteins such as HDAC1, which mediates active transcriptional repression (Magnaghi-Jaulin et al., 1998; Brehm et al., 1998). Interestingly, acetylation of non-Rb-bound E2F-1 has been shown to increase its transcription potential, DNA binding, and protein half-life (Martinez-Balbas et al., 2000).

Concerning E2F DNA binding, mutation of E2F-1 in a helix-loop-helix domain at E138 dramatically inhibits its ability to bind DNA (Cress et al., 1993), and it has also been suggested that free E2F-1 may bind a different subset of genes than Rb-bound E2F-1 (Tao et al., 1997). Importantly, the association between E2F and cyclin A inhibits the ability of E2F to bind DNA, and may provide a negative feedback for E2F activity (Yam et al., 2002). Thus, the E2F family are critical mediators of cell cycle progression, and are primarily regulated by the pocket protein Rb.

6.2 The Pro-Apoptotic Properties of E2F-1

In addition to regulating cell cycle progression, E2F-1 also has apoptotic properties, which make it relatively unique in the E2F family. There are multiple lines of evidence supporting the pro-apoptotic function of E2F-1. Perhaps the strongest evidence is the observation that overexpression of E2F-1 can induce apoptosis following its previously mentioned ability to induce S phase entry (Shan and Lee, 1994; Hunt et al., 1997; Dong et al., 1999). Shan and Lee (1994) used a tetracycline-inducible E2F-1 expression system in fibroblasts and found that E2F-1 induced apoptosis in a dose-dependent manner. Furthermore, they found that overexpression of E2F-1 deletion mutants that were defective for DNA binding did not affect cell cycle progression or apoptosis. These results suggest that the observed apoptosis was not due to non-specific cytotoxic effects of overexpression, but instead depended on the activity of E2F-1 (Shan and Lee, 1994).

In the converse experiment, the absence of E2F-1 in E2F-1^{-/-} mice resulted in tumorigenesis and defects in apoptosis, further suggesting a basal anti-proliferative/tumor

suppressor function for E2F-1 (Yamasaki et al., 1996; Field et al., 1996; Cloud et al., 2002). Mice deficient for Rb demonstrated tumorigenesis in some tissues, and increased apoptosis in others, both of which could be partially rescued by deletion of E2F-1 (Lee et al., 1992; Jacks et al., 1992; Tsai et al., 1998; Yamasaki et al., 1998). Finally, E2F-1 has been shown to transcriptionally activate known pro-apoptotic genes including Apaf-1 (Furukawa et al., 2002; Moroni et al., 2001), BH3-only Bcl-2 family members (Hao et al., 2007), caspases (Nahle et al., 2002), and Arf (Aslanian et al., 2004), which inhibits the degradation of the apoptotic protein p53 (Pomerantz et al., 1998). Indeed, some studies have linked the apoptotic activity of E2F-1 with p53 (Wu and Levine, 1994; Qin et al., 1994), although E2F-1 also reportedly induces apoptosis in an p53-independent manner (Nip et al., 1997; Hunt et al., 1997; Holmberg et al., 1998). Notably, a caspase cleavage site has been found in Rb, suggesting that another consequence of caspase activation may be the activation of pro-apoptotic E2F-1 target genes (Fattman et al., 1997; Chau et al., 2002; Borges et al., 2005).

Thus, there appear to be two faces to E2F-1, one which promotes cellular proliferation, and another that promotes cellular demise (Phillips et al., 1999). The fact that E2F-1 overexpression has been shown to induce S phase entry followed by apoptosis highlights the importance of cellular factors such as Rb or cyclin A, which may blunt or inhibit excessive E2F-1 activity. The mechanism by which the cell determines whether E2F-1 activates proliferative versus apoptotic genes is still being addressed. However, it may have to do with the slight variability in different E2F binding sites (Tao et al., 1997), or with the recruitment of E2F-1-interacting proteins such as Jab1 or even Rb itself to

impart selective activation to a subset of E2F-1 target genes (Hallstrom and Nevins, 2006; Dick and Dyson, 2003; Black et al., 2005).

6.3 The role of E2F-1 in the Heart

Adult cardiomyocytes exist predominantly in G0 (Pasumarthi and Field, 2002), and even a slight increase in the rate of apoptosis in the heart may have deleterious effects on cardiac function (Wencker et al., 2003). Thus, the ability to induce cell cycle entry in cardiac myocytes may be one approach underlying future regenerative therapy. Because E2F-1 overexpression was previously shown to induce S phase entry, it was hypothesized that this may induce cardiomyocyte mitosis and form a basis for cardiac regeneration (Agah et al., 1997). It was shown that adenovirus-mediated overexpression of E2F-1 or the adenoviral protein E1A, which liberates E2F-1 from Rb, induced G1 exit of cardiac myocytes, as measured by DNA synthesis and induction of E2F-1-dependent target genes (Kirshenbaum and Schneider, 1995; Kirshenbaum et al., 1996; Agah et al., 1997).

Unfortunately, like other cell types, E2F-1 overexpression in cardiac myocytes ultimately led to their apoptotic death (Kirshenbaum et al., 1996; Agah et al., 1997). E2F-1-induced apoptosis of cardiac myocytes was independent of p53, and could be inhibited by the adenoviral protein E1B (Kirshenbaum et al., 1996; Agah et al., 1997). However, the underlying mechanisms of E2F-1-dependent cell death in cardiomyocytes are still not completely understood. Intriguingly, E2F was indirectly implicated in hypoxia-induced apoptosis of cardiomyocytes because Rb expression rescued hypoxic cells from death (Hauck et al., 2002).

As an exciting side note, co-expression of the anti-apoptotic proteins Bcl-2 or E1B with E2F-1 did not result in increased cell number, suggesting that other innate mechanisms in cardiomyocytes resist progression past G2 (Kirshenbaum et al., 1996; Agah et al., 1997). More recent work has shown that the overexpression of both E1A and Cdc5, a protein involved in mitotic entry, was sufficient to induce cardiomyocyte mitosis and increased cell number (Williams et al., 2006b). Furthermore, overexpression of cyclin A2 has also been shown to induce cardiomyocyte mitosis (Chaudhry et al., 2004). In place of E2F-1, one group has concluded that E2F-2 expression can induce cardiomyocyte mitosis, without inducing any apoptosis (Ebelt et al., 2005; Ebelt et al., 2008). Surprisingly, this group also observed cardiac myocyte mitosis with expression of E2F-1 alone (Ebelt et al., 2005), and an explanation for why E2F-1-induced mitosis was seen here, but not in other heart studies (Williams et al., 2006b; Agah et al., 1997; Kirshenbaum et al., 1996) is not available at present, but may depend on methodology. Finally, work over the last number of years has revealed the existence of mitotic events in the heart after an MI (Beltrami et al., 2001) and discovered cardiac stem cells, which may be capable of cardiac regeneration (Bearzi et al., 2007). This body of work challenges the dogma that all adult myocytes are terminally differentiated. Future work may develop regenerative therapies based on these results.

Briefly, in the context of cardiac hypertrophy, stimulation of cardiac myocytes with angiotensin II was inferred to induce E2F activity (Sadoshima et al., 1997).

Concordantly, inhibition of E2F activity prevented phenylephrine- and serum-induced hypertrophy, although these effects may have been dependent on the block of E2F-3a and

E2F-4 activities, in addition to that of E2F-1 (Vara et al., 2003). These studies suggest that E2F activity may be involved in hypertrophy. Basal E2F activity may be critical for normal cardiac function because it was recently demonstrated that cardiac-specific knockout of E2F-3 resulted in congestive heart failure and death (Cloud et al., 2002). Thus, future therapies involving modulation of E2F activity should proceed with caution.

7.0 Bcl-2 Family

7.1 Domains of Bcl-2 Family Members

Bcl-2 is the mammalian homolog of the *C. elegans* protein Ced-9 (Hengartner and Horvitz, 1994), and was initially identified as an oncogene in leukemic B-cells (Tsujiimoto et al., 1984). While Bcl-2 itself is anti-apoptotic, the family contains both pro- and anti-apoptotic members that share homology at Bcl-2 homology (BH) domains, of which there are four (BH1-4). The BH1, BH2, and BH3 domains are involved in protein-protein interactions between family members (Yin et al., 1994; Petros et al., 2004), and the BH3 domain is also important for the cytotoxicity of the pro-death proteins (Letai et al., 2002; Narita et al., 1998). The BH4 domain has been reported to play a role in the cytoprotective effects of anti-apoptotic members, and is notably absent from the pro-apoptotic members (Huang et al., 1998; Shimizu et al., 2000; De Moissac et al., 1999). Anti-apoptotic members include Bcl-2, Bcl-X_L, Bcl-w, and Mcl-1, and pro-apoptotic members include the multidomain proteins Bax and Bak, as well as the BH3-only proteins Bid, Bim, Bnip3, Bad, Puma, Noxa, and Bik, reviewed in (Youle and Strasser, 2008).

7.2 Bcl-2 Family Members and the Regulation of Apoptosis

The Bcl-2 family of proteins regulate cell survival and cell death. An early simple model suggested that the balance of pro- vs. anti-apoptotic Bcl-2 proteins, measured by a Bcl-2/Bax ratio, would decide its fate (Korsmeyer et al., 1993; Oltvai et al., 1993; Yang et al., 1995). More recent evidence has prompted an expansion of this model to allow for a more complex dynamic between BH3-only proteins, Bax and Bak, anti-apoptotic members, and the details surrounding mitochondrial outer membrane permeabilization (MOMP) (Chipuk and Green, 2008).

Apoptosis induced by the pro-apoptotic Bcl-2 members depends on MOMP, an important event in the initiation of apoptosis via the mitochondria. MOMP results in the liberation of normally sequestered proteins from the intermembrane space such as cytochrome c, Smac/DIABLO, AIF, and others, which continues the apoptotic program as discussed in section 2.2.3 (van Gurp et al., 2003). The major effectors of MOMP are the “effector” pro-apoptotic Bcl-2 proteins Bax and Bak, but the mechanism of action is somewhat controversial (Chipuk and Green, 2008).

MOMP may be induced via the PTP or by the formation of Bax or Bak channels.

Alternatively, it is possible that both have a role in a model describing MOMP. For example, Bax has been shown to induce PTP opening, perhaps via interactions with ANT and/or VDAC (Vieira et al., 2000; Shimizu et al., 1999; Narita et al., 1998; Marzo et al., 1998a). PTP opening depolarizes the inner mitochondrial membrane, induces swelling of the mitochondrial matrix, and leads to MOMP via physical rupture of the outer membrane (Vieira et al., 2000). Notably, cytochrome c and other apoptotic proteins

cannot pass through the PTP itself due to size limitations; however, Shimizu et al reported that Bax induced the transfer of cytochrome c through a VDAC channel (Shimizu et al., 1999). Thus, Bax-induced permeability transition is one possible mechanism for MOMP.

Another model for MOMP is the formation of outer mitochondrial membrane channels by Bax oligomers, independent of the PTP, following Bax translocation and membrane integration via its carboxy-terminal transmembrane domain (Antonsson et al., 2000; Eskes et al., 1998; Jurgensmeier et al., 1998; Antonsson et al., 1997; Goping et al., 1998; Gross et al., 1998). Bak functions similarly to Bax in this regard, but is already found at the mitochondria (Wei et al., 2000; Korsmeyer et al., 2000; Wei et al., 2001). These channels are reportedly large enough to accommodate the release of apoptotic proteins from the mitochondria (Kuwana et al., 2002; Korsmeyer et al., 2000) although this is not universally agreed upon (Roucou et al., 2002).

In addition to understanding how MOMP arises, it is important to understand the dynamic interplay between Bcl-2 family members. According to one model, the activation and oligomerization of Bax and Bak is induced by the “direct activator” BH3-only proteins Bim and t-Bid (Kuwana et al., 2005; Letai et al., 2002), which is important because Bax and Bak have been shown to be dormant without activation (Roucou and Martinou, 2001). In this model, the primary role of the anti-apoptotic members is to antagonize the “direct activator” BH3-only proteins, since apoptosis rests on the activation of Bax and Bak by these molecules. In contrast to Bid and Bim, the other BH3-only proteins are classified as “de-repressors”, meaning that instead of directly

activating Bax or Bak, they are thought to sequester the anti-apoptotic proteins that otherwise inhibit the direct activators Bid and Bim (Kuwana et al., 2005; Letai et al., 2002). This de-repressor group includes Bad, Bik, Bnip3, Puma, and Noxa (Chipuk and Green, 2008).

A slightly different model states that instead of the anti-apoptotic members antagonizing the direct activator BH3-only proteins, they inhibit Bax and Bak activation directly, as supported by the direct interaction between Bax and Bcl-2 (Oltvai et al., 1993). The role for the BH3-only proteins in this model is to sequester the anti-apoptotic proteins to derepress constitutive Bax and Bak activity (Chipuk and Green, 2008). The major difference between the models is that in the first case the anti-apoptotic proteins target the BH3-only proteins Bid and Bim, and Bax/Bak are assumed neutral without activation. In the second model the anti-apoptotic proteins target Bax and Bak, which are assumed constitutively active. It is likely that the truth is a blend of both models, perhaps depending on cell type and other context-dependent factors. Thus, the big picture has been worked out with respect to how Bcl-2 members regulate apoptosis, but the details concerning functional interactions between family members are not yet fully elucidated.

Regarding activation of the BH3-only proteins, each one may be induced by different toxic stimuli. For example, Bnip3 transcription is induced by hypoxia (Bruick, 2000; Guo et al., 2001; Regula et al., 2002b), Bad is dephosphorylated and activated by withdrawal of growth factors (Zha et al., 1996; Hashimoto et al., 2005; Datta et al., 1997), and Bid cleavage and mitochondrial translocation is induced by TNF α -family ligand binding and caspase-8 activity as previously discussed (Luo et al., 1998; Gross et

al., 1999). Thus, the activation of the BH3-only proteins serves to connect these death signals to the mitochondrial apoptotic pathway.

7.3 Bcl-2 Family Members in the Heart

In the heart, the Bcl-2 family members are strongly linked to the regulation of apoptosis. In human hearts post-MI, elevated Bax protein levels and TUNEL positive myocytes were observed in similar locations in the infarct region (Baldi et al., 2002). Moreover, Bax/Bcl-2 ratios were increased in certain areas of the heart during dilated cardiomyopathy compared to controls (Di Napoli et al., 2003). Increased expression of Bax, Bak, Bcl-2, and Bcl-X_L was observed in patient hearts with dilated cardiomyopathy and ischemic heart disease (Latif et al., 2000; Olivetti et al., 1997). In a rat model of pressure overload, Bax protein was elevated while Bcl-2 protein was decreased compared to sham-operated controls (Condorelli et al., 1999). Furthermore, stretch, hypoxia, and pacing have also been shown to upregulate Bax and downregulate Bcl-2 (Leri et al., 1998a; Jung et al., 2001; Leri et al., 1998b). These descriptive studies underscore the notion that the Bcl-2 proteins are critical regulators of apoptosis in the heart.

Interventional studies have confirmed that changes in Bcl-2 proteins can affect cardiac endpoints after ischemia/reperfusion in the heart. Isolated hearts from Bax^{-/-} mice were subjected to ischemia followed by reperfusion and had improved cardiac function, smaller infarcts, and fewer TUNEL positive cells compared to wild type controls (Hochhauser et al., 2003). These results were subsequently corroborated by the same group using LAD ligations *in vivo* (Hochhauser et al., 2007). In a different line of

experimentation, overexpression of Bcl-2 has been characterized as cardioprotective and antagonized cardiac injury following ischemia/reperfusion, both *in vivo* and in isolated hearts (Brocheriou et al., 2000; Imahashi et al., 2004; Chen et al., 2001c). A similar result was obtained with the overexpression of Bcl-X_L (Huang et al., 2003). Interestingly, Bcl-2 was shown to be upregulated during ischemic preconditioning as a direct result of the induced NF- κ B activity (Maulik et al., 1999). Notably, the relationship between NF- κ B and Bcl-2 is one of mutual activation because Bcl-2 has also been shown to promote NF- κ B activation through MAP-kinase-mediated induction of IKK β (Regula et al., 2002a).

Regarding the BH3-only proteins, Bid cleavage and Bad dephosphorylation have been observed following ischemia/reperfusion (Chen et al., 2001b; Murriel et al., 2004). Moreover, Puma was upregulated during ischemia/reperfusion in isolated cardiomyocytes, and Puma-deficient hearts had smaller infarcts and improved cardiac function compared to controls (Toth et al., 2006). The role of Bnip3 and its homolog Nix/Bnip3L will be discussed in section 8.

Taken together, these preliminary results suggest that the Bcl-2 proteins are attractive targets for interventional therapies in the treatment of heart disease in humans. Either augmentation of the anti-apoptotic proteins or inhibition of the pro-apoptotic members may be effective for preserving cardiomyocyte number post MI or during HF in humans. One method for modulating Bcl-2 family activity in the context of cancer therapy is via small molecules that either mimic the presence of a BH3-only protein (Walensky et al., 2004; Oh et al., 2006), or inhibit the actions of Bcl-2 or Bcl-X_L (Oltersdorf et al., 2005;

Wang et al., 2000c; Petros et al., 2006; Becattini et al., 2004). The specific targeting of cardiac myocytes as opposed to other cardiac cell types may be an important issue in the future.

8.0 Bnip3

8.1 Characterization of Bnip3

Bnip3 is best characterized as a pro-death protein; however, the process by which Bnip3 induces cell death is controversial in the literature (see section 8.2). Bnip3 was initially characterized as a binding partner with the anti-apoptotic protein Bcl-2 and with E1B-19K, which is an anti-apoptotic adenoviral protein (Boyd et al., 1994). The selection of Bnip3 by adenoviral evolution, along with other death-inducing proteins such as Bax, Bak, and p53 (Yew and Berk, 1992; Han et al., 1996; Farrow et al., 1995; Chen et al., 1996a) suggests that it is an important cellular mediator of cell death (Boyd et al., 1994; Chen et al., 1997; Yasuda et al., 1998b). Bnip3 was cloned by the Kirshenbaum laboratory, and they were the first to identify Bnip3 as an interacting partner with Bcl-2 in the human heart (Regula et al., 2002b)

Bnip3 is a 194 amino acid protein with a predicted molecular weight of 21.54 kDa (Chen et al., 1997). During SDS-PAGE, Bnip3 migrates as a 30 kDa band and, due to strong homodimerization, an additional 60 kDa band (Chen et al., 1997). Recently, it was revealed that Bnip3 is a phosphoprotein, which likely accounts for why Bnip3 runs slightly higher than predicted on a gel (Graham et al., 2007). Bnip3 contains a PEST domain for proteasomal degradation (Rogers et al., 1986), a BH3-like domain, a

conserved domain between the human protein and the *C. elegans* homolog ceBnip3, and a transmembrane domain (TM), as shown in figure 4 (Chen et al., 1997; Cizeau et al., 2000; Yasuda et al., 1998b; Yasuda et al., 1998a). The amino-terminus and TM regions of Bnip3 are important for interaction with Bcl-2 (Ray et al., 2000). Unlike other BH3-only proteins, the BH3 domain is not required for the pro-death activity of Bnip3, but instead, deletion of the carboxy-terminal TM domain (Δ TM) prevents Bnip3 from targeting the mitochondria and inducing cell death, and also converts the wild type protein into a dominant-negative inhibitor of Bnip3-induced cell death (Chen et al., 1997; Ray et al., 2000; Regula et al., 2002b). The TM domain is also required for homodimerization, but this is not necessary for Bnip3 to kill cells (Ray et al., 2000; Regula et al., 2002b).

Bnip3 is transcribed in select tissues including the heart and liver; however, the protein level remains very low in most tissues including the heart (Galvez et al., 2006; Regula et al., 2002b; Chen et al., 1997; Vande et al., 2000; Yasuda et al., 1999). Within the cell the protein localizes predominantly to the mitochondria (Chen et al., 1997), but it has also been observed in the nucleus (Schmidt-Kastner et al., 2004; Burton et al., 2006). Bnip3 can integrate into the mitochondrial outer membrane via its TM domain to induce the opening of the PTP and the subsequent loss of inner mitochondrial membrane potential ($\Delta\psi_m$) (Vande et al., 2000; Regula et al., 2002b). Although this link with the mitochondria is well accepted, the mechanisms of Bnip3-induced cell death have not been fully elucidated, and are the subject of current research.

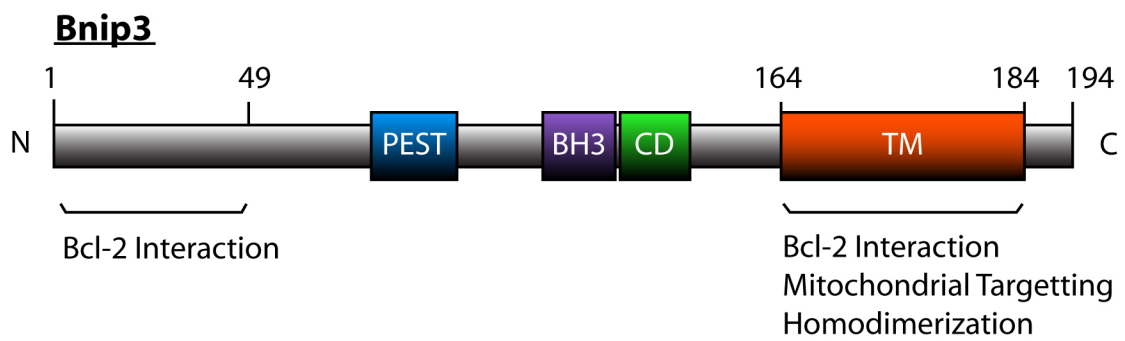


Figure 4. Schematic diagram of the Bnip3 protein. Shown are the PEST domain (blue), BH3-like domain (purple), conserved domain (CD, green), and transmembrane domain (TM, red).

8.2 Bnip3-Induced Cell Death

Evidence exists to support contentions that Bnip3 induces apoptosis, autophagy, and an atypical necrosis-like type of death. It has even been suggested that Bnip3-induced autophagy may be cytoprotective in certain cases.

One hallmark of apoptotic cell death is nuclear condensation and DNA fragmentation, both of which have been observed in cells overexpressing Bnip3 (Regula et al., 2002b; Chen et al., 1997; Ray et al., 2000; Vande et al., 2000). Dr. Kirshenbaum's laboratory was the first to demonstrate that Bnip3-induced cell death was caspase dependent in the heart (Regula et al., 2002b). For this, they incubated Bnip3-expressing cells with the pan caspase inhibitor z-VAD-fmk, and observed a dose-dependent rescue of Bnip3-induced cell death compared with vehicle-treated cells (Regula et al., 2002b). Because caspase activation is such an integral component of the apoptotic program, this evidence strongly supported that Bnip3 induced an apoptotic form of cell death. It was also shown by Regula et al (Regula et al., 2002b) that hypoxia triggered the integration of Bnip3 into the mitochondrial membrane, whereas under normoxia conditions, Bnip3 was only loosely associated with the mitochondria. This was consistent with a similar observation concerning hypoxia-induced Bax mitochondrial membrane integration (Goping et al., 1998), and suggested that Bnip3 was a mediator of hypoxia-induced mitochondrial perturbations, including PTP opening (Regula et al., 2002b). It was recently shown that Bax/Bak knockout cells were more resistant to the pro-death actions of Bnip3, implying that Bnip3 kills at least partially through a mechanism involving Bax and Bak, which further implies an apoptotic death mechanism (Kubli et al., 2007). Consistent with the

notion that Bnip3 induced apoptosis was the finding that Bnip3-transfected cells had cytoplasmic cytochrome c, implying that Bnip3 expression alone was sufficient to induce not only mitochondrial perturbations but also the initiation of the intrinsic apoptotic program (Regula et al., 2002b; Kubli et al., 2007). Taken together, Bnip3-dependent mitochondrial integration and perturbations, release of cytochrome c into the cytoplasm, caspase activation, and nuclear condensation all support the notion that Bnip3 induces apoptosis.

In contrast, other investigators have not observed cytochrome c release following Bnip3 expression, nor have they observed that a caspase inhibitor had any effect on the death process induced by Bnip3 (Vande et al., 2000; Guo et al., 2001). Additionally, no phosphatidyl serine was observed on the surface of cells overexpressing Bnip3 (Vande et al., 2000), which often occurs during apoptosis as a signal for phagocytosis (Wolf et al., 2007). Guo et al (Guo et al., 2001) and Vande Velde et al (Vande et al., 2000) concluded that Bnip3 induced a caspase-independent apoptosis, while Vande Velde et al further observed the loss of plasma membrane integrity prior to chromatin condensation, and termed this mode of death “necrosis-like” (Vande et al., 2000). Using an electron microscope, inspection of cells overexpressing Bnip3 revealed the presence of cytoplasmic vacuoles, consistent with necrosis, and autophagosomes, consistent with autophagy. Together these results suggest that Bnip3-induced death is generated by a caspase-independent mechanism similar to necrosis, and that Bnip3-induced chromatin condensation did not occur by normal apoptotic means in these studies (Vande et al., 2000).

In addition to necrosis and apoptosis, there is also evidence that Bnip3 induces autophagy. Indeed, Bnip3 overexpression in HL-1 cardiac myocytes and other cell types has resulted in upregulated autophagy (Hamacher-Brady et al., 2007; Tracy et al., 2007). Bnip3 was further implicated as an inducer of autophagy because ischemia-reperfusion-induced autophagy was inhibited by the Δ TM mutation of Bnip3 (Hamacher-Brady et al., 2007). Interestingly, this study proposed that Bnip3-induced autophagy may be a cytoprotective response because inhibition of autophagy by a dominant-negative mutant of Atg5 increased Bnip3-induced cell death, and reduced Bnip3-induced autophagy (Hamacher-Brady et al., 2007). Once again, the interaction between the mitochondria and Bnip3 is important because permeability transition has been shown to induce autophagy (Elmore et al., 2001). Thus, Bnip3-induced PTP opening may induce autophagy, which may subsequently remove damaged mitochondria and delay the onset of Bnip3-induced cell death. In this sense, Bnip3 may initially protect the cell from itself, but with time the death-promoting actions of Bnip3 prevail. However, whether Bnip3 actively induces autophagy, or if autophagy occurs in response to Bnip3-induced PTP opening is still being investigated. Thus, more research is required to fully understand the role of autophagy in the heart, and how this is impacted by Bnip3. This is the subject of ongoing studies.

It is apparent that the mode of cell death induced by Bnip3 is still not fully understood. The discrepancies about cytochrome c release and caspase independence may be cell type specific, and possibly an artifact of the transformed cell lines used in some experiments. Cytochrome c release and caspase dependence were observed in primary cultures of

postnatal cardiac myocytes (Regula et al., 2002b). Alternatively, it may be that Bnip3-induced cell death is genuinely “atypical” in a general sense, but depending on circumstances such as timing and cell type it may resemble apoptosis, autophagy, and/or necrosis. Interestingly, Bnip3 has been shown to inhibit Rheb, an upstream activator of mTOR, which is a positive regulator of cell growth. Thus, in addition to actively inducing death, Bnip3 may also be responsible for the inhibition of the growth promoting signals of mTOR in the TORC1 complex during hypoxia (Li et al., 2007).

Interestingly, the mechanism by which the Δ TM mutation of Bnip3 acts as a dominant negative protein is not fully understood. The mechanism must involve a way to neutralize or inhibit the endogenous wild type Bnip3. It has been shown that the TM domain is critical for mitochondrial targeting, Bnip3 homodimerization, and cell death (Chen et al., 1997; Ray et al., 2000; Regula et al., 2002b), so it follows that without the ability to dimerize with the wild type protein, it is unlikely that Bnip3 Δ TM competitively inhibits endogenous Bnip3 homodimerization. Further to this point, it is known that the Bnip3 monomer can kill cells without dimerization because point mutations of Bnip3 within the TM domain prevented homodimerization, yet they did not affect the death-inducing potential of the protein (Ray et al., 2000). Ray et al (Ray et al., 2000) determined that the death-inducing domains of Bnip3 resided outside the TM domain, and that the major role of the TM domain was simply to mediate integration into the mitochondrial membrane. Notably, Regula et al. (2002b) showed for the first time that Bnip3 Δ TM could inhibit hypoxia-induced mitochondrial perturbations and cell death of ventricular myocytes (Regula et al., 2002b). The molecular mechanism underlying the

dominant-negative properties of the Δ TM mutation are not yet fully understood.

8.3 Regulation of *Bnip3*

Among all of the BH3-only death factors, Bnip3 is relatively unique for at least two reasons. The first is that Bnip3 expression is strongly induced by hypoxia, whereas others such as Bad, Bak, and Bax are not (Regula et al., 2002b; Bruick, 2000). This induction is thought to be mediated by the transcription factor HIF-1 α , and analysis of the Bnip3 promoter has revealed a hypoxia response element (Bruick, 2000). Bnip Δ TM has been shown to attenuate hypoxia-induced cell death of ventricular myocytes, highlighting Bnip3 as a central regulator of cell death by hypoxia (Regula et al., 2002b).

The second reason Bnip3 is unique is because of the presence of both an NF- κ B and an E2F response element within the promoter, which regulate Bnip3 transcription (Baetz et al., 2005; Tracy et al., 2007). Using postnatal ventricular myocytes, Baetz et al (Baetz et al., 2005) revealed that Bnip3 transcription was basally repressed by p65 NF- κ B, and that loss of Bnip3 protein attenuated cell death induced by the inhibition of NF- κ B signalling. During hypoxia, the reduction in p65 protein abundance contributed to hypoxia-induced Bnip3 expression (Baetz et al., 2005). It has also been shown that Rb/E2F complexes may regulate Bnip3 transcription (Tracy et al., 2007). Under basal conditions the repressive E2F-4 was associated with the Bnip3 promoter, but it was replaced by E2F-1 during hypoxia, promoting transcriptional activation (Tracy et al., 2007). Overexpression of E2F-1 induced Bnip3 transcription, but it surprisingly repressed Bnip3 transcription induced by simulated hypoxia, and this was explained by the E2F-1-mediated recruitment

of Rb (Tracy et al., 2007). However, Rb may be cleaved during genuine, physiological hypoxia, and loss of Rb is associated with elevated Bnip3 expression. Nonetheless, this implicated Rb as a transcriptional repressor of Bnip3, via E2F-1 (Tracy et al., 2007).

Bnip3 transcription is also regulated by a number of other upstream signals. Arsenic trioxide (As_2O_3) has been shown to upregulate Bnip3, and As_2O_3 -induced autophagy was inhibited by Bnip3 Δ TM (Kanzawa et al., 2005). Additionally, cyanide has been shown to increase Bnip3 transcription via HIF-1 α , and Bnip3 siRNA or Bnip3 Δ TM inhibited cyanide-induced mitochondrial perturbations and apoptosis (Zhang et al., 2007b). Nitric oxide-induced apoptosis, in response to bacterial toxins such as LPS and subsequent iNOS induction, was found to be Bnip3-dependent in macrophages via transcriptional upregulation of Bnip3 (Yook et al., 2004). Bnip3 transcription is induced by dexamethasone in cultured neurons, and this may be mediated via glucocorticoid-dependent inhibition of NF- κ B (Sandau and Handa, 2007; Ramdas and Harmon, 1998). Amyloid beta (A β) also induced Bnip3 expression in neurons, perhaps via HIF-1 α activation, and Bnip3 shRNA attenuated A β -induced cell death (Zhang et al., 2007c). Finally, the Bnip3 promoter is also inhibited by DNA methylation at CpG islands, which has been observed in several cancer cell lines, and the methyltransferase inhibitor 5-aza-2' deoxycytidine derepressed Bnip3 and sensitized these cells to hypoxia-induced cell death (Abe et al., 2005; Murai et al., 2005a; Murai et al., 2005b; Okami et al., 2004).

In fact, it is thought that downregulation of Bnip3 expression may contribute to a more aggressive tumor phenotype (Okami et al., 2004; de Angelis et al., 2004). Cells at the centre of hypoxic tumors adapt and become more aggressive, and this may be mediated

in part by selection for cells with silenced Bnip3 expression (Mellor and Harris, 2007; Heller et al., 2008). Indeed, Bnip3 was observed to be downregulated in pancreatic cancer, and this correlated with reduced patient survival (Erkan et al., 2005). Thus, any interventions that derepress Bnip3 may sensitize certain tumors to hypoxia and chemotherapeutics. Concordantly, knockdown of Bnip3 has been shown to increase resistance to the chemotherapeutics gemcitabine or 5-fluoro-uracil in pancreatic cancer cell lines (Akada et al., 2005; Erkan et al., 2005). However, Bnip3 expression can not be used as a predictor of chemosensitivity (Ishida et al., 2007). The inhibition of Bnip3 in pancreatic, colorectal, and hematopoietic cancer cells has been shown to contribute to tumour pathology (Mellor and Harris, 2007). It is important to note that in direct contrast to the above data, tumors in the breast, lung, and brain, have been characterized as having elevated Bnip3 expression (Mellor and Harris, 2007). Paradoxically, high Bnip3 expression correlated with poor prognosis for non-small cell lung cancer (Giatromanolaki et al., 2004). Nuclear localization of Bnip3 in these cells may explain how the expression of Bnip3 did not result in tumor cell death (Giatromanolaki et al., 2004).

Because Bnip3 is a potent pro-death protein, it follows that it is not only regulated transcriptionally, but also that the protein is subject to additional measures of control. Bcl-2 and Bcl-X_L interact with Bnip3 and inhibit its pro-death activity (Yasuda et al., 1998b; Chen et al., 1997; Ray et al., 2000), and this likely contributes to basal survival of cells. The growth factors EGF and IGF inhibited Bnip3-induced cell death, which interestingly required the BH3 domain of Bnip3 (Kothari et al., 2003). Kubasiak et al (Kubasiak et al., 2002) observed that acidosis during hypoxia was required for Bnip3 to

open the mitochondrial PTP and induce cell death, implying either that acidosis stabilized Bnip3 protein or that it provided some kind of activation signal for Bnip3. At neutral pH, despite the presence of hypoxia, Bnip3 did not induce cell death in cardiac myocytes (Kubasiak et al., 2002). It was later shown by the same group that acidosis extended the protein half-life of Bnip3 (Frazier et al., 2006). In contrast to this, Bnip3 transfection alone, without acidosis, has been shown to include dramatic apoptosis in the same cell type (Regula et al., 2002b). Despite this debate, the recent observation that Bnip3 can be reversibly phosphorylated is exciting, and may reveal another regulatory point for Bnip3 in the future (Graham et al., 2007).

Taken together, it is readily apparent that Bnip3 is highly regulated and under tight transcriptional control, which follows from the notion that deregulated Bnip3 expression would otherwise be lethal. The discrepancies between Bnip3 expression and cell death in tumour tissues, and between characteristics of Bnip3-induced cell death may be explained by unidentified cell-type specific factors that regulate the activity of Bnip3.

8.4 Role of Bnip3 in the Heart

In this section, the effects of Bnip3 on cell death in the heart will be discussed in the context of hypoxia and ischemia followed by reperfusion (I/R). Bnip3 and cardiomyocyte autophagy will not be discussed in this section, because that was mentioned in section 8.2, paragraph 4.

The main ideas presented in sections 8.1 to 8.3 above apply in the heart, and indeed, some studies already mentioned were performed in cardiac cells. To summarize, in the

heart, Bnip3 is upregulated by hypoxia and Bnip3-induced death involves the mitochondrial PTP and is caspase-dependent (Regula et al., 2002b; Hamacher-Brady et al., 2007), although some evidence indicates that it may be caspase-independent (Kubasiak et al., 2002). Additionally, there is debate surrounding Bnip3-induced cytochrome c release in the heart, and evidence for (Hamacher-Brady et al., 2007; Regula et al., 2002b) and against this as well (Kubasiak et al., 2002). In the heart, Bax activation may at least partially underlie Bnip3-induced cell death because Bnip3 activates Bax, and functional inactivation of Bnip3 resulted in the inhibition of simulated I/R-induced Bax translocation and mitochondrial perturbations (Kubli et al., 2007). Notably, Bnip3 has been shown to be transcriptionally repressed in the heart by NF- κ B, and IKK β -mediated NF- κ B activation is sufficient to attenuate hypoxia-induced cell death (Baetz et al., 2005).

Isolated hearts subjected to I/R and transduced with the Bnip3 Δ TM mutation demonstrated reductions in infarct size, ROS production, cytochrome c release, caspase-3 cleavage, and apoptosis (Hamacher-Brady et al., 2007). This implies that Bnip3 otherwise promotes these effects during I/R. Similar results were observed in this study using HL-1 myocytes, including the inhibition of Bnip3-induced death by a caspase inhibitor (Hamacher-Brady et al., 2007). As previously mentioned, this mutation has also been shown to inhibit hypoxia-induced apoptosis of ventricular myocytes (Regula et al., 2002b). Because functional inhibition of Bnip3 reduced the cytotoxic effects of hypoxia and I/R, these results suggest that Bnip3 is an important mediator of hypoxia- and I/R-induced apoptosis in the heart.

It has been shown that Bnip3 is upregulated in heart failure (Regula et al., 2002b), and recently, Diwan et al (Diwan et al., 2007) verified that Bnip3 was a critical mediator of pathological cardiac apoptosis using transgenic mice subjected to one hour of LAD ligation followed by reperfusion. Initially, Bnip3^{-/-} mice had similarly sized infarcts compared to wild type mice but less apoptosis in both the infarct border zone and the remote myocardium. After 3 weeks the Bnip3^{-/-} mice demonstrated a significant reduction in ventricular remodelling compared to controls. These results highlight the importance of Bnip3 as a mediator of apoptosis and ventricular remodelling post-MI. In the reciprocal experiment, cardiac-specific overexpression of Bnip3 caused mice to have larger infarcts and more apoptotic cells compared to controls. Notably, in the absence of any other stimuli, mice expressing Bnip3 from birth exhibited an increase in the basal level of apoptosis as well as poorer contractility compared to wild types (Diwan et al., 2007).

The closest homolog to Bnip3 is a protein called Nix/Bnip3L. While both proteins induce apoptosis in the heart via the intrinsic mitochondrial pathway (Yussman et al., 2002; Regula et al., 2002b), they are not regulated in the same manner (Galvez et al., 2006). Nix is upregulated by hypertrophic signals through activation of Gαq, which may be elevated during pressure-overload, whereas Bnip3 is upregulated by hypoxia (Galvez et al., 2006). Nix was not induced by hypoxia, and Bnip3 was not induced by hypertrophic stimuli (Galvez et al., 2006). Transcriptionally, Bnip3 is subject to positive regulation by HIF-1α, E2F-1, and inhibition by p65 NF-κB (Bruick, 2000; Tracy et al., 2007; Baetz et al., 2005). With respect to Nix, there are reports that it is transcriptionally

activated by EGR2 and FoxO3a (Unoki and Nakamura, 2003; Real et al., 2005).

Overexpression of Nix in the heart resulted in cardiac defects including impaired contractility and elevated apoptosis, and was associated with death one to two weeks after birth (Yussman et al., 2002). Interestingly, an alternative splice of Nix, sNix, which is truncated at the C-terminus did not cause any cardiac defects, and delayed Gαq-induced death (Yussman et al., 2002). This mutation is analogous to the ΔTM mutation of Bnip3, except that sNix interacts with Nix, and it is believed that the mechanism for sNix-mediated antagonism of Nix-induced apoptosis is through heterodimerization (Yussman et al., 2002).

Bnip3 is an important mediator of hypoxia- and I/R- induced cell death in the heart.

Bnip3 may be involved in several types of cell death, all of which contribute to poorer cardiac function following an ischemic or hypoxic insult. Thus, it is a strong candidate gene that may be therapeutically inhibited following an MI. However, it is important to understand exactly how Bnip3 is endogenously regulated.

IV. MANUSCRIPTS

1.0 TRANSCRIPTIONAL SILENCING OF THE DEATH GENE BNIP3 BY COOPERATIVE ACTION OF NF- κ B AND HISTONE DEACETYLASE 1 IN VENTRICULAR MYOCYTES

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James Shaw contributed to data acquisition, data analysis, data interpretation, and manuscript preparation

1.1 Abstract

Earlier we identified a survival role for NF- κ B in ventricular myocytes, however, the underlying mechanism was undefined. In this report we provide new mechanistic evidence that the hypoxia-inducible death factor BNIP3 is transcriptionally silenced by NF- κ B through a mechanism that involves the cooperative actions of HDAC1. Activation of the NF- κ B signaling pathway in ventricular myocytes suppressed basal and hypoxia-inducible BNIP3 gene activity. Basal Bnip3 gene expression was increased in cells derived from p65^{-/-} deficient mice. The histone deacetylase (HDAC) inhibitor Trichostatin A (TSA 10 nM) suppressed the inhibitory actions of NF- κ B on Bnip3 gene transcription. Basal and hypoxia-induced Bnip3 transcription was repressed by wild type but not a catalytically inactive mutant of HDAC1. Immunoprecipitation assays verified interaction of HDAC1 with wild type p65 NF- κ B and mutations of p65 defective for transactivation in ventricular myocytes. Deletion analysis revealed canonical NF- κ B elements within the Bnip3 promoter to be important for repression of Bnip3 gene expression by HDAC1. Further, the ability of HDAC1 to repress Bnip3 gene transcription was lost in cells derived from p65^{-/-} deficient mice but was restored by repletion of p65 NF- κ B into p65^{-/-} cells. Mutations of p65 NF- κ B defective for DNA binding but not for transactivation abrogated the inhibitory actions of HDAC1 on the Bnip3 gene transcription. Together, our findings provide new mechanistic insight into the cytoprotective actions conferred by NF- κ B that extend to the active transcriptional repression of the death factor Bnip3 through a mechanism that is mutually dependent upon HDAC-1.

1.2 Introduction

Nuclear Factor- κ B (NF- κ B) regulates a wide range of cellular processes including T-cell maturation, inflammation, and cell survival. In cells, NF- κ B exists as a heterodimer comprised of p50 and p65 KDa protein subunits. In absence of activating signals, NF- κ B is sequestered in the cytoplasm by the inhibitor protein I κ B α . Biological signals that lead to NF- κ B activation involve the phosphorylation dependent degradation of I κ B α mediated by the IKK signaling complex. In particular, IKK β is the principle kinase responsible for NF- κ B activation. IKK β phosphorylates I κ B α at critical serine residues 32 and 36 resulting in degradation of I κ B α by the proteasome (Li et al., 1999d; Verma et al., 1995). Ostensibly, the phosphorylation dependent loss of I κ B α activity permits NF- κ B to translocate to the nucleus and affect gene transcription (Beg and Baltimore, 1996). Despite this well accepted and proven model for signal induced NF- κ B activity, there are several reports documenting the presence of NF- κ B in the nucleus of non-stimulated cells (Carlotti et al., 2000; Ashburner et al., 2001). The significance of this finding is unknown, but raises the intriguing possibility that the basal activity of certain promoters may be regulated by NF- κ B. In this context, much of the known biological actions of NF- κ B including its anti-apoptosis properties have been ascribed to its well known and proven role as a transcriptional activator (Wang et al., 1998; Chu et al., 1997). However, recent data from our laboratory as well as others suggest that NF- κ B may also act as a transcriptional repressor (Ashburner et al., 2001; Baetz et al., 2005). The biological significance of this feature and its relation to cell death is unknown and has not been formally tested.

Gene transcription is a dynamic process governed by the extent and level of histone activity at a given promoter. Among the best studied histone modifications known to influence gene transcription is histone acetylation, which is governed by the balanced actions of histone acetylases and deacetylases (HDACs) (Seto, 2003). The class I HDACs which include HDAC 1, HDAC 2, HDAC 3, and HDAC 8 are recruited to DNA as part of a large multiprotein repressor complex comprised of Sin3 or Mi-2-NuRD proteins (Heinzel et al., 1997; Johnstone, 2002). One paradigm purports that HDACs inhibit gene transcription by deacetylating histone core proteins (de Ruijter et al., 2003), this compacts the nucleosome sterically impairing access of transcription factors to DNA, reviewed in (Sengupta and Seto, 2004; Seto, 2003; Johnstone, 2002). In addition to histone modification, HDACs can reportedly influence gene transcription by modifying nonhistone DNA binding factors (Yao et al., 2001). Indeed, the ability of HDACs to impair gene transcription by interacting with sequence specific transcription factors has been reported (Ashburner et al., 2001; Zhang and Kone, 2002; McKinsey et al., 2001; Kao et al., 2003).

Previously we showed that IKK β -mediated NF- κ B activation was sufficient to suppress hypoxia-induced mitochondrial perturbations and cell death of ventricular myocytes (Regula et al., 2004). We further demonstrated that NF- κ B averted cell death by transcriptionally silencing the mitochondrial death factor Bnip3 (Baetz et al., 2005). In this report we provide new evidence that NF- κ B mediated repression of BNIP3 occurs through a mechanism that involves the recruitment and deacetylase activity of HDAC-1. We further demonstrate the inhibitory actions of HDAC1 are contingent upon the DNA

binding properties of p65 NF- κ B and mediated through the NF- κ B elements within the Bnip3 promoter. Our data provide novel evidence that survival signals mediated by NF- κ B involve the active transcriptional repression of the death factor Bnip3 through cooperative actions of HDAC 1.

1.3 Materials and Methods

1.3.1 Cell Culture and Transfection

Post-natal ventricular myocytes from 2-day old Sprague Dawley rat hearts were isolated and submitted to primary culture as previously described (Kirshenbaum and Schneider, 1995; Regula and Kirshenbaum, 2001; Regula et al., 2002b). Myocytes or mouse embryonic fibroblasts (MEF) were transfected with the wild type 2.3 kBp Bnip3 promoter luciferase reporter (Bnip3 wt) and Bnip3 promoter in which the canonical NF- κ B elements (NRE) had been deleted and designated (Bnip3 mt) as previously described (Regula et al., 2002b; Kirshenbaum and Schneider, 1995; Kothari et al., 2003; Baetz et al., 2005). Mouse embryonic fibroblasts derived from wild type p65^{+/+} and p65^{-/-} mice were generously provided by Dr. D. Baltimore (Hoffmann et al., 2003). Expression plasmids encoding epitope-FLAG-tagged derivatives of wild type p65 and transactivation defective mutations (p65S529A, p65S36A) were kindly provided by Dr. A. Baldwin,(Madrid et al., 2001); the p65 NF- κ B DNA binding mutant (p65Y23E26) designated p65-DB was kindly provided by Dr. G. Natoli (Saccani et al., 2004). Eukaryotic expression plasmids encoding wild type histone deacetylase 1 (HDAC1) and catalytically inactive mutant HDAC H141A have been previously described (Hassig et

al., 1998). Cells were transfected with the CMV driven eukaryotic expression vector without complementary DNA insert for all transfection controls. Myocytes were maintained in serum free DMEM for 24 to 48 hours. Data were obtained from at least n=4 independent cultures using replicates of n=3 for each condition tested. Luciferase activity was normalized to β -galactosidase activity to control for differences in transfection efficiency and data are expressed as relative light units (Kirshenbaum and Schneider, 1995; Regula et al., 2002b).

1.3.2 Immunoprecipitation and Western Blot

For immunodetection of p65-NF- κ B and HDAC1 proteins, cardiac myocytes were harvested in 1.0% NP-40, 0.5% sodium dodecyl sulfate, 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4, (NP40 buffer). Cell lysates (100 μ g) were resolved on a 10 % sodium dodecyl sulfate-polyacrylamide gel at 140 V for 4 hours and electrophoretically transferred to polyvinylidene difluoride membrane PVDF (Roche Diagnostics). For detection of p65 NF- κ B, the filter was probed with an anti-p65 antibody, clone C20, cat# sc-372 (200 μ g/ml Santa Cruz Biotechnology, Calif) in NP-40 lysis buffer, pH 7.4, containing 0.3% Tween-20, 0.1% bovine serum albumin (TBS-Tween). For detection of HDAC1 the filter was probed with anti-HDAC 1 antibody, clone H51, cat # sc-7872 (200 μ g/ml Santa Cruz Biotechnology) in TBS-Tween. For detection of p65 NF- κ B - HDAC1 interactions, lysates from cells transfected with p65-FLAG tagged proteins were incubated with an anti-FLAG M2 antibody (Sigma, Oakville, Ontario, Canada) and immunoprecipitated with protein A-G agarose beads (Pharmacia, New York, NY). Bound proteins were detected by chemiluminescence reaction by enhanced ECL reagents

(Amersham, Piscataway, NJ).

1.3.3 Cell Culture and Hypoxia

Post-natal ventricular myocytes from 1- to 2-day old Sprague-Dawley rats were subjected to hypoxia for 24 hours in an air-tight chamber under serum free culture conditions continually gassed with 95% N₂ to 5% CO₂, pO₂ < 10 mmHg as previously described (Baetz et al., 2005; Regula et al., 2002b; Gurevich et al., 2001).

1.3.4 Recombinant Adenovirus

Myocytes were infected with adenoviruses encoding wild type IKK β (Ad IKK β wt) (Regula et al., 2004), or control adenovirus containing the CMV promoter (Ad CMV) at multiplicity of infection (MOI) of 10 which achieves >90 % of gene delivery to ventricular myocytes (Kirshenbaum et al., 1993).

1.3.5 RNA and Semi-quantitative RT-PCR

RT-PCR was performed using 0.5 μ g of total RNA using the Promega Access RT-PCR System (Promega Corporation, Madison, Wis) on a PTC-100 thermocycler (MJ Research, Inc, Waltham, Mass) for BNIP3 or house keeping control gene L32 respectively; BNIP3: forward 5'-GGGTAGAACTGCACTTCAGCAA-3' and reverse 5'-CCTGTTGGTATCTTGTGGTGT-3'; L32 gene: forward 5'-TAAGCGAAACTGGCGGAAAC-3' reverse 5'-GCTGCTCTTTCTACGATGGCTT-3' RT-PCR products were analyzed by 2% gel electrophoresis (Baetz et al., 2005). Relative band intensity was quantified by fluorescence scanning densitometry and normalized to

L32 gene on Storm gel analysis system, (Molecular Dynamics, Sunnyvale, Calif).

1.3.6 Statistical Analysis

Multiple comparisons between groups were determined by ANOVA. Unpaired 2 tailed Students t-test was used to compare mean differences between groups. Differences were considered to be statistically significant to 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.

1.4 Results

Previously we identified canonical binding elements for NF- κ B in the Bnip3 promoter (base pairs -1075 to -1069) that were found to be crucial for the inhibitory actions of NF- κ B on Bnip3 gene expression. To begin to assess the underlying mechanisms by which NF- κ B represses Bnip3 transcription, we focused our attention on the p65 NF- κ B subunit because the biological properties conferred by NF- κ B have largely been attributed to the actions of the p65, and our earlier work demonstrated the importance of the p65 for suppressing apoptosis in ventricular myocytes (Baetz et al., 2005; Regula et al., 2002a). We reasoned that NF- κ B may signal through inhibitory factors to repress Bnip3 gene activity. Because HDACs are a major class of chromatin modifying proteins that can reportedly influence NF- κ B transcription (Ashburner et al., 2001), we tested the possibility that HDAC proteins may be involved in the NF- κ B mediated repression of Bnip3 gene transcription. For these experiments the effect of trichostatin A (TSA, 10 nM) a known HDAC inhibitor was tested on Bnip3 gene transcription in the presence and

absence of p65 NF- κ B. As shown in Figure 5 (panel A), a 4.0 fold ($P<0.01$) reduction in Bnip3 gene transcription was observed in cells expressing the p65 NF- κ B. However, the HDAC inhibitor TSA interfered with the inhibitory actions of p65 on Bnip3 gene transcription. Further, a 2.5-fold increase in basal Bnip3 gene transcription was observed in myocytes in the presence of TSA (Figure 5, panel A). To verify that the derepression of the Bnip3 promoter by TSA was not related to spurious effects or restricted to the Bnip3 luciferase reporter, we examined whether TSA would abrogate the repressive effects of NF- κ B on the endogenous Bnip3 gene. For these experiments, ventricular myocytes were infected with an adenovirus encoding IKK β as means to activate NF- κ B. As shown in Figure 5 (panel B), a 2.0 fold reduction in endogenous Bnip3 expression was observed in ventricular myocytes expressing IKK β , a finding concordant with our earlier work (Baetz et al., 2005; Regula et al., 2004). Importantly, the repressive effects of IKK β -mediated NF- κ B activation on Bnip3 gene transcription were suppressed by TSA, a finding consistent with our Bnip3 transcription data. Collectively, our data support the notion that HDACs may be involved in the transcriptional repression of Bnip3 by NF- κ B.

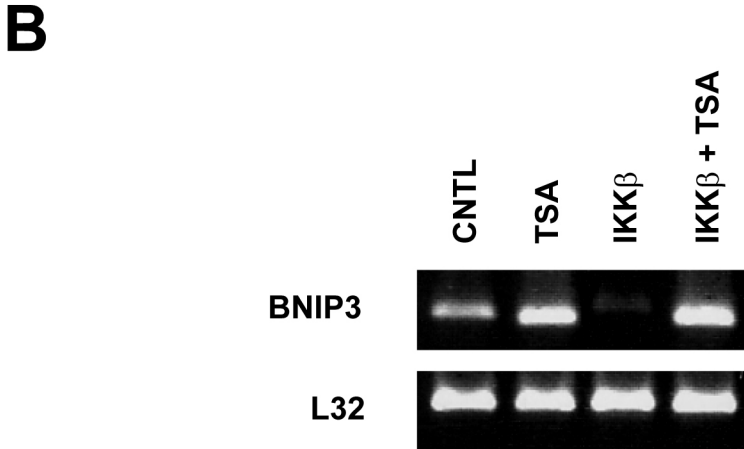
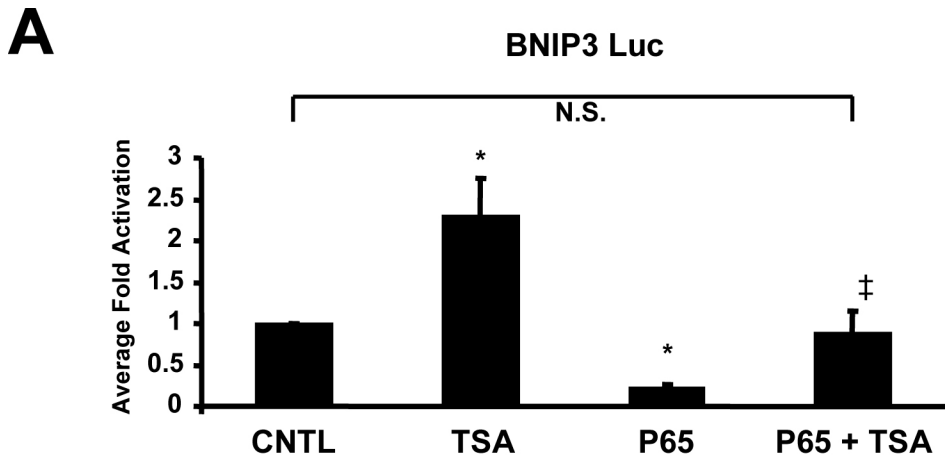


Figure 5. HDAC inhibition disrupts p65-mediated repression of Bnip3 gene transcription. **Panel A**, Postnatal ventricular myocytes were transfected with a Bnip3 luciferase reporter with and without a p65 NF- κ B expression vector in the presence and absence of trichostatin A (TSA, 10 nM). **Panel B**, IKK β -mediated NF- κ B activation represses Bnip3 gene transcription in ventricular myocytes. Postnatal ventricular myocytes were infected with adenoviruses encoding IKK β (IKK β) in the presence and absence TSA (10 nM). Endogenous Bnip3 in ventricular myocytes was examined by semiquantitative RT-PCR. Data were normalized to house keeping control gene L32. Control cells (CNTL) were transfected with the eukaryotic expression vector pcDNA3 (panel A) or infected with control adenovirus (panel B). Data are expressed as mean \pm S.E. ($P < 0.05$). Experiments were repeated at least $n = 6$ with independent culture conditions using replicates of $n = 3$ for each condition tested, * = statistically different from CNTL; ‡ = statistically different from p65; N.S. = not significant.

Our preliminary findings (Baetz et al., 2005) and immunoprecipitation experiments in ventricular myocytes revealed the interaction of the p65 subunit of NF- κ B with only the class I HDAC, HDAC1, (Figure 6, panels A and B). These findings provide cogent evidence that HDAC1 is involved in the transcriptional repression of Bnip3 by p65 NF- κ B. Therefore, we focused our attention on HDAC1 and tested whether NF- κ B signals through HDAC1 and tested whether NF- κ B signals through HDAC1 to repress Bnip3 gene transcription.

For these experiments, cells were transfected with p65 NF- κ B in the presence and absence of wild type or a catalytically inactive form of HDAC1 (HDAC1 H141A) defective for histone deacetylase activity. As shown in Figure 7 (panel A), wild type HDAC1 repressed basal BNIP3 gene transcription by 5.0 fold compared with control cells or cells expressing the catalytically inactive HDAC1 H141A. The inhibitory actions of the p65 NF- κ B on Bnip3 gene transcription were suppressed in cells expressing the catalytically inactive HDAC 1, Figure 7 (panel B), a finding consistent with the operation of HDAC1 in repression of Bnip3 by NF- κ B.

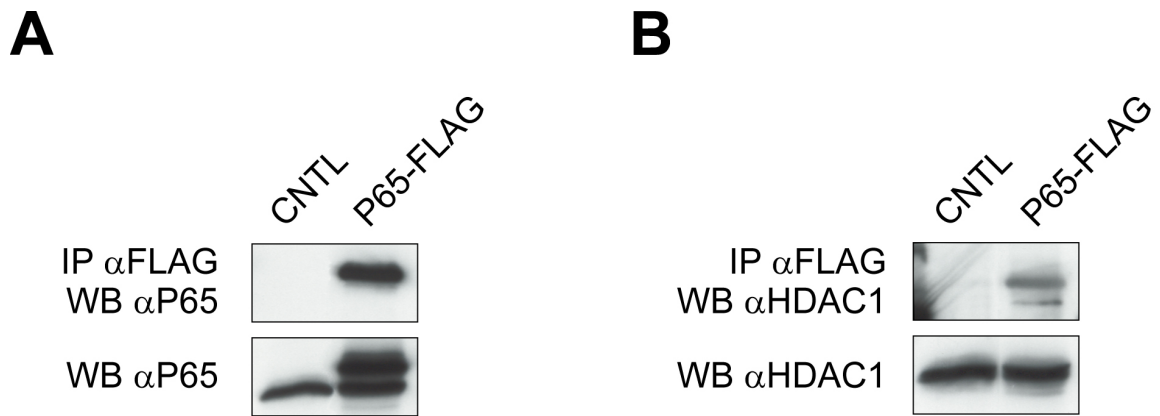


Figure 6. Interaction of p5 NF-κB with HDAC1 in ventricular myocytes. Panel A, upper, Western blot analysis (WB) of cells that were transfected with Flag-Tagged p5 NF-κB and immunoprecipitated (IP) with an antibody directed against Flag (αFLAG). The filter was probed with antibody directed against p5 to verify the expression of p5 NF-κB. Lower panel, Western Blot analysis of total cell lysate for p5. **Panel B,** upper, Western Blot analysis of cell lysate from panel A immunoprecipitated with anti-Flag antibody, the filter was probed with a murine antibody directed against HDAC1. Lower panel, Western Blot analysis of total cell lysate for HDAC1.

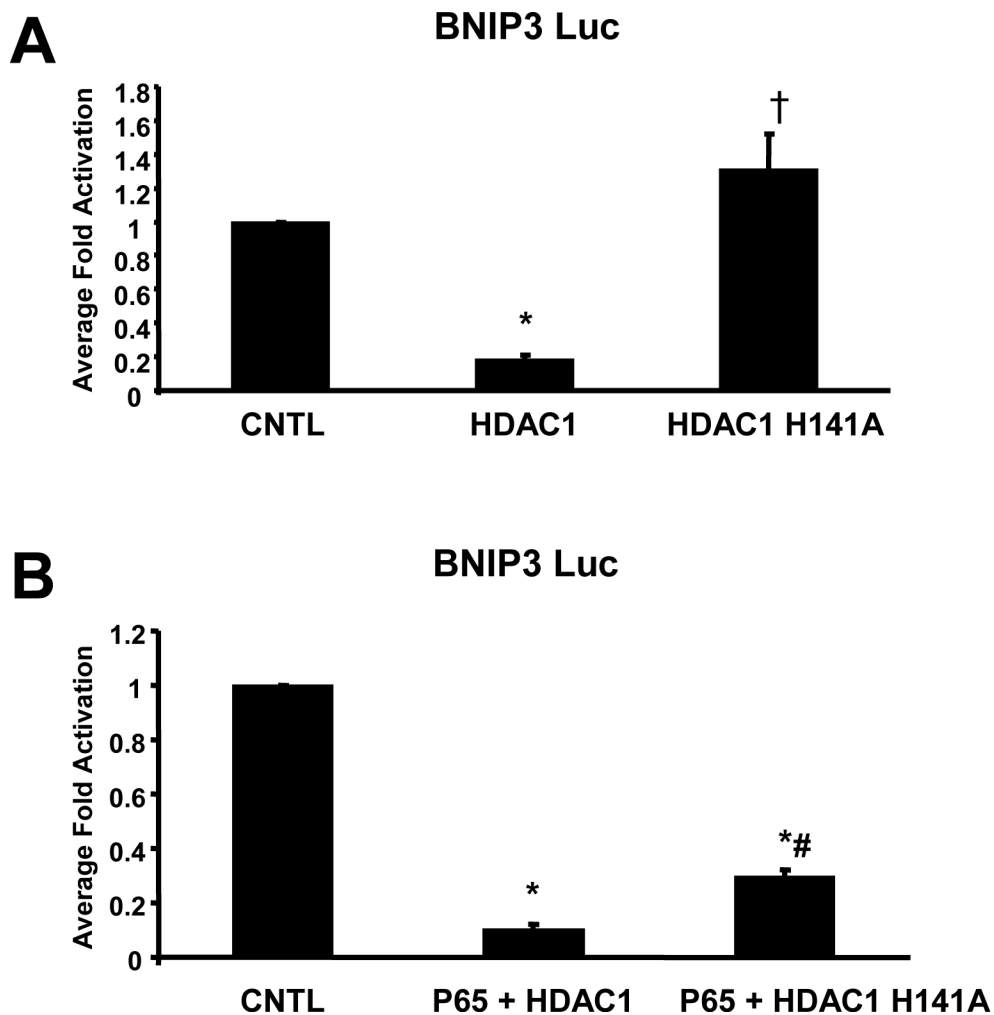


Figure 7. Regulation of Bnip3 gene transcription by HDAC1. **Panel A**, Bnip3 gene transcription in the presence of wild type HDAC1 and catalytically inactive HDAC1 mutant (HDAC1 H141A). **Panel B**, Regulation of Bnip3 gene transcription by p65 NF- κ B in the presence and absence of wild type HDAC1 and mutant HDAC1 H141A. Data are expressed as mean \pm S.E. ($P < 0.05$). Experiments were repeated at least $n=6$ with independent culture conditions using replicates of $n=3$ for each condition tested, *=statistically different from CNTL; †=statistically different from HDAC1; #=statistically different from p65+HDAC1.

To gain further insight into the mechanism underlying the repression of Bnip3 transcription by p50NF- κ B, we assessed whether the inhibitory actions of HDAC1 on Bnip3 gene transcription were related to and functionally dependent on the p50 NF- κ B. For these studies we tested the impact of HDAC1 in the presence of wild type p50 NF- κ B and mutations of p50 defective for transactivation (Baetz et al., 2005; Ashburner et al., 2001). As shown in Figure 8 (panel A), Bnip3 gene transcription was repressed in cells expressing HDAC1 in the presence of either wild type p50 NF- κ B or mutations of p50 NF- κ B defective for transactivation, suggesting that repression of Bnip3 gene transcription is related to the deacetylase activity of HDAC1 and not the transactivation potential of p50 NF- κ B. Further, the p50 NF- κ B proteins tested were expressed to comparable levels and interacted equivalently with HDAC1, Figure 8, panel B. Importantly, expression of the endogenous HDAC1 protein in cells expressing p50 proteins was comparable to vector transfected control cells, Figure 8, panel C. Because HDACs do not directly bind DNA, we next assessed whether DNA binding properties of p50 NF- κ B were necessary for the inhibitory effects of HDAC1 on Bnip3 transcription. As shown in Figure 8 panel A, the ability of HDAC1 to repress Bnip3 transcription was abrogated in cells expressing a p50 NF- κ B defective for DNA binding, supporting the notion that the DNA binding properties of p50 are required for repressive effects of HDAC1 on Bnip3 transcription. Furthermore, deletion of the NF- κ B consensus elements within the Bnip3 promoter suppressed the inhibitory actions of p50 and HDAC1 on Bnip3 transcription, Figure 9. Collectively, our data support the involvement of HDAC1 and p50 NF- κ B in repression of Bnip3 gene activation.

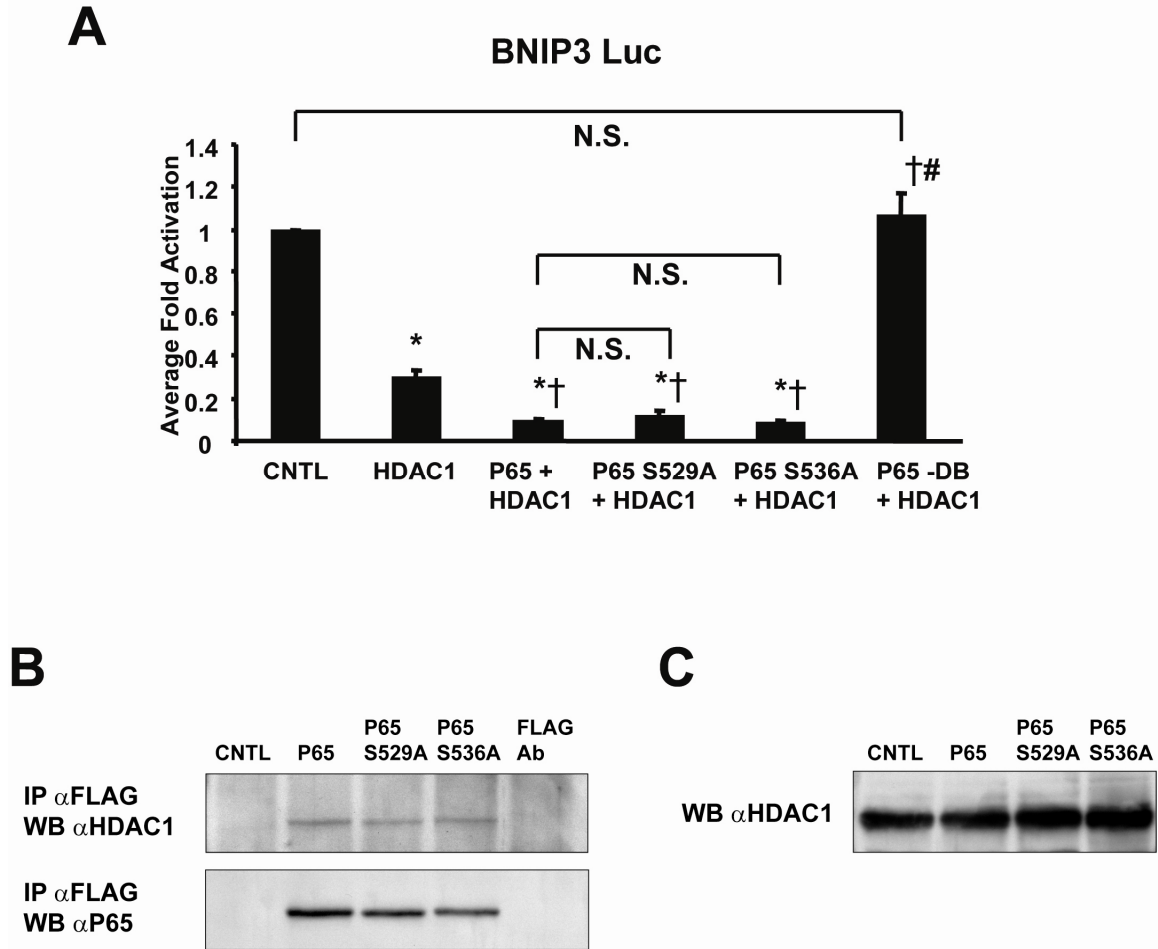


Figure 8. HDAC1 mediated repression of *Bnip3* gene transcription requires the DNA binding properties of NF- κ B. **Panel A**, Regulation of *Bnip3* gene transcription by HDAC1 in the presence and absence of wild type p65 NF- κ B designated (p65) and mutations of p65 NF- κ B defective for transactivation designated (p65S529A, p65S536A) or mutations of p65 NF- κ B defective for DNA binding designated (p65-DB). Control cells (CNTL) were transfected with the eukaryotic expression vector pcDNA3. **Panel B**, Western blot (WB) analysis of cells transfected with either Flag-tagged p65 NF- κ B, p65S529A, or p65S536A and immunoprecipitated (IP) with a murine antibody directed against Flag epitope (α FLAG). The filter was probed for HDAC1 (upper) and p65 (lower). **Panel C**, Western Blot analysis of total cell lysate from conditions shown in B, probed for HDAC1. Data are expressed as mean \pm S.E. ($P < 0.05$). Experiments were repeated at least $n=6$ with independent culture conditions using replicates of $n=3$ for each condition tested, *=statistically different from CNTL; †=statistically different from HDAC1; #=statistically different from p65+HDAC1, N.S.=not significant.

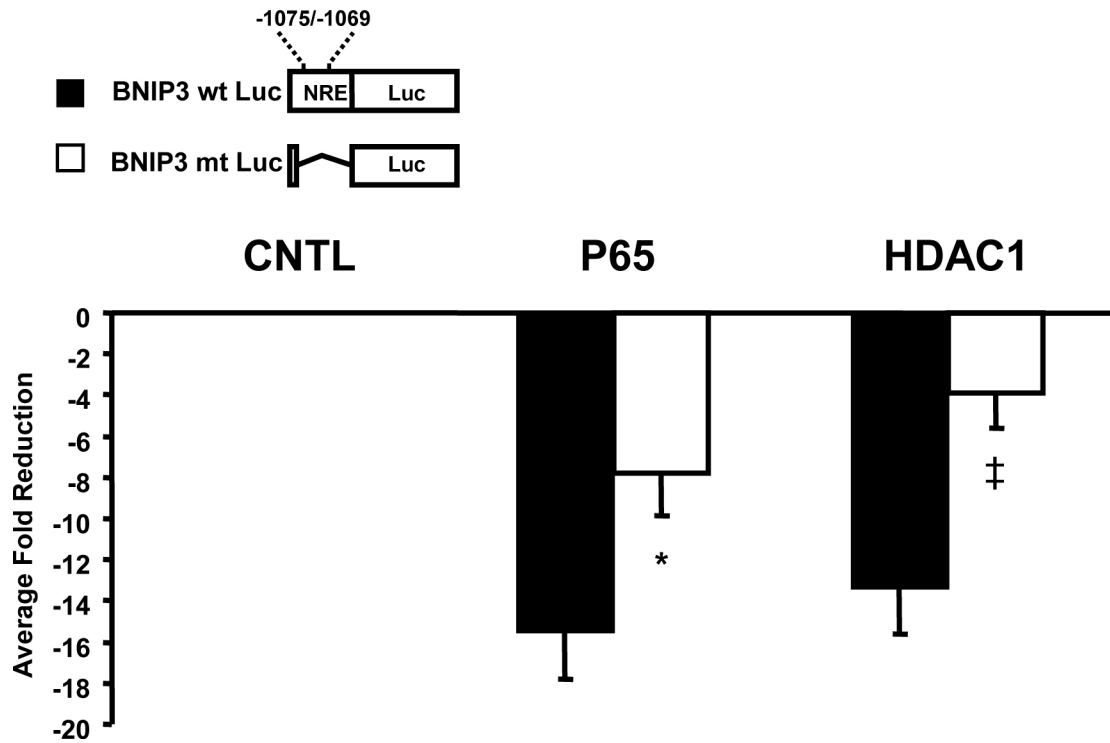


Figure 9. NF-κB elements are required for repression of BNIP3 expression.

Schematic of the BNIP3 promoter depicting the NF-κB response elements (NRE) base pairs (-1075/-1069). Postnatal ventricular myocytes were transfected with luciferase reporter plasmids containing wild type Bnip3 promoter (BNIP3 wt) or mutant Bnip3 promoter, in which the NF-κB elements were deleted and designated (BNIP3 mt), in the presence and absence of p65 NF-κB and HDAC1. Data are expressed as mean ± S.E. fold decrease from their respective controls (BNIP3 wt or BNIP3 mt). Experiments were repeated at least n=4 with independent culture conditions using replicates of n=3 for each condition, *=statistically different from Bnip3 wt +p65 NF-κB; ‡=statistically different from Bnip3wt +HDAC1. Legend; ■= Bnip3 wt; □=Bnip3mt.

To further prove that the inhibitory actions of HDAC1 were functionally contingent on p65 NF- κ B, we next tested whether HDAC1 would repress Bnip3 gene transcription in cells derived from p65^{-/-} deficient mice. As shown in Figure 10 (panel A and B), in contrast to wild type controls, basal transcription of the Bnip3 luciferase reporter as well as the endogenous Bnip3 gene were increased in p65^{-/-} cells compared to wild type cells, a finding concordant with the repression of Bnip3 by p65 NF- κ B and our earlier work (Baetz et al., 2005). Interestingly, the ability of HDAC1 to repress Bnip3 gene transcription was lost in p65^{-/-} cells but was restored by repletion of the p65NF- κ B into p65^{-/-} cells, Figure 10 panel A. In agreement with these findings was an increase in the endogenous basal Bnip3 gene transcription in wild type p65 cells treated with TSA but not in p65^{-/-} cells, a finding consistent with a requirement for p65 NF- κ B for the activity of HDAC1. Moreover, IKK β mediated NF- κ B activation repressed endogenous Bnip3 transcription in wild type p65 cells but not in cells deficient for p65 NF- κ B. Importantly, the repressive effects of IKK β mediated NF- κ B activation on Bnip3 transcription were suppressed in wild type p65 NF- κ B cells by TSA but not in p65^{-/-} cells. These findings are in agreement with the dependency of HDAC1 on p65NF- κ B for the repression of Bnip3 gene activity, Figure 10 panel B. Taken together, the data strongly suggest that Bnip3 gene transcription is regulated by the cooperative actions of NF- κ B and HDAC1.

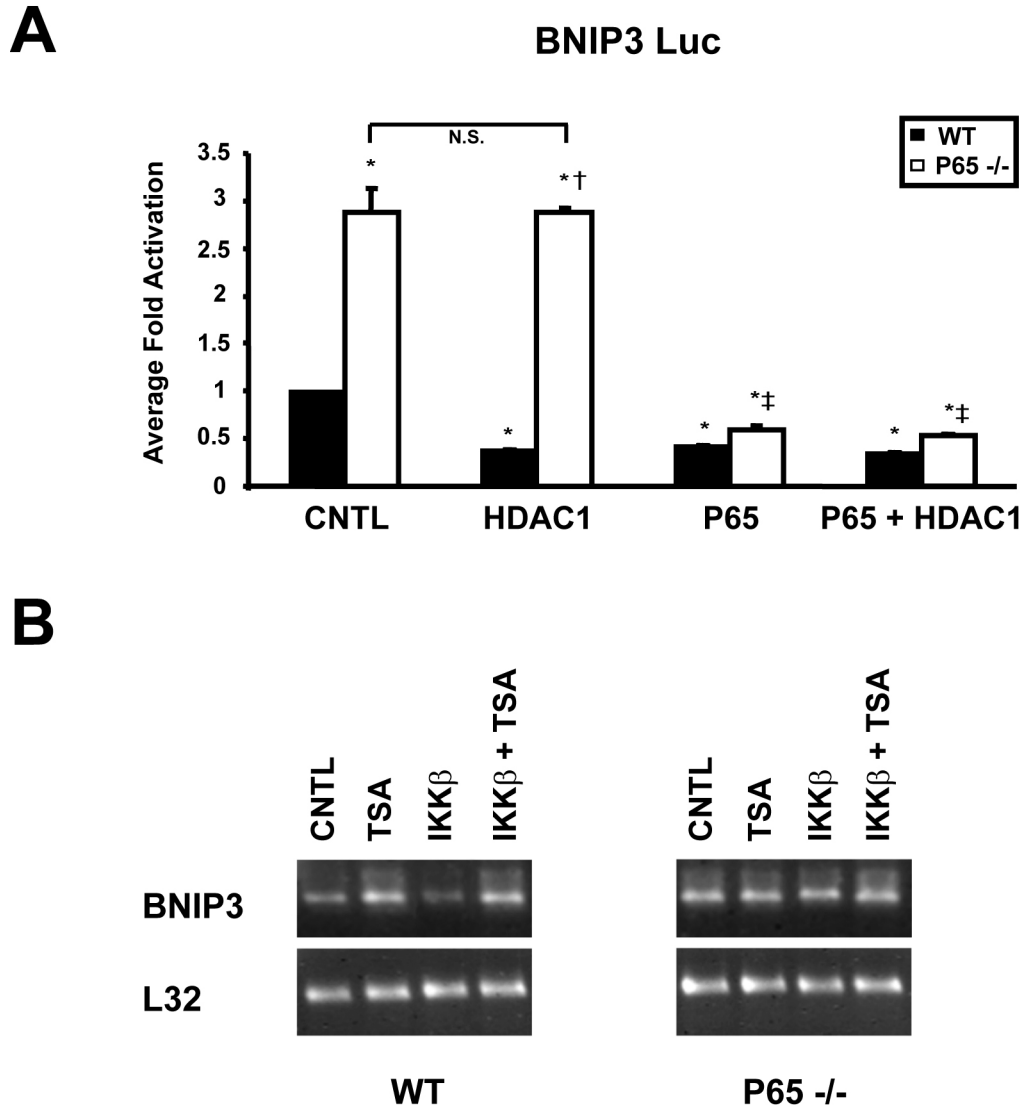


Figure 10. Bnip3 gene expression in wild type and p65 deficient cells. Panel A, Wild type and p65^{-/-} MEF cells (Hoffmann et al., 2003) were transfected with a BNIP3 Luciferase reporter plasmid, in the presence and absence of HDAC1 and p65. A 3.0-fold ($P < 0.001$) increase in basal BNIP3 gene expression was observed in p65^{-/-} cells compared with wild type cells (CNTL). Bnip3 transcription is repressed by HDAC1 in p65^{+/+} cells but not in p65^{-/-} cells. Repletion of p65 NF- κ B into p65^{-/-} cells restored HDAC1 mediated repression of Bnip3 gene transcription. **Panel B,** Semiquantitative RT-PCR analysis of endogenous Bnip3 gene transcription in wild type and p65^{-/-} MEF cells. Data are normalized to L32 expression. Data are expressed as mean \pm S.E. ($P < 0.05$). Experiments were repeated at least n=6 with independent culture conditions using replicates of n=3 for each condition tested; *=statistically different from wild type CNTL; ‡=statistically different from CNTL p65^{-/-} cells; †=statistically different from wild type CNTL with HDAC1; N.S.=not significant. Legend: ■= wild type p65^{+/+}; □= p65^{-/-} cells.

Previously we reported that Bnip3 promoter activity was increased in ventricular myocytes during hypoxia. To ascertain whether the hypoxia-induced activation of Bnip3 is related to functional changes in HDAC1 activity, we monitored the status of HDAC activity in ventricular myocytes under normoxic and hypoxic conditions.

As shown in Figure 11, TSA inhibited HDAC activity in ventricular myocytes by 36% ($P<0.05$) compared with vehicle control cells. Interestingly, in contrast to normoxic cells, a significant 21% ($P<0.01$) reduction in HDAC activity was observed in cells subjected to hypoxia. As predicted, the combination of hypoxia plus TSA had greater repressive effect on HDAC activity than hypoxia alone. Importantly, the hypoxia-induced decrease in HDAC activity was accompanied by a concomitant 4.8-fold increase ($P<0.01$) in Bnip3 gene transcription, Figure 11 (panel B). Importantly, hypoxia-induced activation of Bnip3 gene was suppressed in cells expressing the wild type HDAC1 but not in cells expressing the catalytically inactive HDAC1, Figure 11 (panel B).

Furthermore, deletion of the NF- κ B elements within the Bnip3 promoter elements disrupted the inhibitory actions of HDAC 1 on Bnip3 gene transcription during hypoxia, Figure 11 (panel C).

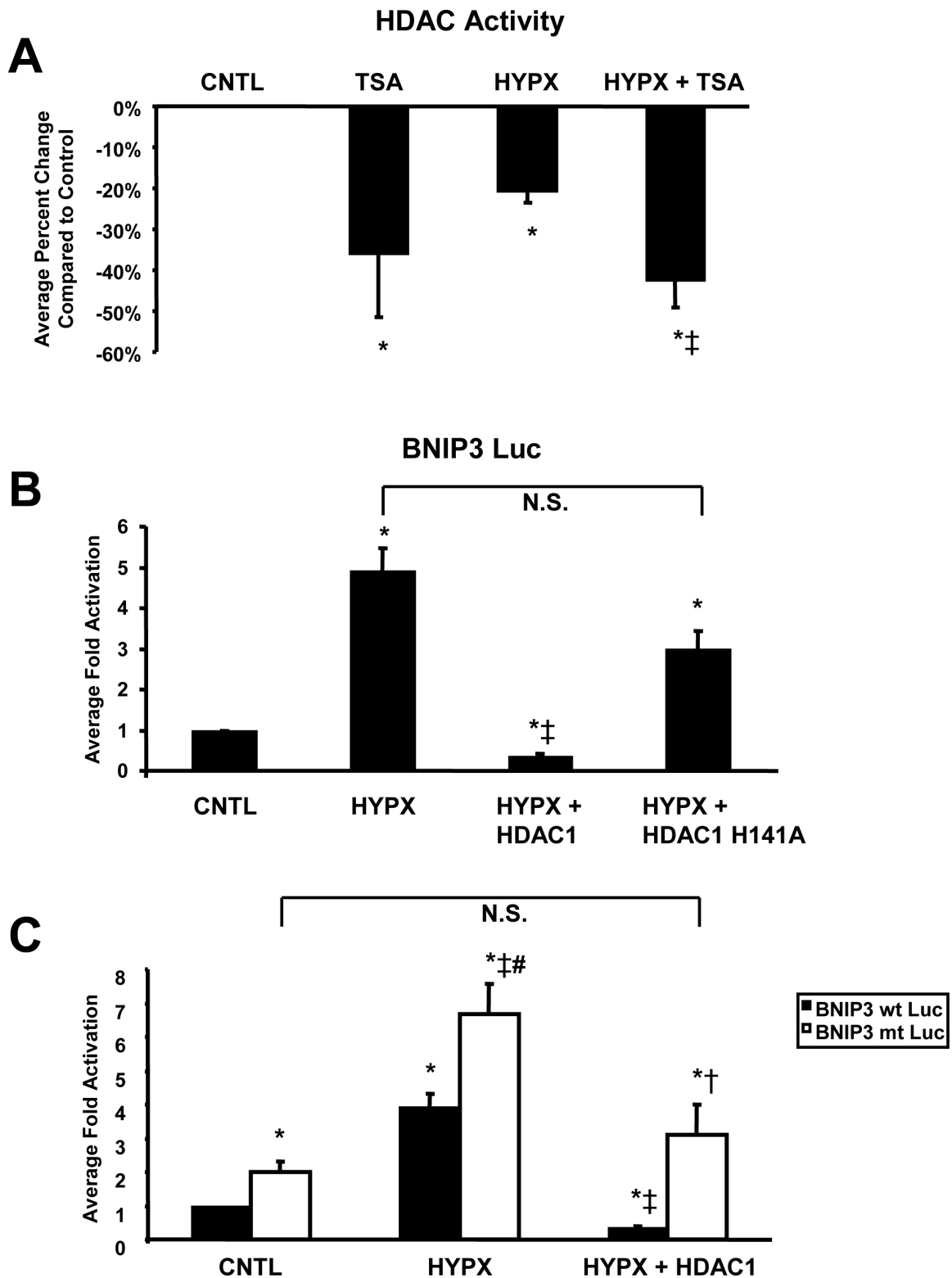


Figure 11. HDAC1 activity and Bnip3 expression during hypoxia. Panel A, Normoxic control cells (CNTL) or cells subjected to hypoxia (HYPX) in the presence and absence of TSA (10 nM) were assessed for HDAC activity. HDAC1 activity was

monitored by following the deacetylation of recombinant fluorometric HDAC substrate HOS3 (Ito et al., 2002) and is expressed as $\mu\text{mol}/\text{min}/\mu\text{g}$ protein; control cells = 1.01×10^{-6} $\mu\text{mol}/\text{min}/\mu\text{g}$ protein; cells treated with TSA = 6.42×10^{-7} $\mu\text{mol}/\text{min}/\mu\text{g}$ protein; cells subjected to hypoxia = 7.91×10^{-7} $\mu\text{mol}/\text{min}/\mu\text{g}$ protein; cells treated with TSA and subjected to hypoxia = 5.70×10^{-7} $\mu\text{mol}/\text{min}/\mu\text{g}$ protein. Data are presented as percent change from control using $n=3$ independent experiments and replicates of at least $n=3$ for each condition tested. **Panel B**, Bnip3 transcription during hypoxia; Bnip3 gene transcription is increased by 5.0-fold ($P<0.001$) in cells subjected to hypoxia compared with normoxic control cells; hypoxia-induced Bnip3 gene activation is repressed by wild type HDAC1 but not the catalytically inactive mutant HDAC1H141A; Panels A and B, *=statistically different from normoxic control cells (CNTL); ‡=statistically different from HYPX, N.S.=not significant. **Panel C**, The NF- κ B element is required for suppression of Bnip3 transcription by HDAC1 during hypoxia; Postnatal ventricular myocytes were transfected with luciferase reporter plasmids containing wild type BNIP3 promoter (BNIP3wt) or mutant Bnip3 promoter in which the NF- κ B site was deleted and designated (BNIP3 mt) as detailed in Figure 9; *=statistically different from Bnip3 wt normoxic control; ‡=statistically different Bnip3 wt HYPX; †=statistically different from Bnip3 wt HYPX+HDAC1; #=statistically different from Bnip3 mt normoxic control. Legend ■=Bnip3 wt; □=Bnip3 mt.

1.5 Discussion

Earlier work by our laboratory established Bnip3 as a critical regulator of mitochondrial perturbations and cell death of ventricular myocytes during hypoxic injury (Regula et al., 2004). Further, we identified that the Bnip3 promoter was strongly repressed by NF- κ B, however, the underlying mechanism was not determined (Baetz et al., 2005). In this report, we provide new mechanistic evidence that the Bnip3 promoter is transcriptionally repressed by p65 NF- κ B through a mechanism that involves the recruitment and catalytic activity of HDAC1. Importantly, we show that the canonical NF- κ B elements within the Bnip3 promoter are crucial for transcriptional repression of Bnip3 by HDAC1.

The antithetical regulation of Bnip3 gene expression by NF- κ B, despite NF- κ B's well established and proven role as a transcriptional activator (Wang et al., 1998; Sen and Baltimore, 1987; Singh et al., 1986; Madrid et al., 2000; Stein et al., 1993), highlights a less defined but emerging role for NF- κ B as a transcriptional repressor (Ashburner et al., 2001; Zhang and Kone, 2002; McCullough et al., 2001; Nozaki et al., 2001). This raises the interesting possibility that NF- κ B may avert cell death by actively repressing certain death genes such as Bnip3. Indeed, our findings that Bnip3 gene transcription was inhibited by NF- κ B is consistent with this notion and in line with our earlier work demonstrating a critical role for NF- κ B for suppression of mitochondrial perturbations and cell death of ventricular myocytes during hypoxia (Baetz et al., 2005; Regula et al., 2004).

The fact that TSA alleviated the inhibitory actions of p65 NF- κ B on Bnip3 gene

transcription is intriguing and strongly suggests the involvement of HDAC proteins in the repression of Bnip3 transcription. Interestingly, however, we noted that TSA completely abrogated the inhibitory actions of NF- κ B on the endogenous Bnip3 gene yet partially disrupted the effects of p65 NF- κ B on the Bnip3 luciferase reporter. This raises the possibility that stoichiometric differences with respect to the efficiency of HDAC inhibition by TSA may exist between the Bnip3 reporter and the endogenous Bnip3 gene. Nonetheless, our data strongly suggest the involvement of HDAC proteins in the repression of Bnip3 gene transcription by NF- κ B.

We focused our attention on HDAC1 for 2 important reasons: first, prior evidence for the regulation of NF- κ B dependent promoters by HDACs (Ashburner et al., 2001; Chen et al., 2002a) and second, perhaps most intriguing was our immunoprecipitation experiments of cardiac cell lysate which revealed interaction of p65 NF- κ B with HDAC1. Our findings that basal and inducible expression of Bnip3 transcription was repressed by HDAC1 verifies the operation of HDAC1 in ventricular myocytes and the notion that HDAC1 may be involved in the NF- κ B mediated repression of Bnip3. The interrelationship between HDAC 1 and p65 NF- κ B becomes even more profound given that we show by not 1 but by 3 independent approaches that functional inactivation of either factor was sufficient to derepress the Bnip3 promoter and increase Bnip3 gene transcription. For example, cells rendered defective for NF- κ B activation or mutations of the p65 defective for DNA binding suppressed the inhibitory actions of HDAC 1 on Bnip3 transcription. The derepression of HDAC1 on Bnip3 gene transcription by the p65 defective for DNA binding, and not by either of the transactivation defective p65 proteins

highlights 2 important features of our study. First, HDAC1 activity is necessary for repression of basal and inducible Bnip3 transcription and second, that the repressive effects of p65 NF- κ B on Bnip3 gene transcription is not dependent on the transactivation potential of p65NF- κ B. The fact that the DNA binding mutation of p65NF- κ B abrogated the ability of HDAC1 to repress Bnip3 transcription is consistent with a model in which p65 NF- κ B serves as a platform for the recruitment of HDAC1 activity. This is in-line with our data in p65^{-/-} cells in which HDAC1 overexpression had no influence on Bnip3 gene transcription. Furthermore, deletion of the NF- κ B elements within the Bnip3 promoter disrupted the repressive effects of HDAC1 on basal and hypoxia-inducible Bnip3 gene activity, substantiating the importance of NF- κ B for repression of Bnip3 gene activity by HDAC1. Importantly, however, we cannot exclude the possibility that the NF- κ B signals through noncanonical or cryptic inhibitory NF- κ B elements nested within the Bnip3 promoter which could easily explain why deletion of the NF- κ B elements did not fully prevent the inhibitory actions of HDAC1.

Our finding that NF- κ B mediated repression of Bnip3 transcription was disrupted by the catalytically inactive HDAC1 was concordant with our TSA data and our contention that HDAC1 is involved in the p65 NF- κ B mediated repression of Bnip3. Together the findings of the present study suggest NF- κ B represses Bnip3 gene transcription through a mechanism that is mutually dependent and obligatorily linked to the catalytic activity of HDAC1. This notion is in agreement with our earlier work demonstrating increased Bnip3 transcription and cell death in ventricular myocytes rendered defective for NF- κ B signaling or in cells derived from p65 deficient mice (Baetz et al., 2005).

Given that Bnip3 gene expression would otherwise provoke mitochondrial defects and cell death, implies that Bnip3 promoter must be highly regulated and under tight transcription control (Baetz et al., 2005). Indeed, Bnip3 is readily distinguished from other BH3 only members of the Bcl-2 family by at least 2 important features. First, Bnip3 expression is predominately restricted to the cardiac lineage (Regula et al., 2002b; Galvez et al., 2006), and second and perhaps the most compelling is the presence of NF- κ B consensus elements within the Bnip3 promoter that are absent from other death factors (Baetz et al., 2005). This unique property of Bnip3 highlights its importance as a key regulator of the intrinsic death pathway in cardiac muscle and its transcriptional divergence from the other death factors (Baetz et al., 2005). Given that earlier work from our laboratory demonstrated a direct linkage between Bcl-2 and NF- κ B activation for the suppression of cell death (Regula et al., 2002a; De Moissac et al., 1998; De Moissac et al., 1999) it is tempting to speculate that survival signals mediated by NF- κ B may in part involve the active transcriptional repression of certain death genes such as Bnip3 through the recruitment of HDACs. The fact that the inhibitory actions of p65 NF- κ B on Bnip3 transcription were suppressed by TSA or catalytically inactive HDAC1 strongly suggests the involvement and importance of the histone deacetylase activity. Whether NF- κ B signals through alternative inhibitory factors or is subject to posttranslational events requisite for repression of Bnip3 is currently unknown and is an area of active investigation.

Nevertheless, under the conditions tested, our data provide the first direct evidence that the death factor Bnip3 is transcriptionally repressed by the cooperative actions of NF- κ B

and HDAC 1. Furthermore, our data provide novel insight into the cytoprotective properties conferred by NF- κ B that now extend to the transcriptional repression of the death gene Bnip3.

2.0 P65-NF- κ B and HDAC1 Associate with the Bnip3 Promoter

2.1 Introduction

The potent, death-inducing properties of Bnip3 imply that its expression is tightly regulated under basal conditions. As previously reported, basal repression of Bnip3 transcription is mediated by p65 NF- κ B (Baetz et al., 2005). As shown in section 1, experimental interventions that interfered with the association between p65 and the Bnip3 promoter, most notably the complete absence of p65 in the p65^{-/-} cells, attenuated HDAC1-mediated Bnip3 transcription. Thus, one proposed mechanism to explain how p65 repressed Bnip3 transcription was through the recruitment of HDAC1 to the Bnip3 promoter. Herein, we add further support to this theory by showing that HDAC1 and p65 are both associated with the Bnip3 promoter under basal conditions.

2.2 Materials and Methods

2.2.1 Cell Culture

Post-natal rat ventricular myocytes were isolated and cultured as in other sections. Please see chapter IV, sections 1.3.1 and 3.3.1.

2.2.2 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed as in section 3.3.7 with antibodies directed against HDAC1 or p65 NF- κ B as described in section 1.3.2.

2.3 Results and Discussion

To test whether HDAC1 and p65 were associated with the Bnip3 promoter under basal conditions, a ChIP assay was performed. As shown in Figure 12, both proteins were present at the Bnip3 promoter under the conditions tested.

The positive result for HDAC1, despite the notion that HDAC proteins do not directly engage DNA suggests that another factor must be recruiting it to the Bnip3 promoter (Mehnert and Kelly, 2007). Because HDAC1 and p65 were previously shown to display protein-protein interactions (chapter IV, section 1), our ChIP results are consistent with our other data supporting the notion that p65 recruits HDAC1 to the Bnip3 promoter. It is predicted that in the absence of p65, or during hypoxia, the association of HDAC1 with the Bnip3 promoter would be markedly reduced compared to control cells. Future experiments will address this issue.

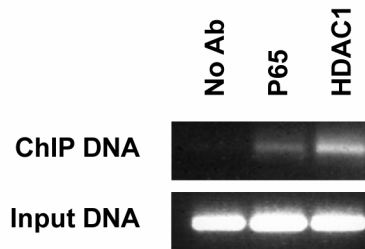


Figure 12. HDAC1 and p65 NF- κ B are associated with the Bnip3 promoter. Top, ChIP of the Bnip3 promoter under basal conditions. Cross-linked and sonicated cell lysates were immunoprecipitated with an antibody directed against p65 NF- κ B (p65) or HDAC1 (HDAC1) followed by PCR amplification of the promoter region known to bind p65. As a negative control, one ChIP condition was performed without an immunoprecipitating antibody (no Ab). Data is representative of at least two independent myocyte isolations. Bottom, input DNA to verify the integrity and uniformity of the DNA used for the conditions tested.

3.0 THE CELL CYCLE FACTOR E2F-1 ACTIVATES BNIP3 AND THE INTRINSIC DEATH PATHWAY IN VENTRICULAR MYOCYTES

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James Shaw contributed to experimental design, data acquisition, data analysis, data interpretation, and manuscript preparation.

3.1 Abstract

The cell cycle factor E2F-1 is known to regulate a variety of cellular processes including apoptosis. Previously we showed that disruption of Rb-E2F-1 complexes provoked apoptosis of postmitotic adult and neonatal ventricular myocytes; however, the underlying mechanism was undetermined. In this report, we show that E2F-1 provokes cell death of ventricular myocytes through a mechanism that directly impinges on the intrinsic death pathway. Furthermore, we show mechanistically that the hypoxia-inducible death factor Bnip3 is a direct transcriptional target of E2F-1 that is necessary and sufficient for E2F-1-induced cell death. Expression of E2F-1 resulted in a 4.9-fold increase ($P<0.001$) in nucleosomal DNA fragmentation and cell death by Hoechst 33258 dye and vital staining. E2F-1 provoked mitochondrial perturbations consistent with permeability transition pore opening. As determined by quantitative real-time PCR analysis, a 6.2-fold increase ($P<0.001$) in endogenous Bnip3 gene transcription was observed in cells expressing wild-type E2F-1 but not in cells expressing a mutation of E2F-1 defective for DNA binding. Rb, the principle regulator of cellular E2F-1 activity, was proteolytically cleaved and inactivated in ventricular myocytes during hypoxia. Consistent with the proteolytic cleavage of Rb, chromatin immunoprecipitation analysis revealed increased binding of E2F-1 to the Bnip3 promoter during hypoxia, a finding concordant with the induction of Bnip3 gene transcription. The Bnip3 homologue Nix/Bnip3L was unaffected in ventricular myocytes by either E2F-1 or hypoxia. Genetic knockdown of E2F-1 or expression of a caspase-resistant form of Rb suppressed basal and hypoxia-inducible Bnip3 gene transcription. Loss-of-function mutations of Bnip3

defective for mitochondrial membrane insertion or small interference RNA directed against Bnip3 suppressed cell death signals elicited by E2F-1. To our knowledge, the data provide the first direct evidence that activation of the intrinsic mitochondrial death pathway by E2F-1 is mutually dependent on and obligatorily linked to the transcriptional activation of Bnip3.

3.2 Introduction

Defects in the regulatory pathways that govern apoptosis have been linked to a variety of human pathologies, including neurodegenerative diseases, cancer, and cardiovascular disease. Mitochondrial perturbations have been postulated to be an underlying feature of the intrinsic cell death pathway (reviewed previously (Green and Reed, 1998)). Oxygen deprivation provokes mitochondrial permeability transition pore (PTP) opening, loss of mitochondrial membrane potential ($\Delta\psi_m$), cytochrome c release, and cell death of ventricular myocytes (Gurevich et al., 2001; Regula et al., 2003). Previously, we have established the mitochondrial death protein Bnip3 as an integral component of the intrinsic death pathway during hypoxic injury of postnatal ventricular myocytes (Regula et al., 2002b; Baetz et al., 2005). However, the signaling pathways and transcriptional processes that regulate Bnip3 transcription remain poorly defined.

The cellular factor E2F-1 is the archetypal member of a family of transcription factors first characterized for their ability to activate genes required for G1 exit and DNA synthesis (Neill et al., 1990). To date at least six homologs of E2F (E2F-1 to E2F-6), together with their dimerization partners DRFT1-polypeptides 1 and 2 (DP-1 and DP-2),

have been identified (Trimarchi and Lees, 2002; Black et al., 2005). Notably, E2F-1 is uniquely distinguished from other E2F proteins by its propensity to provoke apoptosis (Trimarchi and Lees, 2002; Black et al., 2005; Trimarchi et al., 1998), yet the underlying mechanism for this property of E2F-1 is, at best, poorly understood. In cells, E2F-1 interacts with the retinoblastoma gene product Rb via its L-X-C-X-E motif, which is crucial for suppressing cellular E2F activity and E2F-1-dependent promoters (Nevins, 1992). Accordingly, loss-of-function mutations or germ line deletion of Rb in mice results in increased E2F-1 activity and apoptosis (Jacks et al., 1992). Furthermore, the unexpected and counterintuitive tumorigenesis in the E2F-1^{-/-} mice underscores the importance of E2F-1 as a key regulator of apoptosis (Cloud et al., 2002).

Previously, we have shown that displacement of E2F-1 from Rb by viral oncoproteins or overexpression of E2F-1 alone was sufficient to provoke apoptosis of ventricular myocytes; however, the underlying mechanism(s) was not determined (Kirshenbaum and Schneider, 1995). In this report, we provide new compelling evidence that E2F-1 provokes cell death of ventricular myocytes through a mechanism that directly impinges on the intrinsic death pathway. We further show that the death factor Bnip3 is a direct transcriptional target of E2F-1 that elicits death signals downstream of E2F-1 during hypoxia. Our data provide new evidence that operationally links E2F-1 to the intrinsic death pathway via the death factor Bnip3.

3.3 Materials and Methods

3.3.1 Cell culture, Transfection, and Hypoxia

Ventricular myocytes were isolated from 1- to 2-day-old Sprague-Dawley rats and submitted to primary culture as reported (Kirshenbaum and Schneider, 1995). After overnight incubation in DMEM/Ham's nutrient mixture F-12 (1:1), 17 mmol/L HEPES, 3 mmol/L NaHCO₃, 2 mmol/L L-glutamine, 50 µg/ml gentamicin, and 10% FBS, cells were transferred to serum-free medium for 24 to 48 hours as reported previously (Kirshenbaum and Schneider, 1995). Cells were transfected with Bnip3 luciferase reporter plasmids (0.5 µg), along with an expression plasmid for β-galactosidase or small interference (si)RNA (10 nmol/L) using Effectene Transfection Reagent (Qiagen Inc) under serum-free conditions as reported previously (Regula et al., 2002b; Kirshenbaum and Schneider, 1995). Luciferase reporter activity was normalized to β-galactosidase activity to control for potential differences in transfection efficiency between myocyte cultures. Details for 2.3 kb of the human Bnip3 promoter luciferase reporter (Bnip3 Luc) have been reported previously (Regula et al., 2002b). Eukaryotic expression plasmids encoding the wild-type Rb (Rb) and caspase resistant Rb (RbMI) were kindly provided by Dr. J. Wang (University of California at San Diego) (Chau et al., 2002). Expression plasmids encoding cDNAs for wild-type E2F-1 and mutant E2F-1 E138 proteins have been described previously (Johnson et al., 1993). Cells were subjected to hypoxia for 24 hours in an air-tight chamber under serum-free culture conditions continually gassed with 95% N₂, 5% CO₂, with a P_{O₂} of < 5 mmHg, as described previously (Gurevich et al., 2001; Regula et al., 2002b; Baetz et al., 2005).

3.3.2 Recombinant Adenovirus and siRNA

Myocytes were infected with a control adenovirus (AdCMV), adenoviruses encoding E2F-1 (AdE2F-1), or carboxyl-terminal transmembrane domain deletion mutant of Bnip3 defective for mitochondrial targeting (AdBnip3 Δ TM), as reported previously (Regula et al., 2002b; Regula et al., 2004). siRNA directed against E2F-1 was obtained from Santa Cruz Biotechnology (catalog no. cs-29297). Our preliminary findings verified the specificity of siRNA directed against E2F-1 for silencing of E2F-1 gene without influencing other E2F proteins or the housekeeping control gene L32. Earlier, we described the generation of short hairpin interference RNA (shRNAi) directed against Bnip3 (Baetz et al., 2005). Adenoviral delivery of shRNAi against Bnip3 was generated using BLOCK-iT adenoviral RNAi expression system (Invitrogen) using a target sequence of 5'-GATCTACATTGGAAGGCGTCT-3', we previously characterized the authenticity and specificity of the shRNAi knockdown sequence in ventricular myocytes, which achieves > 80% inhibition of endogenous Bnip3 gene expression (Baetz et al., 2005). All adenovirus were used at an multiplicity of infection (MOI) of 10 (Regula et al., 2002b; Kirshenbaum and Schneider, 1995).

3.3.3 Apoptosis and Cell Viability

Cell viability was determined using the vital dyes calcein-acetoxymethyl ester (calcein-AM) (2 μ mol/L; green) to determine the number of living cells and ethidium homodimer-1 (2 μ mol/L; red) to identify the number of dead cells as reported previously (Gurevich et al., 2001; Mustapha et al., 2000). Following interventions, cell viability was assessed by counting > 200 cells from at least 3 to 4 independent myocyte isolations from 3 different

glass coverslips for each condition tested. Apoptotic cell death was assessed by visually inspecting individual myocytes from at least 3 to 4 independent myocyte isolations counting > 200 cells for each condition tested from 3 different glass coverslips. Individual cells were assessed for evidence of nucleosomal DNA fragmentation characterized by pyknotic hyperchromatic staining nuclei by Hoechst 33258 dye (Molecular Probes, Eugene Ore) as reported previously (Gurevich et al., 2001; Mustapha et al., 2000). All fluorescent images were captured using an Olympus AX70 Research grade epifluorescence microscope.

3.3.4 Immunocytochemistry

Immunocytochemistry was performed on fixed cells using a primary murine antibody directed against E2F-1 (clone KH95, Santa Cruz Biotechnology) that was detected by subsequent incubation with a secondary antibody conjugated to the fluorochrome Alexa Fluor 488 (Molecular Probes). Nuclei were visualized with the fluorescent dye TO-PRO-3 (Molecular Probes).

3.3.5 Mitochondrial PTP

Mitochondrial PTP opening was determined by loading ventricular myocytes with 5 $\mu\text{mol/L}$ calcein-AM (Molecular Probes) in the presence of 2 to 5 mmol/L cobalt chloride, as reported previously (Gurevich et al., 2001; Petronilli et al., 1998). Changes in integrated fluoresce intensity is an index of PTP opening. Data are expressed as mean percentages change $\pm\text{SE}$.

3.3.6 Western Blot Analysis

For detection of the Rb 68-kDa cleavage fragment, total cardiac cell lysate (50 µg) was subjected to Western blot analysis using a murine antibody directed toward the 68-kDa Rb cleavage fragment (cleaved [Ab-1], clone 172C1094, catalog no. OP198; Oncogene Research Products) (Chau et al., 2002). The filter was stained with Ponceau-S dye (0.1%) to verify equivalent protein loading of the Western Blot. Bound proteins were detected by enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Inc).

3.3.7 Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP Assay Kit (Upstate, Lake Placid, NY) with slight modifications involving a 2-step cross-linking step as described by Nowak et al (Nowak et al., 2005). DNA and protein were cross-linked in cells by incubation with 2 mmol/L disuccinamidyl glutarate (DSG) for 45 minutes at room temperature and washed 3 times with PBS, followed by incubation with 1% formaldehyde at room temperature for 15 minutes. Sonicated lysates were immunoprecipitated with 2 µg of antibody directed against E2F-1 (clone KH95, Santa Cruz Biotechnology), and incubated overnight at 4 °C with rotation. Purified DNA was amplified by PCR using a primer pair designed to amplify a 278-bp segment spanning the E2F-1 elements within the Bnip3 promoter: forward, 5'-AAAGCGGGAAATGAGAAAGC-3', reverse, 5'-TCGAGCAGAGTCGAAAGAGTC-3'. PCR products were analyzed on a 2% agarose gel.

3.3.8 RNA and Quantitative Real-Time PCR Analysis

Total RNA was extracted from cells using TRIzol Reagent (Invitrogen), followed by treatment with DNase I as reported previously (Baetz et al., 2005). RNA (1 µg) was reverse transcribed with oligo(dT) primers using SuperScript III Reverse Transcriptase (Invitrogen). Gene-specific primers were designed to amplify Bnip3, Nix, and the housekeeping gene L32 by quantitative PCR on a Bio-Rad iCycler thermal cycler using iQSYBR Green Supermix (Bio-Rad). Specific primers were designed using Primer3 version 4.0 software from the GenBank sequence for the rat Bnip3, Nix, and L32 respectively (Baetz et al., 2005): Bnip3 forward, 5'-TGCACTTCAGCAATGGGAAT-3'; Bnip3 reverse, 5'-ACATTTTCTGGCCGAATTGA-3' (accession number no. NM_053420); Nix forward, 5'-CATCCACAATGGAGACATGGAG-3'; Nix reverse, 5'-CTGATACCCAGTCCGCACTTTT-3' (accession no. NM_080888); L32 gene forward, 5'-TAAGCGAAACTGGCGGAAAC-3'; L32 gene reverse, 5'-GCTGCTCTTTCTACGATGGCTT-3' (accession no. XO 6483). All amplifications were performed in the final reaction mixture (25 µL) containing 1x final concentration of SYBR Green Supermix, 500 nmol/L gene-specific primers and 0.5 µL template under the following conditions: 95°C for 5 minutes, followed by 40 cycles consisting of 95.0°C for 10 seconds and 60°C for 30 seconds. Following amplification, iCycler 3.1 software was used to establish the baseline and threshold for each reaction. A cycle threshold (C_t) was assigned at the beginning of the logarithmic phase of PCR amplification. The $2^{-\Delta\Delta C_T}$ (Livak) method was used to calculate the relative gene expression. Expression of each gene relative to the L32 housekeeping gene was calculated as the difference between the C_t values of the 2 genes (ΔC_t). The difference in the ΔC_t of the control and experimental

sample ($\Delta\Delta C_t$) was used to determine the relative expression of the gene in each sample. The generation of specific PCR products was confirmed by melting curve analysis and agarose gel electrophoresis. The values are mean \pm SE from n=3 separate experiments in duplicate for each condition tested.

3.3.9 Semi-quantitative RT-PCR

RT-PCR was performed using 0.5 μ g of total RNA using the Access RT-PCR System (Promega Corporation, Madison, Wis) on an MBS Satellite 0.2G Thermo Fisher Scientific for Bnip3, E2F-1, or housekeeping control gene L32, respectively; the forward and reverse primer set for Bnip3 and L32 genes are described above. The primers for E2F-1 were as follows: forward, 5'-ACGCTATGAAACCTCACTAAA-3'; reverse, 5'-AGGACATTGGTGATGTCATA-3'. RT-PCR products were analyzed by 2% gel electrophoresis as reported earlier (Baetz et al., 2005).

3.3.10 Statistical Analysis

Multiple comparisons between groups were determined by 1-way ANOVA. Unpaired 2-tailed Student's *t* test was used to compare mean differences between groups.

Differences were considered to be statistically significant at $P < 0.05$. In all cases, unless otherwise stated, the data was obtained from at least 3 to 4 independent myocyte isolations, using replicates (n=3 for each condition tested).

3.4 Results

As a step toward understanding the underlying mechanism by which E2F-1 provokes apoptosis in cells, postnatal ventricular myocytes were infected with recombinant adenovirus encoding E2F-1 and assessed for nucleosomal DNA fragmentation by Hoechst 33258 nuclear DNA staining. Apoptotic cell death was determined by visually inspecting individual myocyte nuclei for nucleosomal DNA fragmentation and the appearance of pyknotic hyperchromatic staining nuclei, as reported previously (Gurevich et al., 2001; Mustapha et al., 2000). As shown in Figure 13A and 13B, cells overexpressing E2F-1 displayed a significantly greater percentage of apoptotic nuclei compared with control cells, verifying that E2F-1 is toxic to myocytes and provokes apoptosis. Because perturbations to mitochondria from the opening of the mitochondrial PTP have been suggested to be an underlying feature of the intrinsic death pathway, we assessed whether cell death provoked by E2F-1 involves mitochondrial PTP opening. For these experiments, ventricular myocytes were loaded with calcein-AM in the presence of cobalt chloride to quench the cytoplasmic signal in the presence and absence of E2F-1. The loss of green fluorescence by mitochondria is an index of PTP opening. As shown in Figure 13C and 13D, control cells displayed punctate green staining mitochondria indicative of the PTP in closed configuration; however, a marked reduction in mitochondrial green fluorescence was observed in cells expressing E2F-1, a finding consistent with PTP opening. These findings establish that E2F-1 provokes mitochondrial perturbations, consistent with the intrinsic apoptotic pathway.

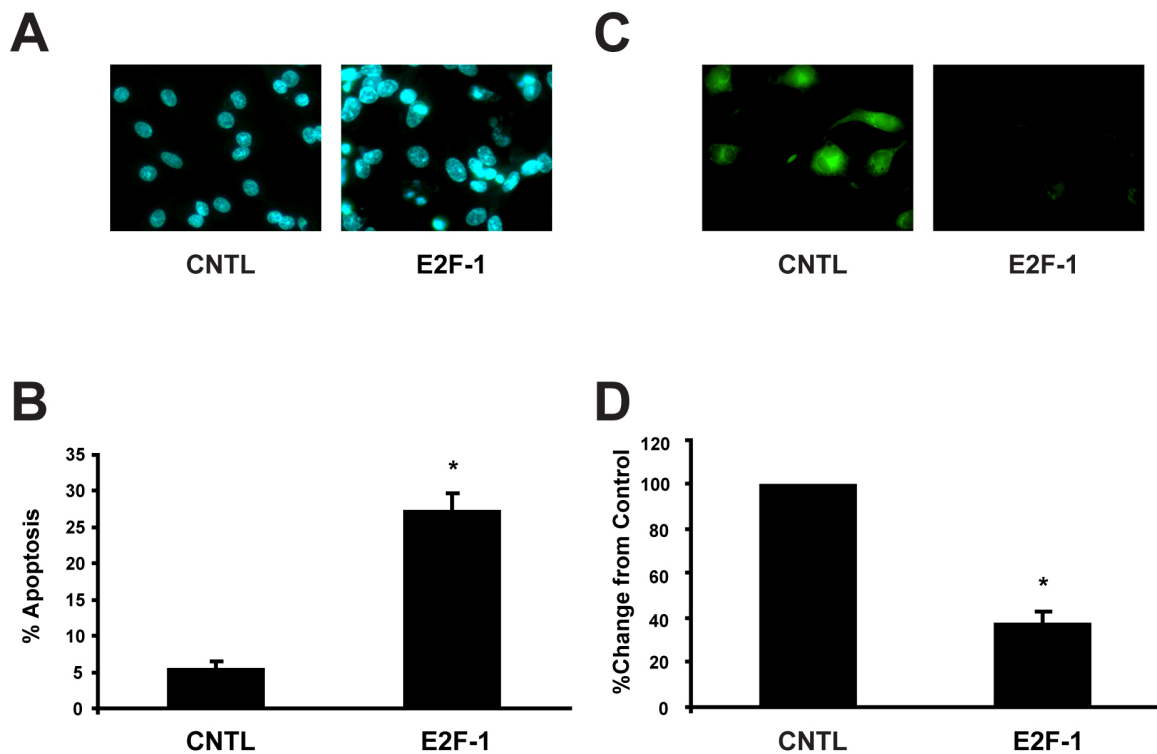


Figure 13. E2F-1 overexpression induces apoptosis and mitochondrial perturbations that are consistent with the intrinsic apoptotic pathway. Cells were infected with recombinant adenovirus encoding E2F-1 cDNA (AdE2F-1) or control virus (CNTL). **A**, Representative epifluorescence images of cells stained with the nuclear dye Hoechst 33258 to visualize apoptotic nuclear morphology. **B**, Quantitative data for **A**. **C**, E2F-1 provokes mitochondrial PTP opening; representative images of cells loaded with calcein-AM in the presence of cobalt chloride to quench the cytoplasmic signal are shown. Loss of green fluorescence is an index of PTP opening. **D**, Quantitative data for **C**. Data were obtained from at least 3 to 4 independent myocyte isolations counting >200 cells for each condition tested. Data are presented as mean percentage changes from control \pm SE. *Statistically different from control virus.

Earlier work from our laboratory and by others established the BH3-only protein Bnip3 as a central component of the intrinsic death pathway in ventricular myocytes during hypoxic injury (Regula et al., 2002b; Kubasiak et al., 2002). Interestingly, sequence analysis of the Bnip3 promoter revealed the presence of canonical DNA elements for E2F-1, raising the intriguing possibility that Bnip3 may be a transcriptional target of E2F-1. To test the possibility that Bnip3 expression was transcriptionally regulated by E2F-1, we monitored Bnip3 gene expression in ventricular myocytes in the presence and absence of E2F-1. As shown in Figure 14A, overexpression of E2F-1 in ventricular myocytes resulted in a 1.5-fold induction ($P < 0.001$) of Bnip3 luciferase reporter gene activity compared with cells expressing a mutation of E2F-1 (E2F-1E138) defective for DNA binding or vector control cells. Concordant with this finding was a marked 6.2-fold increase ($P < 0.001$) in endogenous Bnip3 gene expression in cells expressing E2F-1, as indicated by quantitative real-time PCR (Figure 14B). Interestingly, no apparent change in the expression levels of the Bnip3 homolog protein Nix/Bnip3L was observed in myocytes expressing E2F-1 (Figure 14B), verifying that Bnip3 and not Nix/Bnip3L is transcriptionally activated by E2F-1. To further validate the importance of E2F-1 in the transcriptional activation of Bnip3, we conducted additional experiments in which we assessed the level of Bnip3 gene activation using 2 independent approaches that interfere with cellular E2F-1 activity.

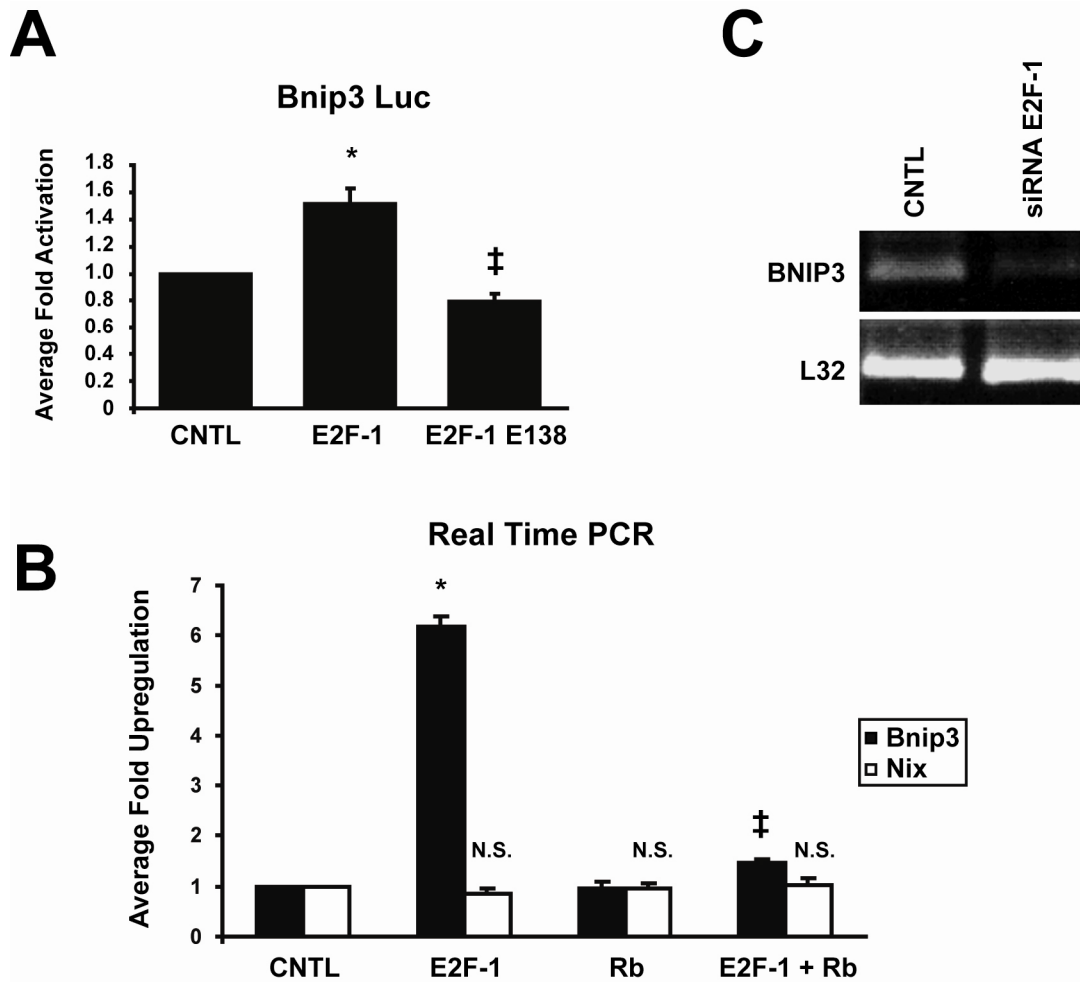


Figure 14. E2F-1 transcriptionally activates Bnip3 in ventricular myocytes. **A**, Bnip3 promoter luciferase reporter assay in the presence of wild type E2F-1 (E2F-1) and a mutant E2F-1 defective for DNA binding (E2F-1 E138). Data were obtained from at least 3 to 4 independent myocyte isolations using replicates of 3 for each experimental condition tested. Data is presented as means \pm SE fold activation from control (CNTL); *Statistically different from control; ‡statistically different from E2F-1. **B**, Quantitative real-time PCR analysis for endogenous Bnip3 (■) and Nix (□) genes, respectively, in the presence and absence of Rb and E2F-1 proteins. *Statistically different from control; ‡statistically different from E2F-1; NS compared with control. **C**, Top, Representative semiquantitative RT-PCR gel depicting the effect of siRNA directed against E2F-1 on endogenous Bnip3 gene transcription. Bottom, Housekeeping control gene L32.

Because cellular E2F-1 is regulated by Rb, we first tested whether expression of Rb itself as a means to sequester E2F-1 activity would influence Bnip3 gene transcription. As shown in Figure 14B, E2F-1-induced Bnip3 gene transcription was markedly suppressed in cells in the presence of Rb compared to E2F-1 alone, supporting our contention for the involvement of E2F-1 in the regulation of Bnip3. To further verify the importance of E2F-1 in the regulation of Bnip3, we next assessed by an alternative method whether siRNA directed against E2F-1, as means to genetically knock-down E2F-1, would impair Bnip3 gene expression. Previously we demonstrated the utility and authenticity RNAi knockdown in ventricular myocytes, which achieves >80% efficiency of inhibition of endogenous gene expression (Baetz et al., 2005). As shown in Figure 14C, in contrast to vector control cells, genetic knockdown of E2F-1 resulted in a significant reduction in endogenous Bnip3 gene transcription, a finding consistent with the repression of Bnip3 gene expression with Rb (Figure 14B). Collectively, our data strongly suggest that E2F-1 is important for the transcriptional regulation of Bnip3.

To test the physiological significance of these findings and to substantiate the possibility that E2F-1-induced cell death involves Bnip3, we next tested whether loss-of-function mutations of Bnip3 would abrogate the cytotoxic actions of E2F-1. For these experiments, we utilized a carboxyl-terminal mutant of Bnip3 (Bnip3 Δ TM), which has been shown previously by our laboratory to be defective for mitochondrial membrane insertion and for provoking cell death. As shown in Figure 15A and 15B in the presence of the Bnip3 Δ TM mutant, E2F-1-induced cell death was dramatically suppressed compared with cells expressing E2F-1 alone. Similarly, E2F-1-induced cell death was

strongly inhibited by genetic knockdown of Bnip3 using RNAi directed against Bnip3. These findings confirm and support our contention that E2F-1-induced cell death is obligatorily linked and mutually dependent on Bnip3.

Because earlier work established that Bnip3 is transcriptionally activated in ventricular myocytes during hypoxia (Regula et al., 2002b; Kubasiak et al., 2002), we next ascertained whether E2F-1 influences Bnip3 transcription during hypoxia. To test this possibility, postnatal ventricular myocytes were subjected to 24 hours of hypoxia and assessed for E2F-1 activity. As shown in Figure 16A, in contrast to normoxic control cells, a marked increase in endogenous E2F-1 gene expression was observed in ventricular myocytes subjected to hypoxia. In accordance with these findings, immunostaining of ventricular myocytes with an antibody directed against E2F-1 protein verified E2F-1 protein was localized to nuclei of hypoxic cells compared to the predominant cytoplasmic distribution observed in normoxic control cells (Figure 16B).

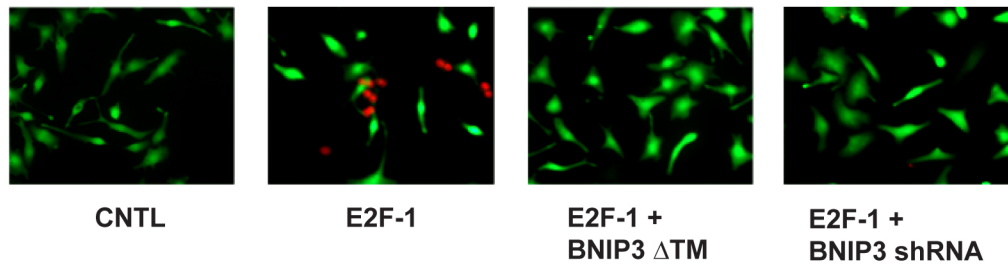
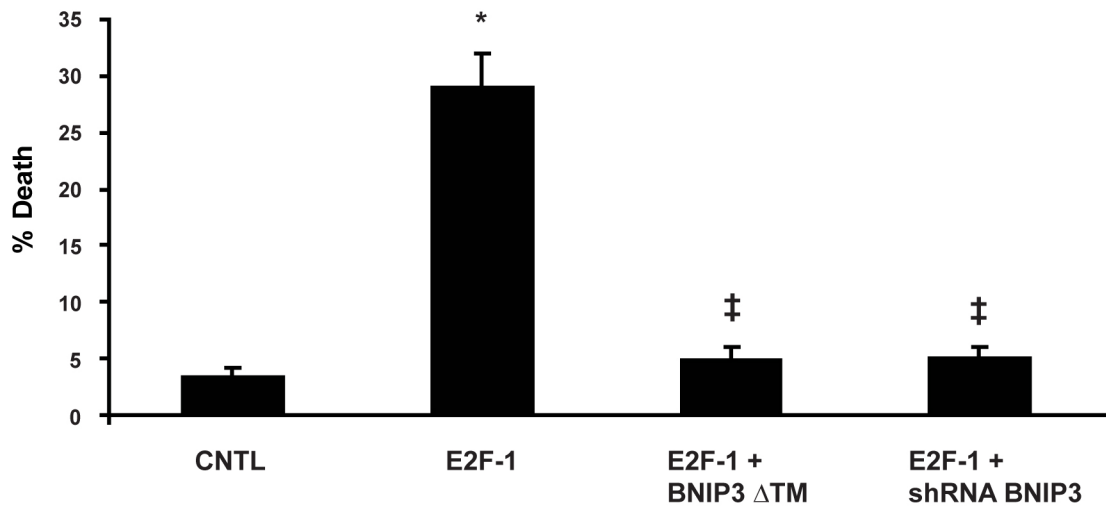
A**B**

Figure 15. E2F-1-induced cell death is contingent on Bnip3. Cells were infected with control virus (CNTL) AdCMV or recombinant adenovirus encoding E2F-1 in the absence or presence of a carboxyl-terminal deletion mutant of Bnip3 defective for mitochondrial targeting (Bnip3 Δ TM) or recombinant adenovirus encoding small hairpin RNA (shRNA) directed against Bnip3 (shRNA Bnip3); specificity and authenticity of small hairpin RNA directed against Bnip3 was reported previously (see Materials and Methods for details) (Baetz et al., 2005). **A**, Representative epifluorescence images of cells stained with vital dyes calcein-AM and ethidium homodimer to visualize live (green) and dead (red) cells, respectively. **B**, Quantitative data for A. Data were obtained from at least 3 to 4 independent myocyte isolations counting >200 cells from 3 glass coverslips for each condition tested. Data are presented as mean percentages of death \pm SE from control. *Statistically different from control; ‡statistically different from E2F-1.

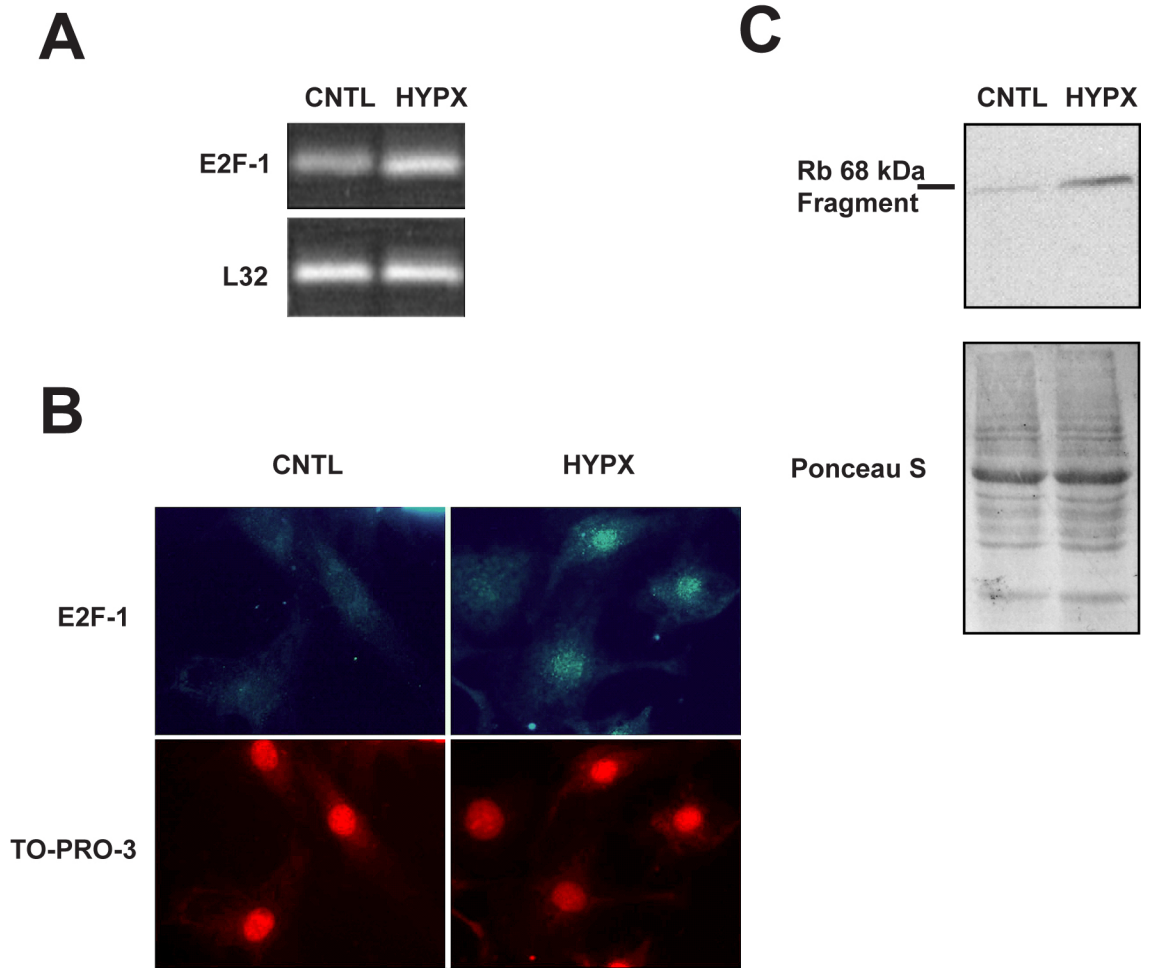


Figure 16. Expression of E2F-1 in ventricular myocytes. **A**, Semi-quantitative RT-PCR analysis of the endogenous E2F-1 gene in normoxic control cells (CNTL) and cells subjected to hypoxia (HYPX). **B**, Representative immunofluorescence images of endogenous E2F-1 protein expression in normoxic control cells and cells subjected to hypoxia. Myocytes were incubated with a murine antibody directed against E2F-1 protein followed by a goat IgG anti-mouse secondary antibody conjugated to the fluorochrome Alexa Fluor 488 (green) (top). Nuclei are identified by TO-PRO-3 staining (red) (bottom). **C**, Top, Western Blot analysis of cardiac cell lysate depicting the 68-kDa cleavage fragment of Rb (Fattman et al., 1997). Note that the antibody detects only the Rb cleavage fragment. Bottom, Ponceau S staining of the filter to demonstrate equivalent protein loading of the Western blot.

Previous work from our laboratory established the involvement of the death effector caspases as an underlying feature of hypoxic injury in cardiac myocytes (Gurevich et al., 2001; De Moissac et al., 2000). This observation, together with the recent finding of a caspase cleavage site in Rb (Borges et al., 2005; Fattman et al., 1997), prompted us to test whether Rb is proteolytically cleaved in ventricular myocytes during hypoxia. For these studies, cardiac cell lysates derived from normoxic and hypoxic myocytes were subjected to Western blot and analyzed for the presence of the 68-kDa Rb caspase cleavage product (Fattman et al., 1997). As shown in Figure 16C, in contrast to normoxic control cells, a marked increase in the 68-kDa Rb cleavage product was detected in myocytes subjected to hypoxia, a finding consistent with the increased E2F-1 activity in hypoxic cells.

To assess whether E2F-1 is involved in the hypoxia-induced activation of Bnip3, we next monitored Bnip3 promoter activity under normoxic and hypoxic conditions in the presence of interventions that suppress or inhibit E2F-1 activity. As shown in Figure 17, a 3.8-fold increase ($P < 0.001$) in Bnip3 luciferase reporter activity was observed in myocytes during hypoxia compared with normoxic control cells. However, hypoxia-induced expression of the Bnip3 luciferase reporter was markedly suppressed by a caspase-resistant form of Rb (RbMI). Furthermore, genetic knockdown of E2F-1 similarly repressed hypoxia-induced Bnip3 promoter luciferase activity, substantiating our contention that E2F-1 is required for activating the Bnip3 promoter during hypoxia.

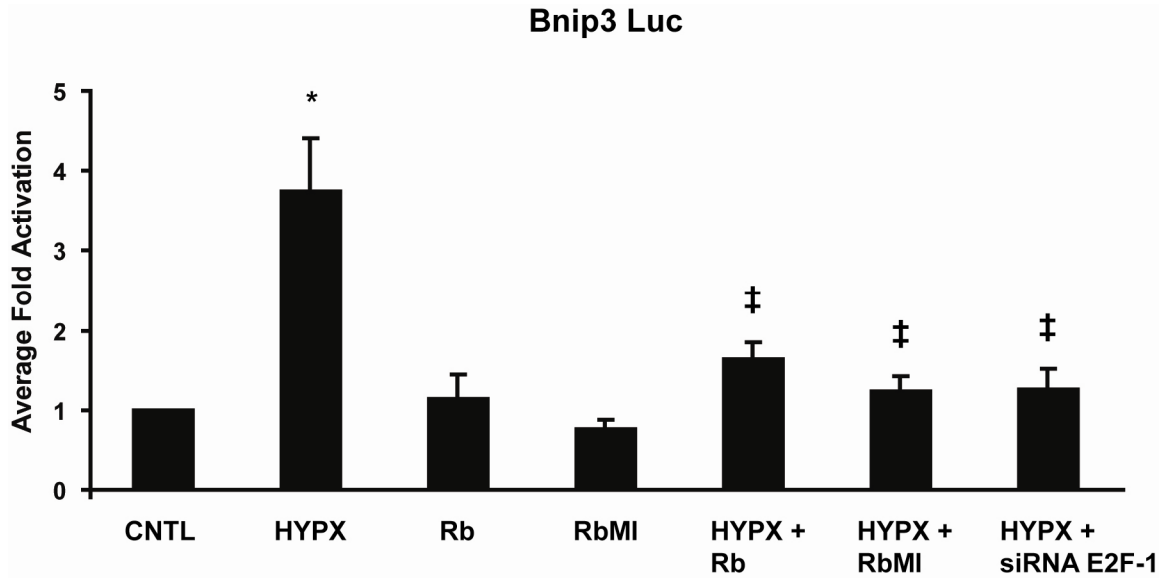


Figure 17. Regulation of Bnip3 promoter activity during hypoxia is dependent on E2F-1. Bnip3 promoter luciferase activity was monitored in normoxic control cells or cells subjected to 24 hours of hypoxia (HYPX) in the presence and absence of Rb (Rb), a caspase resistant Rb (RbMI), or siRNA directed against E2F-1 (siRNAE2F-1). Data were obtained from at least 3 to 4 independent myocyte isolations using replicates of 3 for each experimental condition tested. Data are presented as mean fold activations compared to control \pm SE. *Statistically different from control; ‡statistically different from hypoxia.

To verify that the induction of Bnip3 gene transcription by E2F-1 was directly related to E2F-1 occupying the Bnip3 promoter during hypoxia, we performed ChIP analysis of the Bnip3 promoter under normoxic and hypoxic conditions. As shown in Figure 18, E2F-1 was relatively undetectable at the Bnip3 promoter under normoxic control conditions; however, it readily bound the Bnip3 promoter during hypoxia. Importantly, hypoxia-induced E2F-1 promoter binding was abrogated in cells expressing of Rb. These findings are concordant with our transcription data for Bnip3 and confirm that E2F-1 directly engages the Bnip3 promoter during hypoxia to affect Bnip3 gene transcription. Collectively our data support our contention that E2F-1 is necessary and sufficient for induction of Bnip3 transcription.

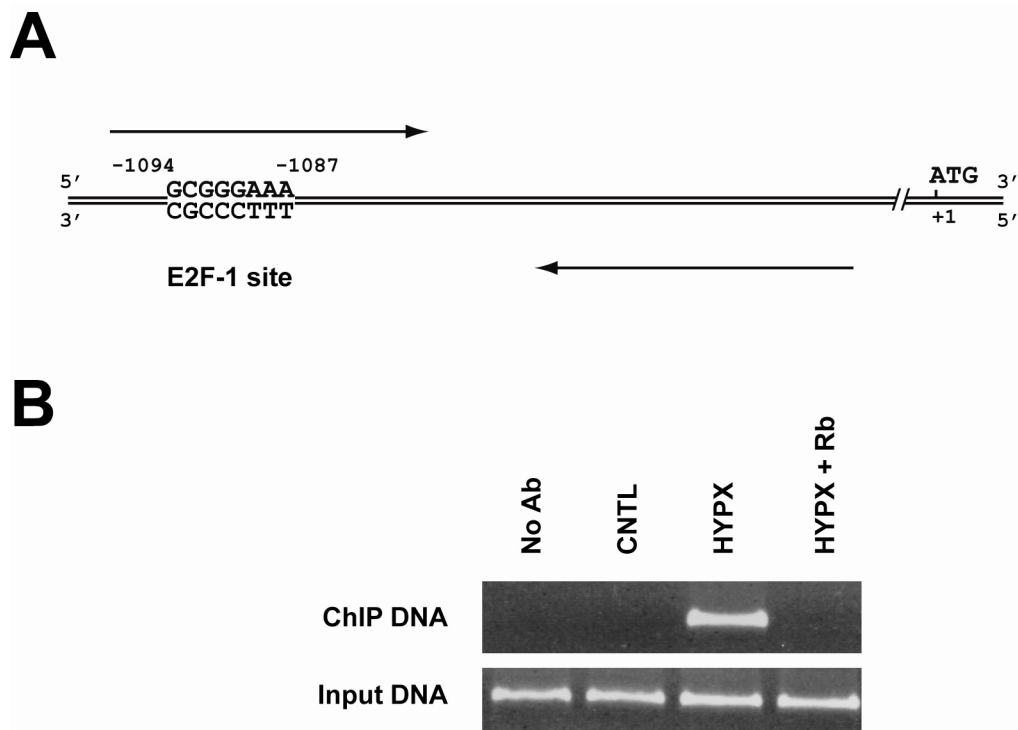


Figure 18. Hypoxia induces E2F-1 binding to the Bnip3 promoter. **A**, Schematic representation of the Bnip3 promoter; arrows depict the location of the forward and reverse PCR primer set used to amplify the consensus E2F-1-binding element within the Bnip3 promoter, which generates a 278-kbp PCR product. **B**, Top, Representative ChIP of the Bnip3 promoter under normoxic control conditions (CNTL) and hypoxia (HYPX) in the presence and absence of Rb. Cross-linked and sonicated cell lysates were immunoprecipitated with an antibody directed against E2F-1, followed by PCR amplification of the consensus E2F-1-binding elements. The ChIP data were repeated at least 2 times from independent myocyte isolations using duplicate samples for each experimental condition tested. Bottom, Input DNA to verify the integrity and uniformity of DNA used for the conditions tested.

3.5 Discussion

Previously we reported that E2F-1 was sufficient to provoke apoptosis of postnatal ventricular myocytes; however, the underlying mechanism was not determined (Agah et al., 1997; Kirshenbaum et al., 1996). In this report, we provide new evidence that E2F-1 provokes cell death of ventricular myocytes through a mechanism that impinges on the intrinsic death pathway. We further show that the death factor Bnip3 is a direct transcriptional target of E2F-1 that is necessary for E2F-1-induced cell death. Bnip3 was first identified as an adenovirus E1B 19-kDa-interacting protein (Boyd et al., 1994; Chen et al., 1997); however, its role in the context of the viral oncoprotein E1A- or E2F-1-induced cell death has not been addressed (Kirshenbaum and Schneider, 1995). Since these initial observations, our laboratory established Bnip3 as a critical component of the intrinsic death pathway during hypoxic injury (Regula et al., 2002b; Baetz et al., 2005). Nix/Bnip3L is the closest homolog to Bnip3 (Chen et al., 1999), but, unlike Bnip3, Nix appears to be regulated by hypertrophic growth signals and not by hypoxia or ischemic injury (Galvez et al., 2006; Yussman et al., 2002), highlighting the diversity of these factors. Bnip3 is further distinguished from other Bcl-2 family members by at least 2 important features. The first is that Bnip3 is transcriptionally activated by hypoxic or ischemic stress (Regula et al., 2002b); the second is the presence of consensus elements for E2F-1 within the Bnip3 promoter, raising the strong possibility that Bnip3 may be a transcriptional target of E2F-1. Indeed, our finding that endogenous Bnip3 gene expression and not Nix/Bnip3L was increased in ventricular myocytes in the presence of E2F-1 supports this contention.

Furthermore, this appears to be a restricted feature of E2F-1, given that neither E2F-2 nor E2F-3 had any influence on Bnip3 gene transcription (N.Y. and L.A.K., unpublished data, 2007). Hence, the unique ability of E2F-1 to regulate Bnip3 may explain, in part, the propensity of E2F-1 for provoking apoptosis over the other E2F factors (Black et al., 2005; Kowalik et al., 1995; Phillips et al., 1999). For this reason, the findings of the present study are intriguing and suggest that E2F-1 induces cell death through a mechanism that involves de novo Bnip3 gene transcription.

The interrelationship between E2F-1 and Bnip3 is profound because we show by not 1 but by 2 independent approaches that functional inhibition of Bnip3 was sufficient to suppress the cytotoxic actions E2F-1. These findings substantiate the importance of Bnip3 as a downstream target of E2F-1 during hypoxia-induced apoptosis of cardiac myocytes. The fact that deregulated Bnip3 would otherwise be lethal to cells and provoke apoptosis implies that it must be under highly regulated and tight transcriptional control. Indeed, earlier work by our laboratory established that the Bnip3 promoter is strongly repressed under basal conditions (Baetz et al., 2005; Shaw et al., 2006).

Therefore, we believe that an inappropriate or untimely Bnip3 gene transcription would have catastrophic consequences on cell survival. The limited E2F-1 binding to the Bnip3 promoter under basal normoxic conditions is consistent with this view. Although unproven, it is tempting to speculate that the inordinate apoptosis and midgestational lethality observed in the Rb^{-/-} mice is a result of increased Bnip3 activation from deregulated E2F-1 activity. This notion is founded on relative resistance of the E2F-1^{-/-} mice to apoptotic signals (Yamasaki et al., 1996) and our own findings demonstrating

that interventions that suppressed E2F-1 activity similarly suppressed Bnip3 gene transcription. The finding that Rb was proteolytically cleaved during hypoxia is intriguing and exemplifies the genetic and functional diversity among other pocket protein members, p107 and p130, that do not contain caspase cleavage sites and do not appear to be involved in apoptosis (Fattman et al., 1997). The finding that overexpression of a caspase-resistant Rb suppressed hypoxia-induced E2F-1 binding to the Bnip3 promoter and Bnip3 gene transcription strongly supports the importance of E2F-1 as a transcriptional activator of Bnip3 and the putative role of Rb as an antideath factor (Hauck et al., 2002).

To our knowledge our data provide the first direct evidence that Bnip3 is transcriptionally activated in ventricular myocytes by E2F-1 and underlies E2F-1-induced mitochondrial defects and cell death. Whether E2F-1 acts in concert with transcription factors such as hypoxia-inducible factor-1 α to activate Bnip3 transcription during hypoxia in ventricular myocytes is unknown and an active area of investigation.

Importantly, our finding that Bnip3 is transcriptionally activated by E2F-1 is in complete agreement with a recent report documenting the regulation of Bnip3 by E2F-1 in non-muscle Rb-deficient cells (Tracy et al., 2007), supporting our contention that Bnip3 is a transcriptional target of E2F-1 in ventricular myocytes. Although we cannot exclude the possibility that other factors regulated by E2F-1 may contribute to cell death induced by Bnip3 (Hershko and Ginsberg, 2004), the notion that E2F-1 causes cell death directly via increased Bnip3 expression is favorable based on our finding that E2F-1 engages the Bnip3 promoter coincidentally with the activation of Bnip3 transcription. Additionally,

the fact that loss-of-function mutations of Bnip3 or genetic knockdown of Bnip3 with siRNA abrogated the ability of E2F-1 to provoke cell death would argue that other death factors are not likely involved in E2F-1 induced cell death seen here. The findings of the present study are intriguing and provide new mechanistic evidence that E2F-1 is necessary and sufficient for induction of Bnip3 gene transcription. Based on the present findings, our data establish a novel paradigm for regulation of the intrinsic death pathway by E2F-1 that is operationally linked and mutually dependent on the transcriptional activation of Bnip3.

V. OVERALL SYNTHESIS AND FUTURE DIRECTIONS

The manuscripts presented in the previous chapter further our understanding of the regulation of Bnip3 expression in the heart under basal and hypoxic conditions. The first study revealed that p65 NF- κ B transcriptionally repressed Bnip3 gene transcription, at least in part, through the recruitment of the corepressor protein HDAC1. The second study demonstrated that E2F-1 was a transcriptional activator of Bnip3 during hypoxia and that the cell death seen upon overexpression of E2F-1 was largely Bnip3-dependent.

One of the next steps in this course of study is to bring together these investigations and ascertain whether the NF- κ B/HDAC1 and Rb/E2F-1 pathways are independent regulators of Bnip3, or if instead they influence each other. For example, one may speculate that the inhibitory signals mediated through IKK β , p65, and HDAC1 may influence the ability of E2F-1 to activate Bnip3. Reciprocally, E2F-1 itself may somehow inhibit NF- κ B signaling. While the two manuscripts in this thesis have independently analyzed Bnip3 regulation in the context of NF- κ B/HDAC1 or Rb/E2F-1, it is important to keep in mind that a given level of Bnip3 expression is the result of a dynamic interrelationship between many cellular factors that do not necessarily act in isolation. Precedents for the above conjectures are presented below.

Acetylation of E2F-1 has been shown to increase its ability to bind DNA and activate transcription (Martinez-Balbas et al., 2000). Consistent with this notion is the observation that association between E2F-1 and the HAT CPB/p300 is important for the ability of E2F-1 to activate transcription (Ait-Si-Ali et al., 2000; Trouche et al., 1996). It is classically thought that Rb-mediated inhibition of E2F-1-dependent transcription may

be mediated through recruitment of an HDAC such as HDAC1 (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). However, in late G1 of the cell cycle, Rb becomes hyperphosphorylated and releases E2F-1 for the activation of target genes (Yam et al., 2002). A mechanism must exist to prevent the expression of death promoting E2F-1-target genes during such circumstances. Previous work has implicated PI3K and Akt as upstream silencers of E2F-1-dependent death genes during the cell cycle (Hallstrom and Nevins, 2003; Hallstrom et al., 2008), as well as the E2F-1-binding protein Jab1, which may impart specificity to the target genes activated by E2F-1 (Hallstrom and Nevins, 2006). In the context of the Bnip3 promoter, it is possible that the constitutive presence of p65 and HDAC1 may inhibit Bnip3 expression through immediate deacetylation of E2F-1 proteins that attempt to associate with the Bnip3 promoter. Given that cardiac myocytes are resistant to cell division, this hypothesis may be extended to the control of basal cell survival by NF- κ B, given that E2F-1 is expressed in the heart, and that not all E2F-1 is Rb-bound. The association of E2F-1 with the Bnip3 promoter could be tested by ChIP assay in the presence and absence of HDAC1 or HDAC activity, via siRNA or TSA, respectively. The experiment with TSA may be more robust because other HDACs may compensate when only HDAC1 is knocked down with the siRNA. One caveat to this hypothesis is that although p65^{-/-} cells have elevated Bnip3 transcription (Baetz et al., 2005), they do not spontaneously die during the cell cycle. Thus, other factors must exist to prevent inappropriate Bnip3-induced cell death; however, it is unknown whether these cytoprotective control mechanisms act at the level of transcription, or if they antagonize the killing-potential of the protein.

Interestingly, IKK β has been shown to inhibit the transactivation potential of E2F-1 in primary human fibroblasts (Araki et al., 2008). This was mediated through p65-dependent inhibition of E2F-1-HAT association and an IKK-dependent increase in the nuclear localization of the repressive E2F-4 in complex with p130 (Araki et al., 2008). Applying these results to the regulation of Bnip3 gives a scenario in which NF- κ B signaling actively prevents the association of E2F-1 with the Bnip3 promoter. This could be tested by a ChIP assay for E2F-1 in the presence and absence of IKK β . Because E2F-1 becomes associated with the Bnip3 promoter during hypoxia, and hypoxia-induced Bnip3 expression and cell death can be inhibited by IKK β , it will be interesting to see if IKK β prevents E2F-1 from engaging the Bnip3 promoter during hypoxia.

In addition to directly activating the transcription of Bnip3, it may be that E2F-1 somehow antagonizes the repressive effects of NF- κ B. Using human endothelial cells, Chen et al (Chen et al., 2002b) showed that E2F-1 inhibited basal- and TNF α -induced NF- κ B nuclear translocation and DNA binding. This was ascribed to an E2F-1-dependent inhibition of the phosphorylation and degradation of I κ B α (Chen et al., 2002b). Further to this point, they concluded that E2F-1 inhibited the DNA binding ability of NF- κ B by competing with p50 for association with p65 (Chen et al., 2002b). Presumably this reduced the formation of a functional p50/p65 NF- κ B heterodimer. Another group of investigators observed that E2F-1 inhibited the activation of the IKK complex by downregulating the adapter protein TRAF2, although no effect was observed on TRAF2 transcription (Phillips et al., 1999). In the context of Bnip3 regulation, the repression of NF- κ B activity by E2F-1 would be consistent with transcriptional activation

of Bnip3 by E2F-1 alone. However, because these experiments were carried out in different cell types, it is unknown whether the trends would be observed in cardiac myocytes. In order to begin to test for the effect of E2F-1 on NF- κ B activity in the heart, the activity of an NF- κ B luciferase reporter could be assayed in the presence and absence of E2F-1 and TNF α to see whether E2F-1 inhibits basal or TNF α -induced NF- κ B-dependent luciferase activity. A parallel experiment may look at p65 subcellular localization by immunocytochemistry and I κ B α protein levels by western blot.

As previously stated, Bnip3 expression is repressed in colorectal and pancreatic cancer cell lines, and this may contribute to a more aggressive phenotype (Mellor and Harris, 2007). It has been shown that overexpression of E2F-1 in these cell lines may confer chemotherapeutic sensitization (Elliott et al., 2002; Dong et al., 2003; Dong et al., 2002; Atienza, Jr. et al., 2000), and this is consistent with E2F-1-dependent activation of Bnip3. However, this must be interpreted with caution because colon cancer cells that were resistant to 5-fluorouracil became sensitive upon transfection with decoy oligonucleotides targeted towards E2F-1, suggesting that E2F-1 activity was important for chemotherapeutic resistance (Obama et al., 2002). In a given cellular context E2F-1 may promote increased proliferation or increased death, so therapeutic interventions that aim to overexpress E2F-1 in cancer cells must proceed with caution and perhaps on a case-by-case basis to avoid creating a worse prognosis. With respect to NF- κ B, interventions that disrupted NF- κ B signalling in cancer cells have been shown to increase sensitivity to anti-cancer therapies (Yokoi and Fidler, 2004; Arlt et al., 2001; Muerkoster et al., 2003; Kunnumakkara et al., 2008; Fujioka et al., 2003), and this is consistent with the

derepression of Bnip3. Future experiments will be necessary to determine the significance of Bnip3 in this role. Regarding HDAC inhibitors, it is tempting to speculate that the derepression of Bnip3 may be a contributing cytotoxic mechanism towards their anti-cancer effects (Masoudi et al., 2008; Zou et al., 2008; Medina et al., 1997; Piacentini et al., 2006; Bai et al., 2006).

It has been shown that acetylation of p65 potentiates its transactivation potential (Chen et al., 2002a; Chen et al., 2001a), and that phosphorylation of p65 may be required for its acetylation (Chen et al., 2005). There is a report stating that dephosphorylation of p65 is coincident with the association between p65 and HDAC1 and that phosphorylated p65 is preferentially associated with the HAT CBP/p300, but only weakly with HDAC1 (Zhong et al., 2002). This suggests that the p65 protein found at the Bnip3 promoter may be dephosphorylated because it interacts with HDAC1 for the transcriptional repression of Bnip3. Further implications are that because of the dephosphorylation, the p65 at the Bnip3 promoter may be deacetylated, and that the deacetylated/dephosphorylated p65 may not be capable of transactivation, again suggesting an underlying mechanism for the repression of Bnip3 by p65. Previous results from Dr. Kirshenbaum's laboratory showed that non-phosphorylatable mutations of p65, defective for transactivation, repressed Bnip3 equivalently to wild type p65 (Baetz et al., 2005). That experiment was designed to show that p65-mediated repression of Bnip3 was a direct effect, and not due to the activation of an NF- κ B target gene. However, it would be interesting to see whether phospho-mimetic mutations of p65 such as S529D or S536D activate Bnip3 transcription, or at least inhibit Bnip3 to a lesser extent than the wild type protein. In addition to

targeting p65 itself, it is also possible that the specifically recruited HDAC deacetylates the histones within the Bnip3 promoter and antagonizes the activation of transcription. This may be explicitly tested using a ChIP assay for acetylated histone H3 or H4 on the Bnip3 promoter in wild type cells and cells overexpressing HDAC1. It remains unclear why HDAC1 is recruited specifically to p65 NF- κ B at the Bnip3 promoter for transcriptional repression, while p65 also activates transcription in association with p300 at the promoters of other more conventional NF- κ B target genes such as IL-6 (Vanden Berghe et al., 1999). Perhaps it has to do with other factors present at the Bnip3 promoter that facilitates the p65-HDAC1 interaction.

It will also be important for further investigations to determine whether therapeutic interventions that inhibit Bnip3 can improve outcomes post-MI in animal models and in human clinical trials. A major contribution of Dr. Kirshebaum and his laboratory has been the characterization of Bnip3 as a critical mediator of hypoxia-induced cell death in cardiac myocytes (Regula et al., 2002b; Baetz et al., 2005). There is already a strong precedent in the literature that modulation of Bnip3 protein will affect clinically relevant endpoints such as left ventricular end diastolic volume and ejection fraction. One such example is from Diwan et al. (Diwan et al., 2007), who showed that the absence of Bnip3 during MI was beneficial and that excess Bnip3 in a transgenic mouse overexpressing Bnip3 in the heart was certainly detrimental, as discussed in chapter III, section 8.4. Another example is the work by Hamacher-Brady et al (Hamacher-Brady et al., 2007), which revealed that functional inhibition of the Bnip3 protein, by transduction of Bnip3 Δ TM, reduced creatine kinase release and decreased infarct size in an ex vivo heart

subjected to ischemia followed by reperfusion. However, the techniques used here (transgenic animals and ex vivo perfusion) are not useful for treating humans.

Thus, it is possible that an anti-Bnip3 cardiac gene therapy may be developed that either specifically knocks down the Bnip3 protein, or negatively regulates the transcription of Bnip3. This may be accomplished through vectors that contain either shRNA against Bnip3, or the dominant negative Bnip3 Δ TM gene. Alternatively, it is conceivable that caspase resistant Rb, p65 NF- κ B, HDAC1, or siRNA against E2F-1 may be useful for inhibiting Bnip3 transcription post-MI, although these candidate transgenes will affect multiple downstream target genes. Future experiments will assess which approach yields the best clinical outcomes, but likely other interventions targeted towards the prevention of ventricular remodelling and arrhythmias may also be important, and perhaps in the future, an anti-Bnip3 gene therapy will be one component in a cocktail of genes delivered post-MI.

Regarding the actual gene delivery, cDNA for the above genes may be delivered within adenoviral or adeno-associated viral vectors. These types of vectors are currently favoured over other types of viruses and naked plasmid DNA based on their efficacy of gene delivery to the heart, and their relative safety (Rissanen and Yla-Herttuala, 2007). One advantage of the adeno-associated viruses is that they do not produce as strong of an immune reaction compared to adenoviruses (Gruchala et al., 2004). Other candidate vectors such as retroviruses have been used in past human gene therapy trials, but insertional mutagenesis induced cancer in some patients (Hacein-Bey-Abina et al., 2003). The issue of cardiac specificity may be addressed by injecting the vector directly into

cardiac tissue (French et al., 1994) or the pericardium (Fromes et al., 1999). However, it would be ideal to generate a vector that preferentially targets cardiac myocytes that could be delivered through an intravenous injection. Research is in progress in this area, and it has been reported that a particular serotype of adeno-associated virus shows selectivity for the heart after intravenous injection (Pacak et al., 2006; Zincarelli et al., 2008). Interestingly, it is possible to include a cardiac specific promoter such as α -myosin heavy chain or myosin light chain 2v within an adenovirus or adeno-associated virus, potentially allowing for increased target specificity (Phillips et al., 2002; Muller et al., 2006; Griscelli et al., 1998). Another interesting possibility is the use of a hypoxia-induced promoter (Pachori et al., 2004; Su et al., 2004; Phillips et al., 2002). These combined measures of cell-type and transcription-based targeting would ensure that even if the virus infected off-target cells, the transcription of the transgene would be limited to the cells or context of choice. However, intravenous delivery may not be appropriate for humans because of the large amount of virus required and the concern for an inflammatory response (Rissanen and Yla-Herttuala, 2007). The future holds great promise for molecular and genetic-based cardiac therapies; however, current work must continue to characterize the roles of cellular proteins so that we not only select optimal target genes, but also fully understand the consequences of our interventions.

VI. CONCLUSIONS

This work provides insight into the mechanism by which p65 NF- κ B transcriptionally represses Bnip3. The data support the notion that p65 recruits the co-repressor protein HDAC1 to the Bnip3 promoter, thereby inhibiting Bnip3 transcription.

The other set of experiments investigated the relationship between E2F-1, Rb, and Bnip3 expression. It was found that E2F-1 transcriptionally activated Bnip3, and E2F-1 was important for hypoxia-induced Bnip3 expression. E2F-1 directly associated with the Bnip3 promoter for this action. The mechanism by which E2F-1 induced apoptosis of cardiac myocytes was determined to be, in large part, via a Bnip3-dependent pathway.

VII. REFERENCES

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