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Regulation of Apoptosis by the Muscle Regulatory Transcription Factor Myod

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REGULATION OF APOPTOSIS BY THE MUSCLE REGULATORY TRANSCRIPTION FACTOR MYOD

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DEDICATION

This dissertation is dedicated to my family, for whom this journey would never have begun, to Frank, who has patiently stood by me, I could not have continued and to Dr. Crystal Weyman, who without her guidance, I could not have completed. Thank you all.

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And as John wrote in his dissertation, I am truly sorry to all of the trees which gave their lives in this endeavor. At least I got recycled paper for my final drafts...

REGULATION OF APOPTOSIS BY THE MUSCLE REGULATORY TRANSCRIPTION FACTOR MYOD

TERRI J. HARFORD

ABSTRACT

We have previously reported that the level of MyoD expression correlates with the level of apoptosis that occurs in a subpopulation of skeletal myoblasts induced to differentiate by serum withdrawal. Herein we document that MyoD expression dictates the apoptotic threshold in myoblasts and fibroblasts in response to a variety of apoptotic stimuli. Specifically, re-expression of MyoD in skeletal myoblasts rendered defective for both differentiation and apoptosis by the expression of oncogenic Ras restores their ability to undergo both differentiation and apoptosis in response to serum withdrawal. Further, using a fibroblast cell line expressing an estrogen receptor:MyoD fusion protein, we have determined that addition of estrogen sensitizes these fibroblasts to apoptosis induced by serum withdrawal, or by treatment with etoposide or thapsigargin. RNAi mediated silencing of MyoD in either 23A2 or C2C12 myoblasts renders these cells resistant to apoptosis induced by serum withdrawal, or by treatment with etoposide or thapsigargin. Finally, MyoD mediated regulation of the apoptotic response to these various stimuli correlates with the level of induction of the pro-apoptotic Bcl-2 family member PUMA.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AIF	Apoptosis inducing factor
Apaf-1	Apoptotic Protease Activating Factor 1
ARF	Alternative Reading Frame
ASSP	Apoptotic stimulating protein of p53
ATF-1	Activating Transcription Factor-1
ATP	Adenosine Triphosphate
A/T	Adenosine/
AU	Adenosine uridine
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 associated killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell Lymphoma 2
Bcl-xL	B-cell lymphoma extra large
Bcl-w	B cell lymphoma -W
BH-3 only	Bcl-2 Homology domain 3 only
bHLH	Basic helix loop helix
Bid	Bcl-2 homology interacting domain death agonist
Bim	BCL-2-interacting mediator of cell death
BME	Beta mercaptoethanol/Basal Medium Eagle
BSA	Bovine Serum Albumin

C	Control
Caspase	Cysteine aspartic acid
cdc25	Cyclin dependent kinase 25
CDK4	Cyclin dependent kinase 4
cDNA	copy deoxyribonucleic acid
CEBPα	CCAAT enhancer binding protein alpha
ced-3	Cell death defective-3
ced-4	Cell death defective-4
ced-9	Cell death defective-9
CHAPS	3-[3-(chloramidopropyl)dimethylammonio]-1-propanesulfonic acid
CHiP	Chromatin immunoprecipitation
CHX	Cyclohexamide
C_t	Coefficient time
DNA	Deoxyribonucleic acid
Diablo	Direct inhibitor of apoptosis binding protein with low pi
DM	Differentiation medium
DMEM	Dulbecco modified eagle medium
dNTPs	Deoxyribonucleotriphosphates
DR5	Death receptor 5
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
egl-1	Egg-laying defective-1
ELISA	Enzyme linked immunosorbant assay

EMSA	Electromobility shift assay
ER	Estrogen receptor
ESCs	Embryonic stem cells
FADD	Fas-associated death domain protein
FasL	Fas ligand
FBS	Fetal bovine serum
FLIP	FLICE-inhibitory protein
FLISP	Fluorescently labeled inhibitor of serine protease
FOXO3a	Forkhead box-3a
GADD45	Growth arrest and DNA-damage-inducible 45
GAPDH	Glyceraldehyde phosphodehydrogenase
GFP	Green fluorescent protein
GM	Growth medium
HATs	Histone acetyl transferases
HDACS	Histone deacetyltransferases
HLH	Helix loop helix
HLH-2	Helix loop helix -2
HLH-3	Helix loop helix-3
HRP	Horse radish peroxidase
Hsp70	Heat shock protein 70
IAPs	Inhibitor of apoptosis
Id	Inhibitor of differentiation protein
IGFs	Insulin-like growth factors

Mcl1	Myeloid cell leukemia sequence 1
MEF2	Myocyte enhancer factor-2
m_{exp}	Experimental median
MHC	Myosin heavy chain
MNase	Micrococcal nuclease
MOMP	Mitochondrial outer membrane potential
MOPS	3-(N-Morpholino)propanesulfonic acid; n-(3-sulfopropyl orpholine);
MRFs	Muscle regulatory factors
MRF4	Muscle Regulatory factor-4
mRNA	Messenger Ribonucleic acid
Myf5	Myogenic factor-5
MyoD	Myogenic differentiation
OD405	Optical density at 405 nm wavelength
P	Parental
PMSF	Phenyl methylsufonylfluoride
PNPP	*p*-Nitrophenyl Phosphate, Disodium Salt
PPARα	Peroxisome proliferators-activated receptor alpha
PPARγ	Peroxisome proliferators-activated receptor gamma
P/S	Penicillin/streptomycin
P-ser	Phosphatidyl serine
P-thr	Phosphatidyl threonine
P-tyr	Phosphatidyl tyrosine
PUMA	p53 upregulated modulator of apoptosis

Puro	Puromycin
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
Ras	Rat sarcoma
Rb	Retinoblastoma
RNAi	Ribonucleic acid interference
RT-PCR	Reverse-transcription-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin ribonucleic acid
Smac	Second mitochondria derived activator of caspases
SMAD3	Mothers against decapentaplegic homolog 3
STAT1	Signal transducer and activator of transcription-1
tBid	Truncated Bid
TGFβ	Transforming growth factor beta
TNF	Tumor necrosis factor
TRAIL	TNF related apoptosis inducing ligand
TBS-T	Tris buffered saline with Tween 20
UCP3	Uncoupling protein 3
XIAP	x linked inhibitor of apoptosis
z-VDVAD-fmk	Benzyloxycarbonyl-Val-Asp(OMe)-Val-Ala-Asp(OMe)-fluoromethylketone

CHAPTER I

INTRODUCTION

While all can agree the study of apoptosis is critical to the understanding of cancer and to elucidate potential therapeutic targets for cures, we must not overlook the importance of understanding the tightly regulated apoptosis as it applies to the healthy development of multicellular organisms as well as in maintaining homeostasis. In understanding the regulation of apoptosis in this context, we can utilize the knowledge gained to improve on stem cell based therapies currently used in the treatment of degenerative diseases such as muscular dystrophies and for the purpose of cardiac repair (Dona, et al. 2003, Menasche 2004, Sandri et al. 2001, Scorsin et al. 2000, Skuk and Tremblay 2003, Suzuki et al. 2001, Taylor et al. 1998). Once we are able to determine the molecular mechanisms which determine the fate of cells during differentiation and the associated apoptosis, we will have potential novel targets that can be manipulated to improve the efficacy of these stem cell based therapies.

1.1 Skeletal Myogenesis

The formation of skeletal muscle during development or in response to muscle damage is achieved through the process of myogenesis. Myogenesis can be divided into two separate temporal events. Determination is the process through which multipotential stem cells are committed to the myogenic lineage. Differentiation is the process through which myoblasts become myotubes (Figure 1). Controlling myogenesis is a family of muscle regulatory transcription factors (MRFs). Although expression of any of these factors will induce the conversion of multipotent stem cells to myoblasts (Tapscott 2005), determination, in vivo is defined by the expression of two MRFs, MyoD and Myf5. Myogenin and MRF4 are required for differentiation (Perry and Rudnicki 2000). Differentiation of skeletal myoblasts is a complex process composed of temporally separate events (de la Serna 2001). Actively dividing myoblasts must first exit the cell cycle at a specific point, during G1, express muscle specific genes such as myosin heavy chain (MHC) and fuse to form multinucleated myotubes (Rudnicki and Jaenisch 1995). Differentiation is positively regulated by the activation of the myogenic regulatory factors MyoD, myogenin, Myf5 and MRF4. These basic helix-loop-helix transcription factors activate the gene expressing of the cell cycle inhibitor $p21^{Waf-1}$, which leads the cell to exit the cell cycle, followed by genes expressing skeletal muscle protein such as myosin heavy chain (MHC) (Zhang et al. 1999).

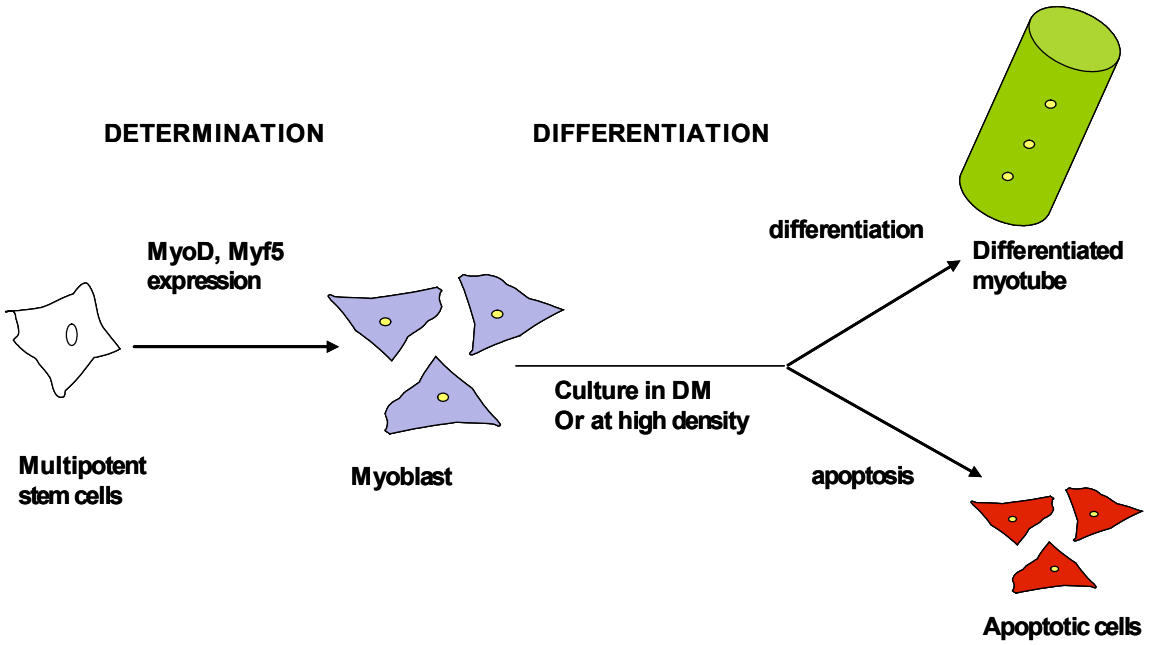


Figure 1. Skeletal myogenesis. Multipotential stem cells upon expression of MyoD or Myf5 are committed to the myogenic lineage. Upon activation of these myogenic transcription factors, actively dividing myoblasts exit the cell cycle and begin to express muscle specific proteins and later fuse to form multinucleated myotubes. A subpopulation of these myoblasts will undergo apoptosis rather than differentiation.

1.2 Myogenic transcription factors

Each of the four MRFs shares two characteristic regions: a helix-loop helix (HLH) region and a basic region. The helix-loop-helix region is required for dimerization and the basic region is required for DNA binding (Davis et al. 1990 and Voronova et al. 1990). There are two classes of bHLH proteins: Class I bHLH proteins, also referred to as E proteins, which include E12, E47, HEBα, and HEBβ and Daughterless and these are expressed in many different tissues. Class II bHLH proteins include MyoD, myogenin, and achaete-scute and are expressed in a tissue specific manner. Class I bHLH proteins can form homo- or hetero-dimers, however the Class II bHLH proteins typically form hetero-dimers with Class I bHLH proteins instead of homo-dimers (Murre et al. 1989). Class I: Class II hetero-dimers can bind both canonical and non-canonical E Boxes MyoD and E proteins contain a conserved structure of a basic region required for binding DNA at specific E boxes and a HLH region required for hetero-dimerization to E proteins. Once the MyoD:E-protein heterodimer binds E boxes found in myogenic promoters and chromatin remodeling occurs, transcription of genes required for differentiation begins. MEF2 proteins are also found to bind A/T rich regions in myogenic promoters and act in a co-operative manner with MyoD to drive transcription of the myogenic program. (Blackwell and Weintraub 1990) (Figure 2).

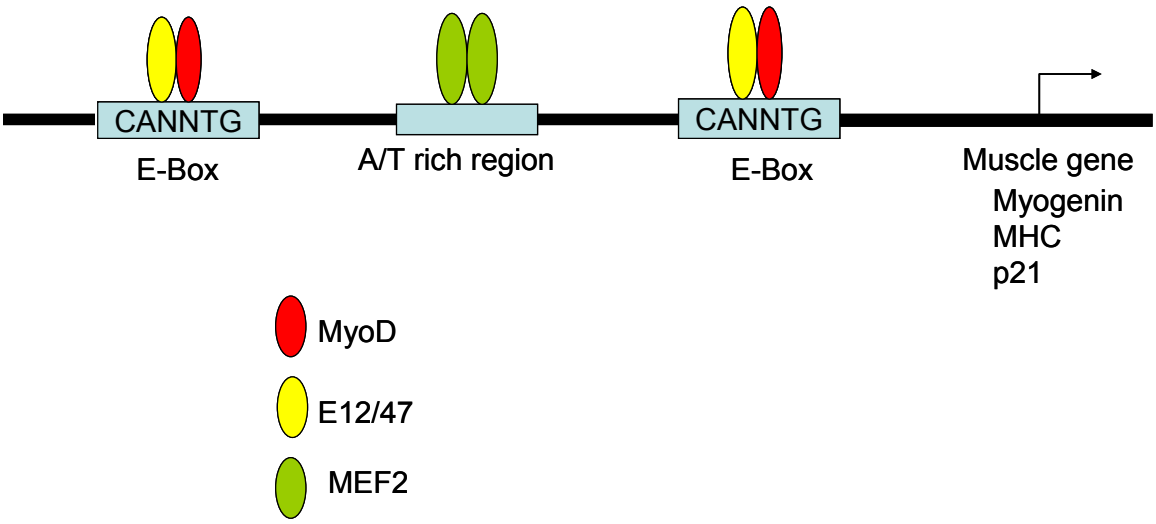


Figure 2. MyoD and E-proteins bind to canonical E box. MyoD and E proteins contain a conserved structure of a basic region required for binding DNA at specific E boxes and a HLH region required for hetero-dimerization to E proteins. Once the MyoD:E-protein heterodimer binds E boxes found in myogenic promoters and chromatin remodeling occurs, transcription of genes required for differentiation begins. MEF2 proteins are also found to bind A/T rich regions in myogenic promoters and act in a co-operative manner with MyoD to drive transcription of the myogenic program.

The MyoD protein shares sequence identity among mammalian species (88-93%) and zebra fish (73%). Additional homologues can be found in *C. elegans* (HLH1), Drosophila (Nautilus) and in jelly fish (Ci-MyoD). In muscle specific promoters, MyoD/E-protein hetero-dimers typically bind two canonical E boxes and work cooperatively with myocyte enhancing factor (MEF) proteins bound to A/T rich regions in promoters also found near the E boxes (Gossett et al. 1989 and Bergstrom et al. 2002). Interestingly, MyoD is found bound to the myogenin promoter in myoblasts cultured in growth media (GM), but is transcriptionally silent. MyoD transcription repression is achieved by the methyltransferase Suv39H1. Suv39H1 methylates the chromatin at the myogenin promoter (Harter and Mal 2003 and Mal 2006). Additionally, histone de-acetylases (HDACs) are also present at the promoters, blocking transcription activation by preventing acetylation of histones (Dillworth et al. 2004). Upon serum withdrawal, histone acetyltransferases (HATs) are recruited to myogenic promoters, chromatin is remodeled and transcription will commence (Sartorelli et al. 2005). Recruitment of chromatin remodeling complexes is required to activate the transcription of the myogenic program. An additional level of regulation of expression of the myogenic program occurs through the Id proteins. Id proteins function to block differentiation by binding E proteins or the MRFs (Friday et al. 2003, and Megeney et al. 1995). Binding of Id to E proteins or MRFs sequesters these factors in the cytosol. Once serum is withdrawn, Id is rapidly degraded, and the E protein or MRF is free to translocate to the nucleus to transcribe the myogenic suite of genes.

1.3 Skeletal myoblast model system

Since the muscle regulatory transcription factor (MRF) family consisting of Myf-5, MyoD, myogenin and MRF4, was discovered, skeletal myogenesis has served as the model for understanding signaling events regulating cell lineage determination and differentiation. Detailed information about the mechanistic regulation of myogenesis has come from studies using established mouse myoblast cell lines. The most commonly used myoblast cell lines are either isolated from C3H mice (C2C12 and BC3H-1) or derived from multipotent non-specified 10T1/2 fibroblasts also isolated from C3H mice (23A2 myoblasts). These fibroblasts were treated with 5 azacytidine, which blocks re-methylation of DNA, then selected for the expression of MyoD and the ability to undergo differentiation as determined by the expression of MHC or myogenin (Pinney et al. 1988). Additionally, 10T1/2 fibroblasts expressing a MyoD: estrogen receptor fusion protein is also utilized, where a MyoD estrogen receptor fusion protein is expressed but kept inactive until addition of estradiol to the culture medium (Pinney et al. 1988). *In vivo*, circulating mitogens keep myoblasts in an actively dividing and undifferentiated state. Differentiation of myoblasts occurs in response to a decrease in mitogens after appropriate migration during development. To imitate this response *in vitro*, skeletal myoblasts are switched from culture in GM (medium plus 10-20% fetal bovine serum) to culture in differentiation medium (DM) (medium with low (2%) or no serum) (Olson 1992).

23A2 skeletal myoblasts are 10T1/2 fibroblasts committed to the myogenic pathway through the expression of MyoD (Pinney et al. 1988). The 23A2 myoblasts can be induced *in vitro* to differentiate by switching cultured cells from GM to DM (Dee et al. 2002). Myoblasts can also be induced to differentiate by allowing cultures to grow to confluence in GM or when cells are grown without a fresh supply of serum (Dee et al. 2002). Growth factors contribute to the regulation of cell differentiation by activating specific transmembrane receptors, leading to the stimulation of multiple intracellular signal transduction pathways. MRFs are kept inactive in undifferentiated actively dividing myoblasts by signaling pathways initiated by growth factors or mitogens, such as fibroblast growth factor-2 (FGF-2) or transforming growth factor-β (TGFβ) (Ludolph and Konieczny 1995, Campbell et al. 1998). Low concentrations of insulin-like growth factors (IGFs) enhance myoblast differentiation whereas high concentrations inhibit their differentiation (Florini and Ewton 1996, Weyman and Wolfman 1998) by acting to promote cell proliferation.

We (Dee et al. 2002) and others (Wang and Walsh 1996) have previously reported that when induced to differentiate in response to mitogen withdrawal, approximately 30% of myoblasts will undergo apoptosis instead of differentiation. While the apoptotic process in other cell systems has been comprehensively investigated, the apoptotic process as a consequence of differentiation in skeletal myoblasts is only now emerging.

1.4 General Apoptosis

Apoptosis is a tightly regulated form of cell death that has the ability to clear unwanted cells without the induction of an immune response. Apoptosis is characterized by chromatin condensation, nuclear fragmentation, membrane blebbing and finally clearance of the apoptotic body via macrophages (Kerr et al. 1972). Apoptosis is important developmentally and in tissue homeostasis. Most of our current understanding of the mechanisms in apoptosis came from studies done in *C. elegans*. (Horvitz 1999). During maturation of the nematode, 1090 cells are formed but 131 of these cells undergo apoptosis at specific times. In exploring the mechanisms responsible for the well orchestrated apoptotic program in *C. elegans*, many genes were uncovered to play an integral part in the cell death program (Ellis and Horvitz 1991). Apoptosis can be triggered by a variety of stimuli, such as genetic damage, cellular stresses, cellular responses to external cues, or by aging. There are two predominant pathways cells utilize to carryout apoptosis; the intrinsic pathway and the extrinsic pathway, which will be discussed next. It is important to note that there is cross talk between these two pathways, both upstream and downstream of the mitochondria.

1.5 Extrinsic apoptotic pathway

The extrinsic apoptotic pathway is triggered by external signals which are transduced to the cell via transmembrane receptors called death receptors. These death receptors are part of the TNF receptor gene family (Locksley et al. 2001). In skeletal myoblasts, the death receptor 5 (DR5) plays an important role in apoptosis (O'Flaherty et al. 2005). Upon binding the TNF related apoptosis-inducing ligand (TRAIL), the DR5 receptor trimerizes signaling the recruitment of cytosolic adapter molecules to bind the cytosolic portion of the receptor (Sartorius 2001). Binding of the adapter molecule Fas associated death domain protein (FADD) to DR5 leads to the association of FADD with pro-caspase 8. Pro-caspase 8 then can auto-activate itself through a proteolytic cleavage (Denault 2002). Once activated, caspase 8 perpetuates the apoptotic cascade by either cleaving caspase 3 or cleaving the proapoptotic Bcl-2 protein Bid which, when activated, translocates to the mitochondria to activate Bax or Bak to initiate mitochondrial membrane permeabilization and cytochrome C release (Figure 3) (Luo 1998).

Extrinsic Apoptotic Pathway

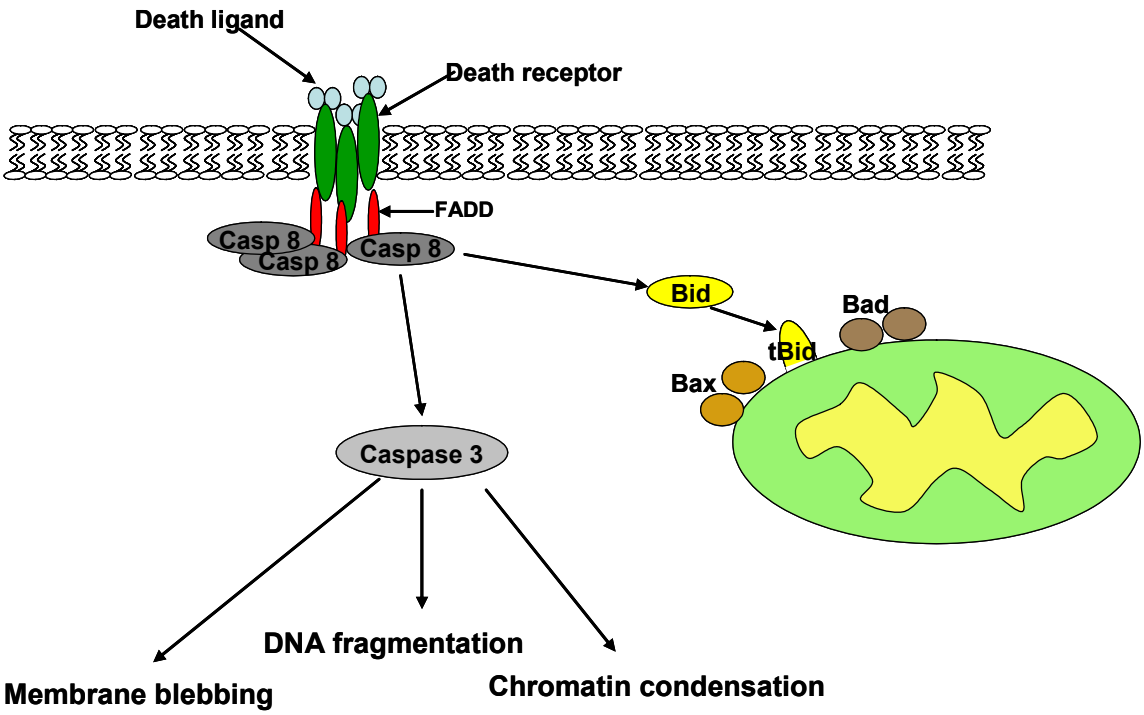


Figure 3. Extrinsic Apoptotic Pathway. The extrinsic apoptotic pathway is triggered by external signaling molecules or death ligands binding to death receptors at the plasma membrane. This binding initiates the trimerization of the receptor which in turn recruits the adapter molecule FADD to the cytosolic portion of the receptor. Once bound, caspase 8 is recruited and activated via an induced proximity self cleavage event. Activated caspase 8 then cleaves and activates caspase 3 leading to completion of the apoptotic program or cleaves the pro-apoptotic Bcl-2 molecule Bid which then translocates to the mitochondria to activate Bax or Bak which will cause disruption of the mitochondrial membrane.

1.6 Intrinsic apoptotic pathway

The Intrinsic apoptotic pathway can be triggered by a multitude of signals including but not limited to: ER stress, DNA damage, and depletion of nutrients. These intrinsic apoptotic stimuli will set into motion intracellular signaling events involving the Bcl-2 family of proteins that result in the disruption of the mitochondrial membrane, followed by the release of cytochrome C.

The Bcl-2 family of proteins makes up a group of both anti-apoptotic members and pro-apoptotic members. The anti-apoptotic members include Bcl-2, Bcl-xL, Mcl1, and Bcl-w. Pro-apoptotic members can be classified as either multidomain, such as Bax and Bak, or as BH-3 only (Bcl-2 Homology domain 3 only) such as PUMA, Noxa, Bim and Bad (Figure 4). Proapototic members can be regulated in a number of ways. For instance, regulation can occur by phosphorylation (Bad) or increased expression in response to trophic factor withdrawal (Bim and PUMA) or in response to genotoxic stress (PUMA, Noxa and Bax) or by cleavage (Bid) in response to death ligand signaling. The primary role of BH3 only members, such as PUMA, is to assist the proapoptotic functions of Bax and Bak (Chao and Korsmeyer 1998, Scorrano and Korsmeyer 2003). Interestingly, the BH-3 only proteins share only a 9 amino acid sequence similarity found in the BH-3 region (Huang and Strasser 1997) and this region is responsible for the pro-apoptotic function. Bax *or* Bak is necessary for the release of cytochrome C whereas truncated Bid (tBid) (Fidzianska and Goebel

1991), as well as distinct but yet unidentified pathway (Kandasamy et al. 2003), is required for additional mitochondrial disruption.

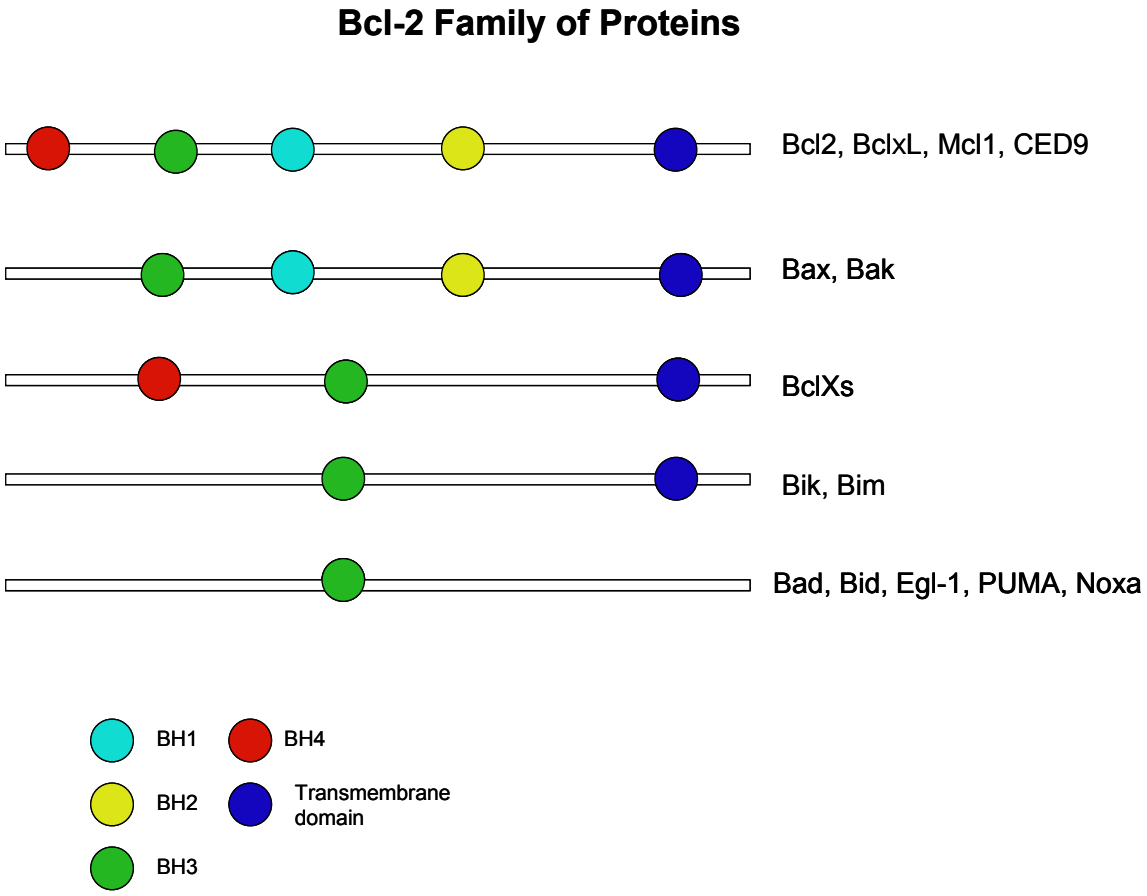


Figure 4. Bcl-2 family of proteins. This family of proteins is the watchdog of apoptosis, guarding the mitochondrial membrane. Most members contain multiple BH domains, however some contain only the BH-3 domain, which is responsible for the pro-apoptotic functions. Additionally, some members contain a transmembrane domain. Although these proteins function mainly at the mitochondria, their cytosolic locations may not be at the mitochondria when not activated.

Hypotheses of upstream events leading to mitochondrial membrane disruption vary but the general consensus is that Bax or Bak dimerization leads to the formation of a mitochondrial permeability transition pore within the mitochondrial membrane (Datta et al. 1997 and Lindsten et al. 2000). These pores cause the membrane potential to become disrupted and to the release of cytochrome C as well as other pro-apoptotic molecules (Scorrano 2002). Bax or Bak dimerization is prevented by binding of Bax or Bak to the anti-apoptotic Bcl-2 or Bcl-X_L molecules. The mechanism responsible for the interruption of the Bcl-2/Bax or Bak interaction is not fully understood, but activated Bid has been shown to play a role (Letai 2002). Additionally, it is thought that PUMA, another pro-apoptotic Bcl-2 member, may act at the mitochondria by binding Bcl-2 or Bcl-X_L (Bouillet and Strasser 2002). Once cytochrome has been released, formation of the apoptosome occurs. The apoptosome consists of the cytosolic adapter protein Apaf-1, ATP, cytochrome C and procaspase 9 (Acehan 2002). Upon formation of the apoptosome, caspase 9, an initiator caspase, is activated, and once activated, will in turn activate the executioner caspases 3, 6 and 7 (Figure 5) (Slee 1999).

Intrinsic Apoptotic Pathway

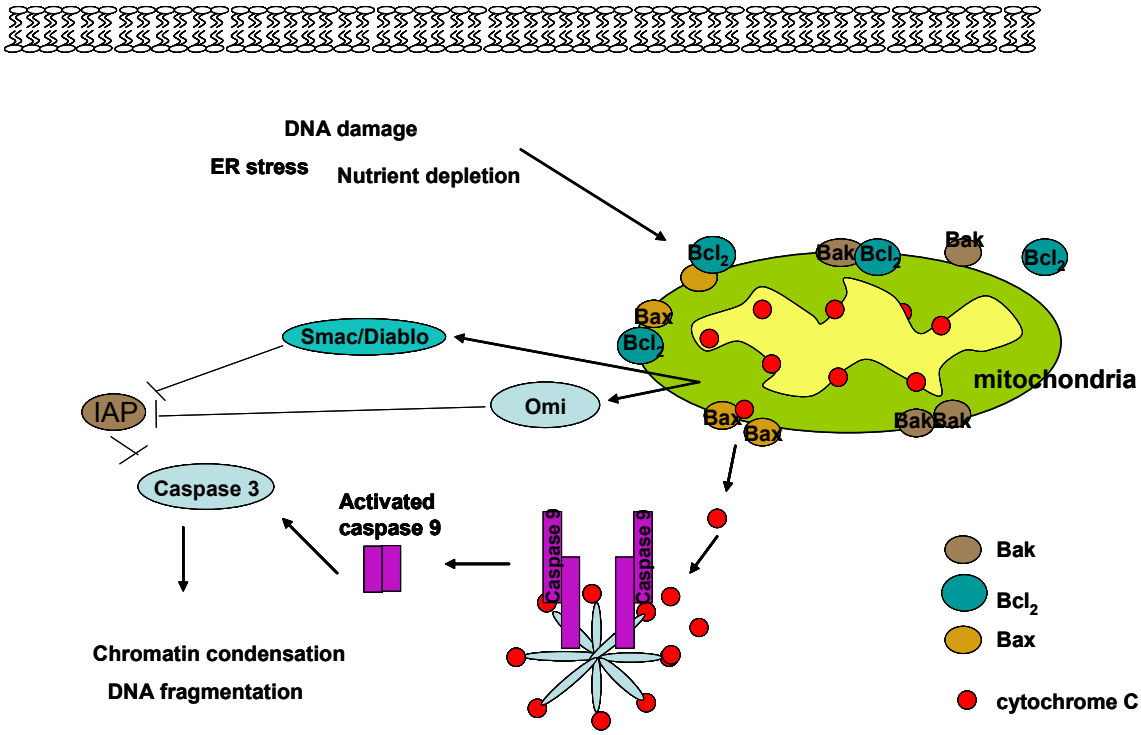


Figure 5. Intrinsic Apoptotic Pathway. Upon apoptotic stimulus, such as DNA damage, ER stress or nutrient depletion, signaling events key to the release pro-apoptotic molecules from the mitochondria. Cytochrome C binds Apaf-1 which recruits caspase 9. This complex, the apoptosome, activates caspase 9 which in turn activates caspase 3 via cleavage event. Smac/Diablo and Omi can also be released, which function to block the anti-apoptotic IAPs that block caspase 3 activation. Once caspase 3 is activated, the final stage of apoptosis occurs as seen by chromatin condensation and DNA fragmentation.

1.7 Overview of mitochondrial disruption in other systems

Apoptosis in other systems is a result of the mitochondrial outer membrane permeabilization (MOMP) followed by the release of pro-apoptotic molecules from the inner membrane space of the mitochondria (Newmeyer and Ferguson-Miller 2003). These molecules include: cytochrome C, Smac/Diablo, Omi, apoptosis inducing factor (AIF), and endonuclease G. Upon release from the mitochondrion, cytochrome c, along with the cytosolic adaptor protein Apaf-1, forms the apoptosome to activate caspase 9, an initiator caspase. A caspase (cysteine-aspartic acid specific protease) cascade is initiated by the release of cytochrome C from the mitochondria (Robertson et al. 2000, Salvensen and Dixit 1997, Thornberry and Lazebnik 1998). Smac/Diablo acts as antagonist through an inhibitory binding of cytosolic inhibitor of apoptosis proteins (IAPs) to enhance this cascade when released, whereas Omi is responsible for cleavage of IAPs. The function of IAPs is to bind and inhibit the activation sites of caspase 3, 7 and 9 and target these for proteosomal degradation. A non-canonical form of apoptosis that is caspase-independent results from the release of apoptosis inducing factor (AIF) and endonuclease G from the mitochondrial inner membrane space (19), in addition to the loss of the mitochondrial membrane potential ($\Delta\psi_m$) (Kroemer 1999). AIF and endonuclease G both can lead to DNA fragmentation and chromatin condensation in the absence of caspase activation.

1.8 Caspases

Caspases (cysteine aspartic acid-specific proteases) are proteases which are expresses as inactive zymogens or pro-caspases. Caspases can be classified as initiator caspases, which include caspases 2, 8 9 and 10, or executioner caspases, which include caspase 3, 6 and 7. The role of the initiator caspases is to activate the downstream executioner caspases. The role of executioner caspases is to carry out the degradation of cellular components including structural proteins and DNA, resulting in membrane blebbing and final clearance (Salvesan et al. 1997 and Savill and Fadok 2000). Structurally, caspases contain a prodomain, and the catalytic caspase domain. The caspase domain is subdivided into large and small subunits. Inactive caspases occur as homodimers, but upon cleavage, associate into a tetrameric complex containing two large and two small subunits (Figure 5). Caspases contain a cysteine residue within the catalytic pocket that participates in the cleavage of the carbonyl end of aspartic acid residues found within target proteins.

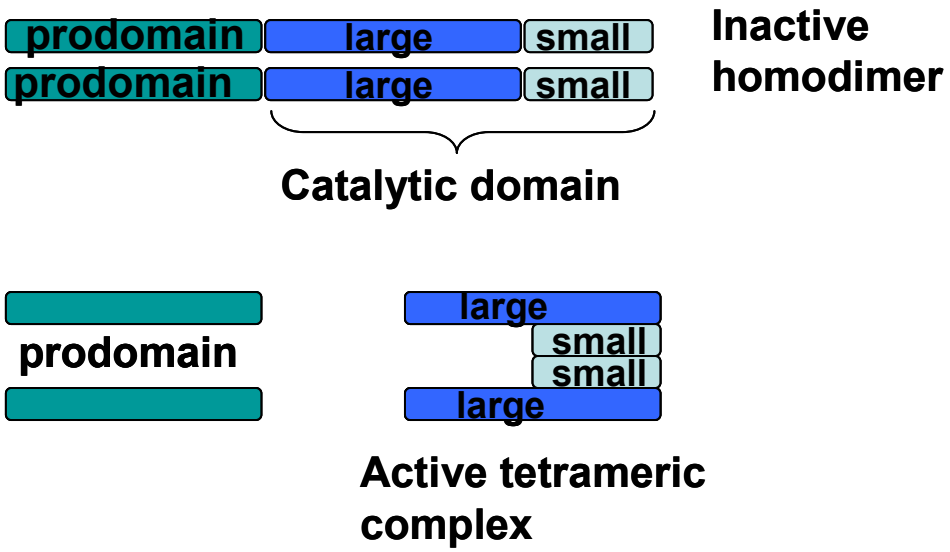


Figure 6. Caspase structure and activation. Caspases are expressed in an inactive form and exist in the cytosol as homodimers. Once an apoptotic signal is received, the procaspase domain is cleaved followed by the formation of the now active tetrameric complex. The large and small domains make up the catalytic subunit

1.9 Serine Proteases in apoptosis

Serine proteases have been implicated in the apoptotic process. Granzyme B, which is a serine protease expressed in T cells also contributes to apoptosis (Talanian et al. 1997). Interestingly, in melanoma cells, serine proteases were found to participate in apoptosis in a caspase independent manner. Omi is known to be released from the mitochondria during apoptosis and functions to cleave IAPs (Miller and Stockdale 1986). Omi is expressed in an inactive form and is activated via autocatalytic process. Once activated, Omi translocates to the cytosol where it binds XIAP (x linked inhibitor of apoptosis) protein (Hedge et al. 2002). The protease function of Omi has not yet been found (Verhagen et al.2002). Utilizing the serine protease inhibitor AEBSF, apoptosis was abrogated upon DNA damage, induced by etoposide and ER stress induced by tunicamycin or brefeldin A (de Bruin et al. 2003). Additionally, blocking caspase activation by treatment with z-VDVAD-fmk, a cell permeable caspase inhibitor that binds to the active site but cannot be cleaved, was unable to inhibit DNA damage or ER stress induced apoptosis. Similar results were also seen in rat fibroblasts, thymocytes, HeLa and neuronal cells (Egger et al. 2003). In the rat fibroblast, over-expression of Bcl-2 was able to block this caspase independent apoptosis (Egger et al. 2003). The mechanism, by which serine proteases can perpetuate apoptosis, is unknown. We have determined that AEBSF blocks the release of cytochrome C in skeletal myoblasts cultured in DM.

A serine protease that functions prior to mitochondrial disruption has not yet been reported.

1.10 Molecular mechanisms of apoptosis in skeletal myoblasts

Upon induction of differentiation in skeletal myoblasts, apoptosis occurs in vertebrate model systems, in primary cultured myoblast systems and in established cell lines, such as C2C12 myoblasts and 23A2 myoblasts (Dee et al. 2002, Wang and Walsh 1996, Fidzianska and Goebel 1991, and Sandri et al. 1996). Our lab has previously reported that when cultured myoblasts are switched from growth medium (GM) to differentiation medium (DM), approximately 30% of myoblasts will undergo to apoptosis, whereas the remaining 70% will exit the cell cycle and differentiate. The apoptotic process will occur within 12 hours while the differentiation process takes 48 hours (Dee et al. 2002). Typically, myoblasts will first exit the cell cycle, and then proceed to differentiation. Differentiation results in survival (Wang and Walsh 1996, Miller and Stockdale 1986).

Our lab has documented a role for PUMA, the TRAIL/DR5/FLIP pathway, serine proteases, release of cytochrome C and activation of caspase 9 and 3 in the apoptotic process of skeletal myoblast induced to differentiate. While regulation of PUMA (Nakanishi and Morishima 2005, Nakano and Vousden 2001, Yu et al. 2001), DR5 (Han et al. 2001) and FLIP (Wu et al. 1997) is attributed to

p53 in other systems, we have found that increased PUMA expression is not a result of p53 signaling in skeletal myoblasts. DR5 and FLIP regulation have not yet been investigated. The transcription factor FOXO3a also regulates PUMA and FLIP (Cerone et al. 2000, You et al. 2006). However, activation of FOXO3a is responsible for the increased expression of proapoptotic member Bim and TRAIL, but this does not occur in our system (Skurk et al. 2004, Wang et al. 2002). We have previously documented cells that express constitutively active forms of G12V H-Ras and Raf will not undergo differentiation and are defective for apoptosis in response to serum withdrawal (Dee et al. 2002, Wang et al. 1997). Thus our data suggests expression of G12V H-Ras leads to a decrease in the expression of MyoD.

1.11 Transcription factors associated with apoptosis

MyoD induces differentiation, but can it also play a role in apoptosis? Transcription factors associated with the expression of proapoptotic molecules include p53 (Scorrano 2003), FOXO3a (Cerone et al. 2000, You et al. 2006, Gilley et al. 2003 and Wildey and Howe 2009), STAT1 (Peschiaroli et al. 2002), SMAD3 (Kumar et al. 1997) and Myc (Wildey et al. 2003). Of these, MyoD activity most closely resembles that of either p53 or FOXO3a.

1.11.1 P53

The well known, intensely studied tumor suppressor p53 has been reported to have an impressive number of roles including regulation of metabolism, proliferation, differentiation and apoptosis. Since this body of work encompasses the study of apoptosis and to some degree, proliferation and differentiation, we will discuss only these functions of p53. One mechanism by which p53 can induce cell death is by the expression of the pro-apoptotic Bcl-2 member PUMA (Nakano et al 2001). Additionally, p53 plays a role in regulation of cell cycle progression. The cyclin dependent kinase inhibitor p21 has been shown to be activated by p53 (el-Diery 1998). Cell cycle and apoptotic regulation by p53 serves dual purposes. Firstly, exit from cell cycle can allow sufficient time to correct any harm caused by genotoxic stress, preventing tumor formation (Gatz and Wiesmuller 2006). Secondly, exit from the cell cycle or apoptosis blocks progression of tumor growth.

Interestingly, p53 has been shown to play a role in myoblast differentiation rather than apoptosis. When cultured in DM, p53-/- myoblasts showed a 50% reduction in differentiation as detected by the expression of myosin heavy chain in comparison to p53+/+ myoblasts (Porrello et al. 2000). This differentiation defective phenotype was attributed to the failure to upregulate Rb, since expression of MyoD, myogenin, p21, cyclinD1, and cyclinD3 showed no changes in expression patterns (Porrello et al. 2000). The role of Rb during differentiation is to induce cell cycle withdrawal and it cooperates in regulating

the expression of late differentiation genes (Gu et al. 1993). Also shown in the previous paper was that p53-/- myoblasts, when cultured in DM, differentiation associated apoptosis levels did not change, supporting our findings that apoptosis in skeletal myoblasts cultured in DM is a non-p53 mediated event (Shaltouki et al. 2007).

1.11.2 FOXO3a

Foxo3A is another transcription factor that serves dual roles in cells. Under certain stress conditions, Foxo3a can regulate apoptosis or cell cycle progression. One way Foxo3a can regulate apoptosis is through the increased expression of the pro-apoptotic Bcl-2 member Bim. Bim pro-apoptotic function is exerted in cells by binding the anti-apoptotic protein Bcl-2. Bcl-2 functions to inhibit apoptosis by preventing the dimerization of Bax or Bak, a step that is critical for the release of cytochrome C during mitochondrial apoptosis. Other pro-apoptotic target genes for Foxo3a are TRAIL and FasL (Obexer et al. 2007). Both FasL and TRAIL are responsible for the activation of the extrinsic apoptotic pathway by binding their respective death receptors Fas and DR5. Foxo3a can also block cell cycle progression by regulating the expression of GADD45 or p21 (Tran et al. 2002). As with p53, expression of cell cycle inhibitors can allow cells with genotoxic DNA damage to repair the damage, rather than undergo apoptosis, thus promoting survival rather than cell death.

MyoD is a helix loop helix transcription factor. To date, the only other helix loop helix transcription factors known to be involved in the apoptotic process is the Daughterless-like (HLH-2) and Achaete-scute-like (HLH-3) transcription factors expressed in *C. elegans*, shown to induce apoptosis during neuronal development (Thellmann et al. 2003). Additionally, preliminary evidence using RNAi (RNA interference) in *C. elegans* has suggested that HLH-2, along with HLH-3, regulates the expression of egl-1, a BH3 only protein similar to PUMA (Peden et al. 2008).

The role of MyoD in skeletal myoblast differentiation has been extensively studied. However, the potential role of MyoD in skeletal myoblast apoptosis is a novel idea. The purpose of this body of work is to explore the role of MyoD in regulation of the apoptosis associated with differentiation as well as in response to other more traditional apoptotic stimuli. Further, we will explore the molecular mechanisms by which MyoD can affect apoptosis. My first hypothesis is that MyoD is sufficient and necessary for the differentiation-associated apoptosis in response to culture in DM in both skeletal myoblasts and in fibroblasts. My second hypothesis is that MyoD plays a role in apoptosis in response to the DNA damaging agent etoposide and thapsigargin, an agent which induces ER stress. Lastly, my third hypothesis is that MyoD plays a role in the expression of the pro-apoptotic Bcl-2 protein PUMA in response to culture in DM and in response to etoposide and thapsigargin treatments in GM.

CHAPTER II

MATERIALS AND METHODS

2.1 Cells and cell culture

The growth and differentiation properties of 23A2 myoblasts and the 23A2 derivatives expressing the G12V:H-Ras (A2:H Ras myoblasts), A2Q61K N-Ras (N-Ras) (Weyman and Wolfman, 1997), 10T1/2 (Weyman et al. 1988) and 10T1/2 ER:MyoD fibroblasts (Hollenberg et al. 1993) have been reported previously. Cells were cultured on gelatin-coated plates and maintained in growth medium (GM), which consists of basal modified Eagle's medium (BME), 10% fetal bovine serum (FBS) and a 1% combination of 10,000 I.U./ml penicillin and 10,000 μg/ml streptomycin (1% P/S). Differentiation was induced by switching cells from growth medium to differentiation medium (DM), which consists of BME, 1% P/S and 0% FBS. Cells were incubated at 37°C in 5% CO_2.

2.2 Transient Transfection

Prior to transfection, cells were plated at 1 X 10^5 on 35 mm 6 well culture dishes or at 4X10^5 on 100 mm culture plates. A plasmid containing the full length cDNA of murine MyoD (pcDNA3:MyoD) was transfected using Lipofectamine and Plus reagents following manufacturers' instructions (GibcoBRL). Briefly, varying amounts as indicated of plasmid DNA was preincubated with Plus reagent in OPTImem low serum media, then combined with Lipofectamine preincubated with OPTImem for 15 minutes. Cells were washed with phosphate buffered saline 7.4 (PBS) followed by the addition of plasmid DNA/Lipofectamine/Plus reagent mixture to the cell culture. After three hours, the OPTImem DNA/Lipofectamine/PLUS mixture was replaced with fresh GM and cells were allowed to incubate overnight.

2.3 Immunoblot analysis

Cells were plated at 4 X 10^5 and the following day, were treated as indicated in each figure legend. Lysates were prepared by adding 50-100 μl of lysis buffer (20 mM MOPS pH 7.4, 5 mM magnesium chloride, 200 mM sucrose, 100 mM EDTA, 0.001% DNAse, 200 mM P-ser , 100 mM P-tyr, 100 mM P-thr , 100 mM PNPP, 1 M phenyl methylsufonylfluoride (PMSF) and 50 μg/ml each of aprotinin, pepstatin, and leupeptin with 1% CHAPS). Protein concentrations of the cell lysates were determined using Coomassie Protein Assay reagent from Pierce per manufacturer's instructions. Following protein determination, lysates

were denatured in 5X sample buffer containing 10% SDS, 50% glycerol, 10% 2-mercaptoethanol, 300 mM Tris HCl, and pH 6.8 and 0.0025% bromphenol blue.

Protein extracts were separated through denaturing polyacrylamide gels following the Laemmli method (Laemmli 1970). Following SDS polyacrylamide gel electrophoresis (SDS-PAGE), samples were transferred electrophoretically from thirty-five minutes to one hour at 1500 milliamps onto Hybond-P polyvinylidene difluoride membrane in transfer buffer (20% methanol, 1 g/L SDS, 5.8 g/L Tris base and 29 g/L glycine. After transfer, membranes were placed in a blocking solution of 5% non-fat dry milk, 10% newborn calf serum in TBS-T buffer (136.9 mM NaCl, 25 μM Tris and 1% Tween20). Immunoblot analysis was performed to detect: p53 by incubating membrane for 1 hour with p53 antibody diluted 1:1000 , MHC by incubating membrane for one hour with a mouse monoclonal antibody MF20 that is specific for skeletal myosin heavy chain protein (Bader et al. 1982), MyoD by incubating membrane overnight with a mouse monoclonal MyoD antibody (BD Biosciences, 554130) diluted 1:1000, PUMA by incubating membrane overnight with a rabbit polyclonal PUMA antibody (Abcam ab-9643) diluted 1:500. Actin or Hsp70 expression levels were monitored to ensure equal loading using monoclonal anti-β actin antibody (Sigma A5441- 0.5ML) diluted 1:30,000 or anti-Hsp70 antibody (BD Biosciences 610608). All membranes were then incubated in an HRP-conjugated secondary antibody to the primary antibody for one hour diluted 1:1000. Following incubation with antibodies and prior to the addition of chemiluminescence

substrate, the membranes were washed 5 times in TBS-T buffer. Membranes were then incubated with SuperSignal West Pico Chemiluminescence Substrate (Pierce) as instructed by the manufacturer and bands were visualized by using Kodak Scientific Imaging film.

2.4 ELISA

The presence of cytosolic nucleosomes is a hallmark of apoptosis and indicates that DNA fragmentation has occurred. Cytosolic nucleosomes were detected using the Cell Death Detector ELISA (Enzyme linked *i*mmunosorbant assay) plus kit (Roche Diagnostics). Briefly, G12V H-Ras myoblasts or 23A2 myoblasts were plated 10^5 per well in 6 well plate. Next day, G12V H-Ras myoblasts were transfected as described in figure legend and next day transferred to culture in GM or DM for 8 hours as noted. Cells were lysed in 100 μl lysis buffer from Cell Death Detection ELISA kit for 30 minutes with rocking at room temperature. The contents of this lysis buffer are unknown (proprietary according to Roche). This lysis buffer only breaks plasma membrane and leaves nuclear membrane intact. Cytosolic extracts were then incubated in 96-well microtiter plate pre-coated with streptavidin. Additionally, a mater mixture containing 72 μl incubation buffer and 4 μl each histone:biotin antibody and DNA:ABTS antibody was added to each well The lysate/antibody mixture was incubated at 4ºC rocking overnight. Next day, plate was washed three times with PBS, followed by addition of 100 μl ABTS substrate prior to reading on plate reader at 405 nm wavelength at 60

second intervals for 30 minutes. Readings were taken at a time point when in linear range.

2.5 Stable transfection of shRNA for gene knockdown

2.5.1 Assembly of lentiviral vector for transduction of myoblasts

We purchased a pKLO.1 vector expressing a shRNA against murine MyoD or no insert. These expression vectors were transformed in DH5α E. coli, then grown up on agar plates containing the selective antibiotic carbenicillin (Sigma C1389). The expression vector was grown in broth culture and purified using Qiagen Midiprep kit.

Next, lentivirus was assembled using HEK293Tcells. 293T cells were transfected with pKLO.1 shRNA vectors along with pCMVdelta 8.2 lentivirus vector and pVSV-G vector expressing viral coat protein. Briefly, 2 million cells were plated on 60mm plate. Next day, media was removed and washed with PBS, followed by transfection with solution containing 300 μl OPTImem, 12 μl Lipfectamine 2000 and 5 μg each pKLO.1, pCMVΔR8.2 and pVSV-G vectors for 10 hours. After 10 hours transfection reagent was removed and 4 ml DMEM containing 10% FBS and 1% penstrep was added. After 24 hours, media containing the assembled shMyoD expressing lentivirus or empty vector was collected, and again fresh DMEM was added. This was repeated for three days. The collected

DMEM was centrifuged to remove cellular debris then flash frozen at -80ºC for future use.

2.5.2 Stable transduction of myoblasts with lentivirus containing pKLO.1 puro-MyoD shRNA or pKLO.1 puro (control vector)

23A2 or C2C12 myoblasts were plated at 400,000 cells per plate and next day transduced with lentivirus. Briefly, 23A2 myoblasts and C2C12 myoblasts were cultured in previously frozen DMEM containing lentivirus plus 10ug/ml polybrene overnight. Next day, DMEM was removed and replaced with fresh GM for 24 hours. Next day, cells were split into 3 150 mm plates and cultured in GM overnight. Next day, cells were cultured in GM plus puromycin (2ug/ml Sigma P8833) for two weeks. After two weeks, clonal populations were selected for further analysis of MyoD expression by immunoblot analysis and RT-PCR.

2.6 Semi-quantitative RT- PCR

Cells were plated at $4x10^5$ /100 mm plate and cultured in GM overnight. Next day cells were cultured as indicated in figure legends. Total RNA was isolated using standard Trizol (Invitrogen) procedures as indicated by manufacturer. Following extraction and quantitation of RNA, 0.5-1ug of total RNA was used in a 20 μl reverse transcription reaction using superscript III (Invitrogen) as indicated in manufacturers instructions with dNTPs, random hexamers, DTT and 5X

manufacturer buffer. 2 μl of the cDNA was used in real time PCR reaction with forward and reverse primers and 10 μl of Platinum® SYBR®Green qPCR SuperMix (Invitrogen). Reactions were performed in triplicate, amplified and quantified using the Opticon 2 quantitative real time PCR system (MJ Research). The cycle number at which fluorescence increased linearly was calculated by the Opticon monitor in the form of C_t values. The C_t value for GAPDH (used as a control reaction) was subtracted from the GAPDH value for the untreated control (GM, vehicle control, etc.) to obtain the correction factor ($\Delta C_{t\ (GAPDH)}$). This correction factor was then subtracted from the C_t value for each experimental mRNA (m_{exp}): ($C_{t(mexp)}$- $\Delta C_{t\ (GAPDH)}$= normalized $C_{t(mexp)}$. The normalized value for each experimental mRNA was then subtracted from the value of the corresponding untreated control to obtain the difference in cycle number. The difference of a single cycle is the equivalent to a 200 percent change in mRNA, so that the normalized value of each experiment is expressed as a change over the untreated sample (set to 100%)

2.7 RT-PCR

Reverse transcription is the same as described above. The PCR reaction differs as follows. Again 2 μl of the cDNA was used in the PCR reaction along with forward and reverse primers plus 10ul of 2X PCR mix (Fermentas). PCR reactions were run for the number of cycles in which amplification of products were still within a linear range as determined by previous reactions. PCR

products were then run on 1% agarose gel stained with ethidium bromide. Images were analyzed using GE Healthcare Life Sciences Typhoon 9410 Imager and band volume quantified using ImageQuant software (v5.2).

Table 1: Primers for PCR

Message	Forward Primer	Reverse Primer
MyoD	GACAGGACAGGACAGGGAGG	GCACCGCAGTAGAGAAGTGT
PUMA	CCAGAAATGGAGCCCAACTA	TATGCTCTTCACAGACCCCC
GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTAGGCCAT

CHAPTER III

RESULTS

3.1 MyoD regulates the apoptosis associated with differentiation

Our lab has shown that switching cultured 23A2 skeletal myoblasts from GM (growth medium) to DM (differentiation medium) induces approximately 30% of cells to undergo apoptosis (DeChant et al. 2002). Our laboratory (Karasarides et al. 2006) and others (Konieczny et al. 1989) have previously shown that skeletal myoblasts expressing oncogenic G12V:H-Ras cause 23A2 myoblasts to become differentiation defective. Additionally, in myoblasts expressing oncogenic G12V H-Ras, levels of both MyoD mRNA and protein are below the level of detection (Konieczny et al. 1989, Karasarides et al. 2006). Re-expression of MyoD in myoblasts expressing G12V:H-Ras can restore differentiation (Koniecnzy et al. 1989). Our lab has recently reported that the apoptotic potential of skeletal myoblasts correlates with expression levels of MyoD (Karasarides et al. 2006). These findings have lead us to hypothesize that MyoD may play a critical role in both the ability to undergo apoptosis or differentiation as a result of

serum withdrawal. To test this hypothesis, we utilized 23A2 skeletal myoblasts stably transfected with oncogenic G12V:H-Ras or Q61K:N-Ras. In the 23A2 myoblasts expressing G12V H-Ras, MyoD expression is below the levels of detection, whereas MyoD expression in Q61K:N-Ras expressing myoblasts is reduced as compared to mock transfected myoblasts (Figure 7). 10T1/2 fibroblasts which do not express MyoD are included as a negative control.

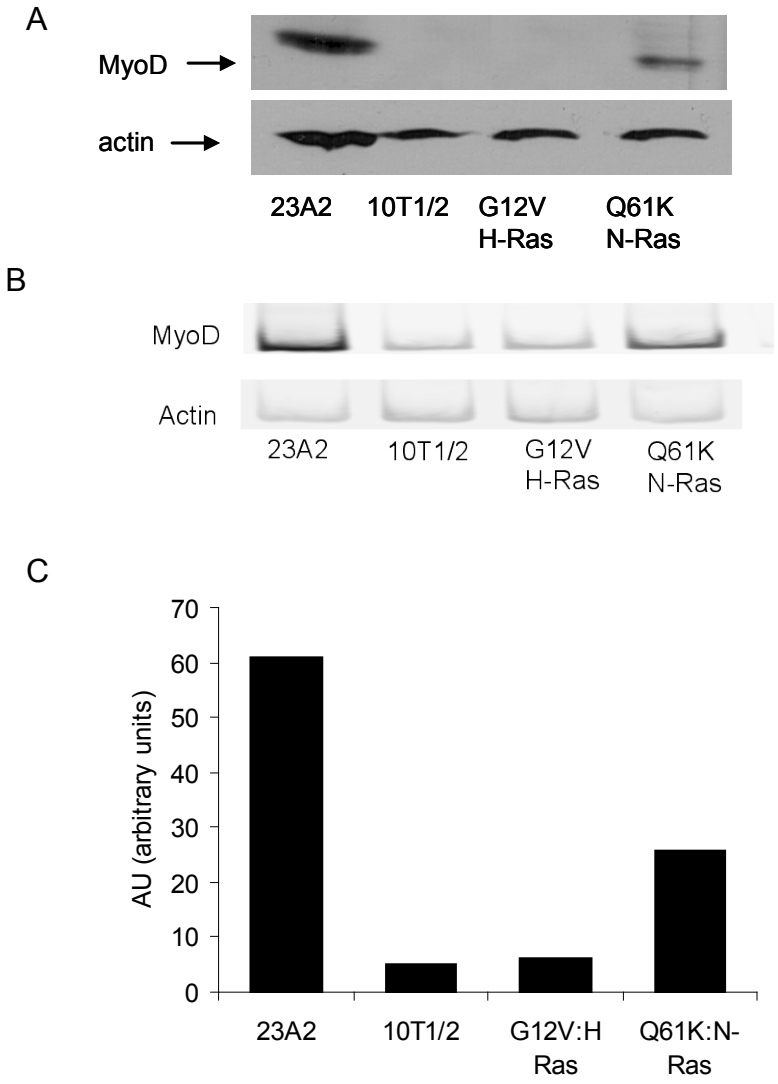


Figure 7. MyoD expression is reduced in oncogenic Ras expressing skeletal myoblasts. Equal numbers of cells were plated and next day, in (A). whole cell lysates were collected, separated by SDS-PAGE prior to electrophoretic transfer to PVDF membrane and immunoblot analysis for MyoD. Shown are results from one experiment that are representative of 2 independent experiments. In (B) total cellular RNA was isolated using standard Trizol method followed by reverse transcription. DNA was subjected to PCR analysis using Alexa Fluor 647 labeled primers for MyoD and actin. In (C) images from B were analyzed using GE Healthcare Life Sciences Typhoon 9410 Imager and band volume quantified using ImageQuant software (v5.2). Shown are results from one experiment that are representative of 2 independent experiments.

In order to measure apoptosis in different oncogenic Ras expressing cell lines, we cultured equal number of cells with DM for 18 hours followed by counting detached cells and total number of cells. Myoblasts undergoing apoptosis will become detached from the tissue culture plate whereas cells which are either quiescent or undergoing differentiation will remain adherent (Dee et al. 2002). The myoblasts expressing different oncogenic Ras isoforms exhibit reduced apoptotic potential as compared to the parental 23A2 myoblast (Figure 8).

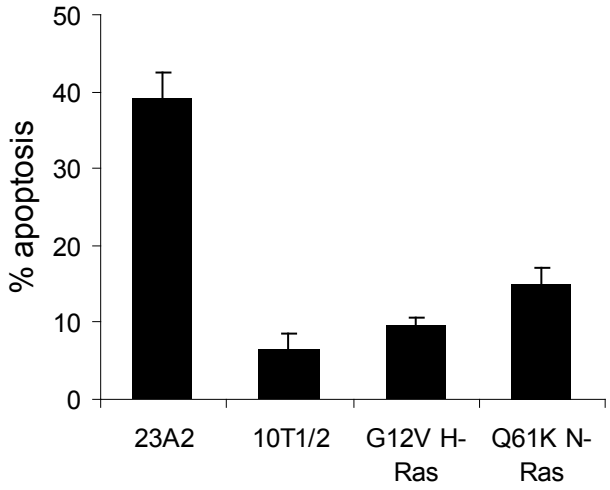


Figure 8. Oncogenic Ras-expressing myoblasts have reduced apoptotic potential. Equal numbers of cells were plated and next day switched to DM for 18 hours. DM was collected and floating cells were counted. Cells remaining on the tissue culture plate were counted. Percent apoptotic cells were calculated by taking detached cells divided by total cells (adherent + detached). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments.

To further support our hypothesis that MyoD plays a role in apoptosis, we transiently re-expressed ectopic MyoD in oncogenic G12V:H-Ras expressing myoblasts at levels comparable to the endogenous levels found in parental 23A2 myoblasts. We decided to use this oncogenic Ras expressing myoblast cell line since endogenous levels of MyoD expression were below the levels of detection, making ectopic expression of MyoD easy to distinguish from any endogenous MyoD expression present. Both differentiation and apoptosis are induced by culture in differentiation medium (DM). This media contains low or no serum and mimics the decreased gradient of growth factors which myoblasts encounter as they migrate away from the notochord during embryonic development (Olson 1992). The next day after transient transfection, we then cultured myoblasts in DM for 24 hours and assayed for the expression of MHC as a marker for differentiation. We performed immunoblot analysis on oncogenic H-Ras expressing myoblasts expressing ectopic MyoD and found that differentiation was restored as evidenced by the expression of MHC (Figure 9).

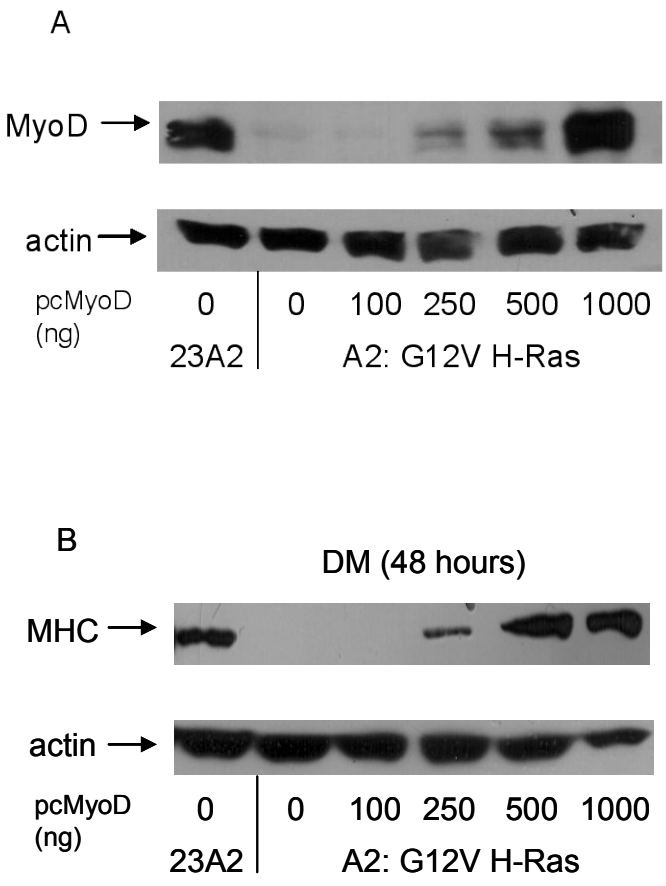


Figure 9. Transient ectopic expression of MyoD in G12V H-Ras myoblasts is sufficient to restore differentiation. Equal numbers of cells were plate and next day transiently transfected with the indicated amount of plasmid for 3 hours prior to switching to GM for 24 hours. In (A) whole cell lysates were collected then separated by SDS-PAGE and followed by immunoblot analysis for MyoD expression. Actin was monitored for equal loading. In (B) cells were cultured in DM for 24 hours followed by collection of whole cell lysates which were then separated by SDS-PAGE prior to electrophoretic transfer to PVDF membrane and immunoblot analysis for MHC expression. Actin was monitored for equal loading. Shown are results from one experiment that are representative of two independent experiments.

As the process of transient transfection can induce cytosolic nucleosome formation, we were required to wait 24 hour post transfection before beginning any apoptosis studies which involved the detection of apoptosis by measuring cytosolic nucleosomes. We previously determined that the optimum time for the detection of cytosolic nucleosomes in parental 23A2 myoblasts was after eight hours of culture in DM. We compared the level of cytosolic nucleosomes using the ELISA kit (enzyme linked immunosorbant assay) (Roche) that utilizes an antibody directed against cytosolic histones and an antibody against DNA conjugated with peroxidase, which when activated in the presence of ABTS, results in a colorimetric change that that can be quantitative. Myoblasts undergoing apoptosis will contain DNA/histone fragments in the cytosol. Only cytosolic lysates are used in this assay. We found that ectopic expression of MyoD in oncogenic H-Ras expressing myoblasts restores their apoptotic potential (Figure 10). Thus, MyoD confers the ability to undergo both differentiation and the associated apoptosis.

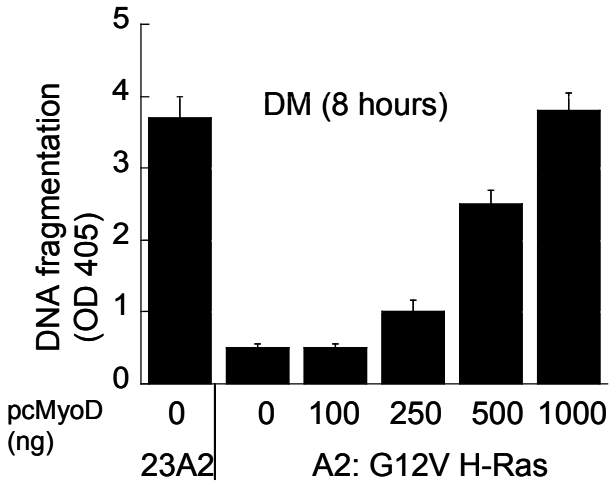
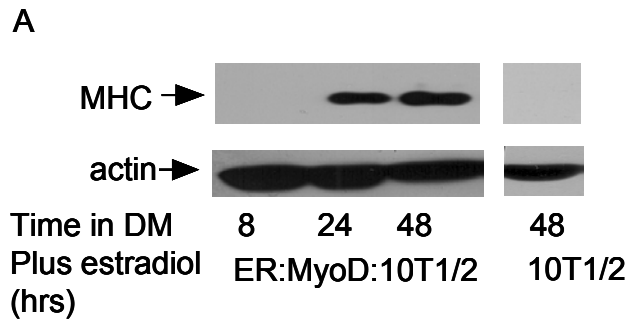


Figure 10. Ectopic expression of MyoD in G12V H-Ras expressing myoblasts restores apoptotic potential in response to serum withdrawal. Equal numbers of cells were plated and next day transiently transfected with the indicated amount of plasmid as indicated for 3 hours prior to switching to GM for 24 hours. Cells were treated with fresh GM or DM as indicated for 8 hours. DNA fragmentation was assayed using the Cell Death Detection ELISAPLUS kit. Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments.

Next, we determined if MyoD expression could induce apoptosis in fibroblasts in response to serum withdrawal by culture in DM. We utilized 10T1/2 fibroblasts that stably express an inducible estrogen receptor: MyoD fusion protein (ER-MyoD:10T1/2) to perform the next set of experiments. The ER-MyoD fusion protein is expressed but cannot induce transcription unless treated first with estradiol. When estradiol is added to the culture medium, this allows ER-MyoD fusion protein to translocate to the nucleus. We assessed the ability of these fibroblasts to undergo differentiation as evidenced by the expression of myosin heavy chain. This was included as a control to show that the ER-MyoD fusion protein could induce differentiation. Immunoblot analysis shows when cultured in DM with estradiol, these fibroblasts express myosin heavy chain (Figure 11A). To determine the apoptotic potential, fibroblasts were cultured in DM for 24 hours with or without estradiol. In the absence of estradiol, the ER-MyoD:10T1/2 fibroblasts contained no more cytosolic nucleosomes than the parental 10T1/2 fibroblasts cultured in DM. However, the apoptotic potential was increased seven fold in ER-MyoD: 10T1/2 fibroblasts cultured in DM plus estradiol as compared to the parental 10T1/2 fibroblasts cultured in DM plus estradiol (Figure 11B). We also compared the expression of MyoD protein when cultured in GM as well as MHC when cultured in DM between 23A2 and C2C12 myoblasts, 10T1/2 and ER-MyoD10T1/2 fibroblasts +/- estradiol as noted. MyoD levels between each cell line correlated to the expression of MHC (Figure 12).



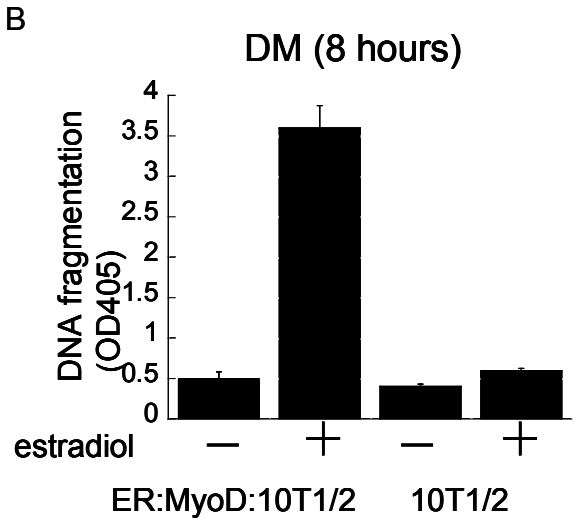


Figure 11. MyoD activation in 10T1/2 fibroblasts expressing an estrogen receptor MyoD fusion protein sensitizes to the induction of differentiation or the associated apoptosis in response to serum withdrawal. In (A) parental 10T1/2 fibroblasts or ER-MyoD:10T1/2 fibroblasts were cultured in DM 48 hrs +/- estradiol prior to collection of whole cell lysates. Immunoblot analysis for myosin heavy chain (MHC) expression was performed using 100 μg of total protein. Actin was monitored for equal loading. Shown are results from one experiment that are representative of 2 independent experiments. In (B) 10T1/2 or ER-MyoD:10T1/2 fibroblasts were cultured in DM +/- estradiol for 8 hours prior to analysis of DNA fragmentation using the Cell Death ELISAplus kit. Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments.

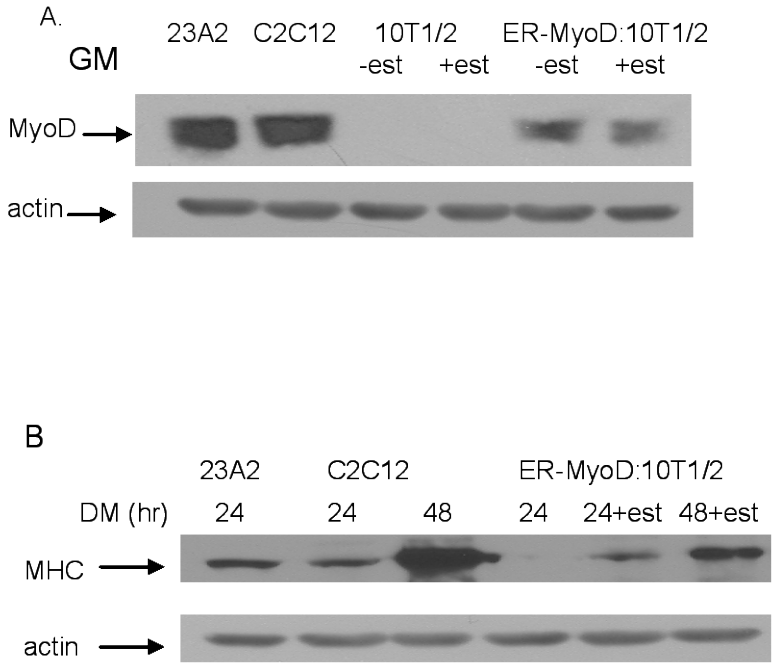


Figure 12. MyoD protein expression correlates with MHC expression between cell lines utilized. Equal numbers of cells were plated. Immunoblot analysis was performed for the expression of (A) MyoD or (B) MHC between all cell lines as noted in (A) GM or (B) DM for various times, using 100 μg whole cell lysates separated by SDS-PAGE prior to electrophoretic transfer to PVDF membrane. Shown are results from one experiment that are representative of 2 independent experiments.

Having determined that expression of MyoD plays a role in the ability of myoblasts and fibroblasts to undergo apoptosis, in addition to its well known role in differentiation, we next hypothesized that absence of MyoD in skeletal myoblasts would render these cells defective for both differentiation and the associated apoptosis. To test this hypothesis, we utilized RNAi technology. We transduced 23A2 and C2C12 myoblasts with a lentivirus carrying a pKLO.1 puro-murine MyoD specific short hairpin RNA (shRNA) or control vector pKLO.1 puro and selected using the antibiotic puromycin for two weeks. After the selection period, we selected clonal populations to analyze for the knock down of MyoD expression. MyoD expression in untreated parental 23A2 or C2C12 myoblasts (each designated as “P”), or a vector alone transduced 23A2 or C2C12 myoblasts clone (each designated as “C”) as well as multiple clones was analyzed (23A2:shMyoD clones or C2C12:shMyoD clones). Monitoring MyoD expression by both immunoblot analysis and qRT-PCR, we were able to successfully knock down MyoD to varying degrees in 23A2 myoblasts and in C2C12 myoblasts using the shRNA directed against murine MyoD (Open Biosystems) (Figures 13 and 14). Having determining the expression level of MyoD, we selected clones 23A2:shMyoD 13, 15 and 16 and C2C12:shMyoD 16, 17 and 19, for further analysis.

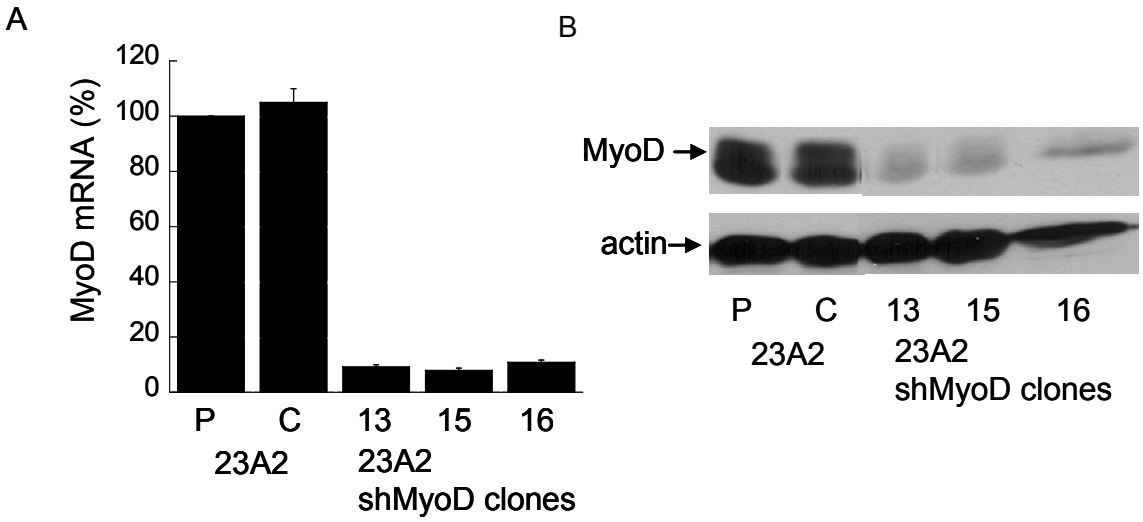


Figure 13. Lentiviral transduction of shRNA against MyoD reduces the expression of MyoD in 23A2 myoblasts. 23A2 myoblasts were transduced with shRNA against MyoD and various clones were screened for the reduction in MyoD expression levels. In (A) equal numbers of cells were plated and the next day total cellular RNA was isolated using the standard TRIZOL method. Quantitative RT-PCR was performed using the Quantitech Sybr Green PCR kit (Qiagen). Fluorescence was detected using an Opticon Monitor (MJ Research) Opticon software was used to calculate cycle threshold (Ct) values. The Ct value for MyoD PCR product was normalized to the Ct value for actin PCR product run parallel. Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments. In (B) equal numbers of cells were plated. Immunoblot analysis of MyoD protein expression was performed. Actin was monitored for equal loading. Shown is the result from one experiment that is representative of three independent experiments.

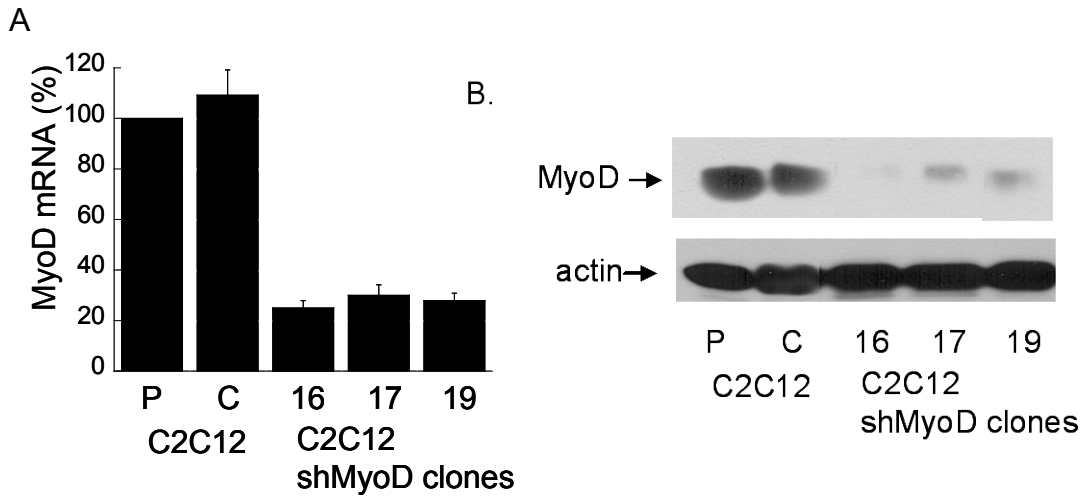


Figure 14. Lentiviral transduction of shRNA against MyoD reduces the expression of MyoD in C2C12 myoblasts. C2C12 myoblasts were transduced with shRNA against MyoD and various clones were screened for the reduction in MyoD expression levels. In (A) equal numbers of cells were plated and the next day total cellular RNA was isolated using the standard TRIZOL method. RT-PCR was performed for analysis of MyoD mRNA expression. PCR product was separated on an ethidium bromide stained agarose gel. Images were analyzed using GE Healthcare Life Sciences Typhoon 9410 Imager and band volume quantified using ImageQuant software (v5.2). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments. In (B) an equal numbers of cells were plated. Immunoblot analysis of MyoD protein expression was performed. Actin was monitored for equal loading. Shown are results from one experiment that are representative of 2 independent experiments.

As a control, these clones were assessed for their ability to undergo differentiation. We hypothesized that myoblasts will be rendered differentiation defective in the absence of MyoD expression. We assayed the ability of these myoblasts to undergo differentiation as evidenced by the expression of myosin heavy chain after culture in DM for 48 hours. As anticipated, immunoblot analyses shows both that the 23A2:shMyoD clones and the C2C12:shMyoD clones with reduced MyoD expression fail to undergo differentiation when cultured in DM (Figure 15). Finally, we also monitored these clones for their ability to undergo apoptosis in response to culture in DM. As predicted, 23A2:shMyoD and C2C12:shMyoD myoblasts with reduced MyoD expression were resistant to the induction of apoptosis, in response to culture in DM (Figure 16).

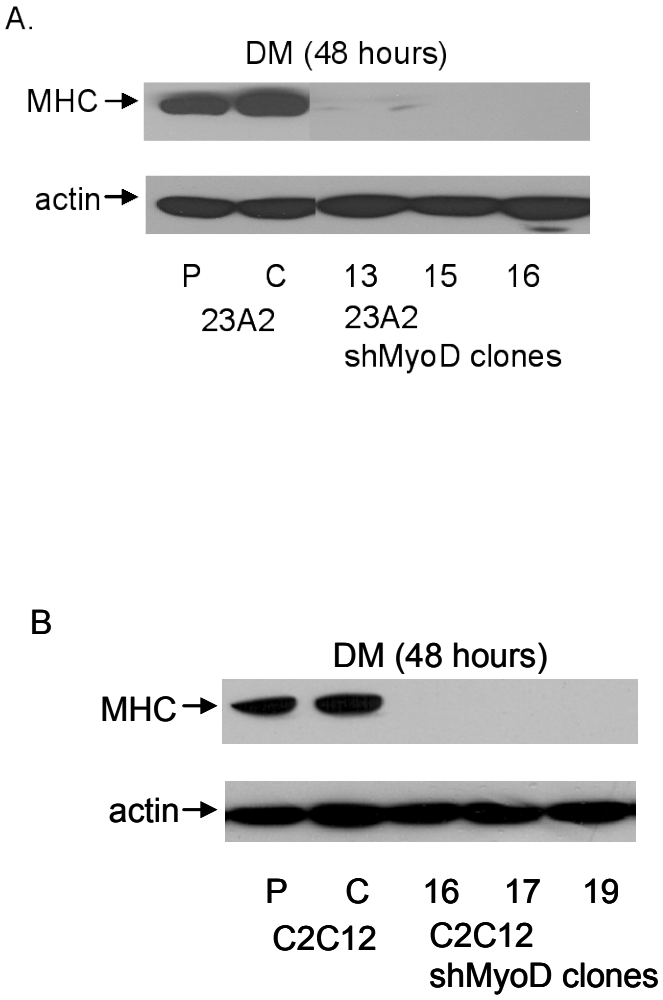


Figure 15. Stable silencing of MyoD expression in 23A2 and C2C12 skeletal myoblasts represses differentiation. Equal numbers of cells were plated and next day cultured in DM for 48 hours. Whole cell lysates were collected then 100 μg of total protein was separated by SDS-PAGE prior to electrophoretic transfer to PVDF membrane and immunoblot analysis for MHC expression in (A) 23A2 myoblasts parental, control and shMyoD clones and MHC expression in (B) C2C12 myoblasts parental, control and shMyoD clones. Actin was monitored for equal loading. Shown is the result of one experiment that is representative of three independent experiments.

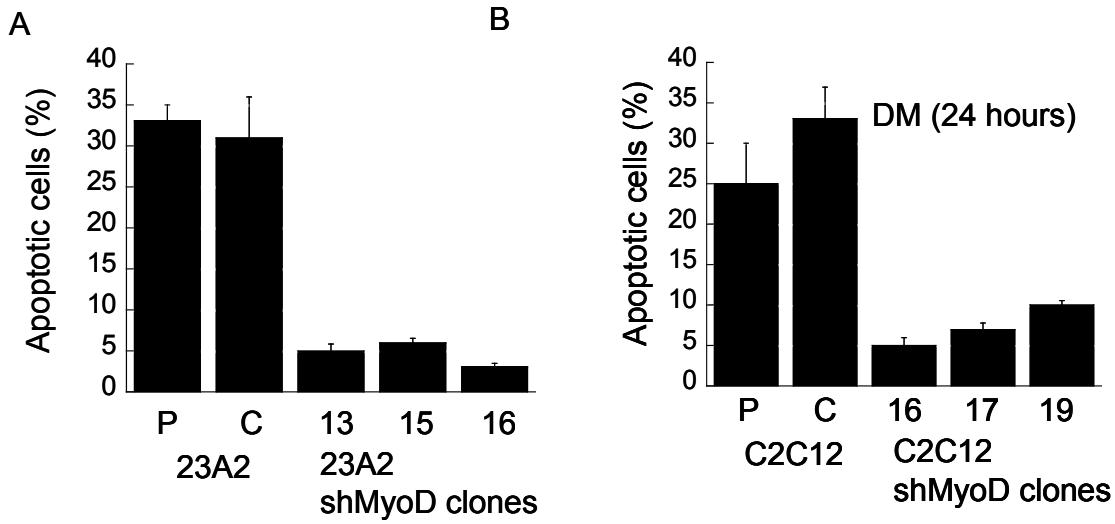


Figure 16. Stable silencing of MyoD expression in skeletal myoblasts represses apoptosis as a consequence of serum withdrawal. Equal numbers of cells were plated and next day switched to fresh GM or DM for 24 hours. In (A), 23A2 myoblast parental, vector, and shMyoD clones and in (B) C2C12 myoblast parental, vector and shMyoD clones, next day, detached cells and adherent cells were collected and then counted. Percent apoptosis was calculated (number of detached cells/ detached cells plus adherent cells). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments.

3.2 MyoD regulates apoptosis in response to other apoptotic stimuli

Since we have determined that MyoD plays a role in the apoptosis associated with differentiation and since this can also be considered as apoptosis induced in response to serum withdrawal, we next hypothesized that MyoD might regulate apoptosis in response to other apoptotic stimuli. We next wanted to analyze apoptosis in response to DNA damage or ER stress.

Prior to testing this hypothesis, we performed time and concentration course analysis for the treatment with etoposide, an agent that induces double- and single-strand breaks in DNA in intact cells or thapsigargin, a compound that causes the release of stored calcium from the ER and blocks calcium re-uptake, leading to toxic intracellular levels. We determined that the optimum time and concentration for treatment to easily detect apoptotic cells with etoposide was 200 μM for 24 hours and with thapsigargin it was 3 μM for 24 hours (Data not shown). We then assessed the effect of MyoD expression in myoblasts on the apoptosis induced by etoposide or thapsigargin. As predicted by the previous experiments, etoposide and thapsigargin each induced apoptosis in the 23A2 and C2C12 parental (P) or empty vector transduced (C) myoblasts. As hypothesized, the 23A2:shMyoD and C2C12:shMyoD clones, where MyoD expression was reduced, were resistant to the induction of apoptosis induced by etoposide or thapsigargin (Figures 17 and 18 respectively).

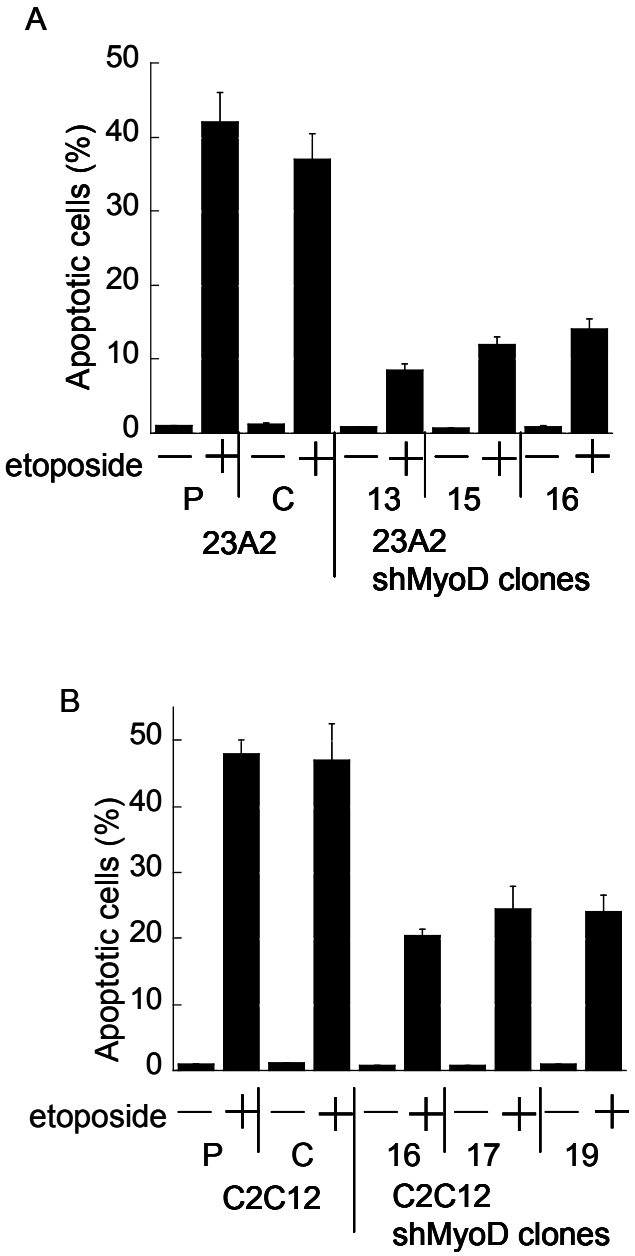


Figure 17. MyoD expression enhances apoptosis induced by agents which cause DNA damage. Equal numbers of cells were plated and next day treated with vehicle (DMSO) or 200 uM etoposide in GM for 24 hours. In (A), 23A2 myoblast parental, vector and shMyoD clones and in (B) C2C12 myoblast parental, vector and shMyoD clones, next day, detached cells and adherent cells were collected, then counted. Percent apoptosis was calculated (number of detached cells/ detached cells + adherent cells). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments.

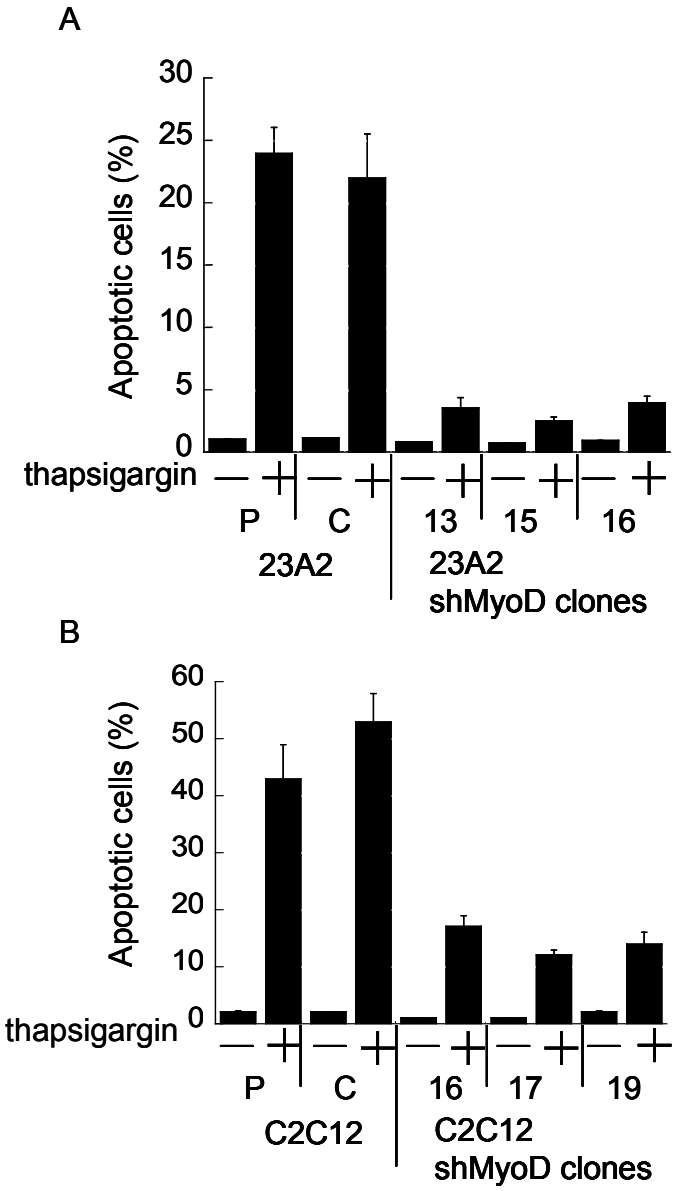


Figure 18. **MyoD expression enhances apoptosis induced by agents which cause ER-stress.** Equal numbers of cells were plated and treated with vehicle or 3μM thapsigargin for 24 hours. In (A), 23A2 myoblast parental, vector and shMyoD clones and in (B) C2C12 myoblast parental, vector and shMyoD clones, next day, detached cells and adherent cells were counted and percent apoptosis was calculated (number of detached cells/ detached cells plus adherent cells). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments.

We next assessed the effect of MyoD in fibroblasts on the apoptosis induced by etoposide or thapsigargin treatment. Using the same conditions as previously described for the myoblasts, we treated 10T1/2 fibroblasts and ER:MyoD 10T1/2 fibroblasts for 24 hours with 200 μM etoposide or 3μM thapsigargin and assessed apoptosis. As expected, the etoposide and thapsigargin treatments each induced apoptosis in the 10T1/2 fibroblasts and ER-MyoD:10T1/2 fibroblasts, both in the absence of estradiol. However, while the addition of estradiol had no effect on the apoptosis induced by etoposide or thapsigargin in the 10T1/2 fibroblasts, the apoptotic response was increased over two times by the presence of estradiol in ER-MyoD:10T1/2 fibroblasts (Figure 19).

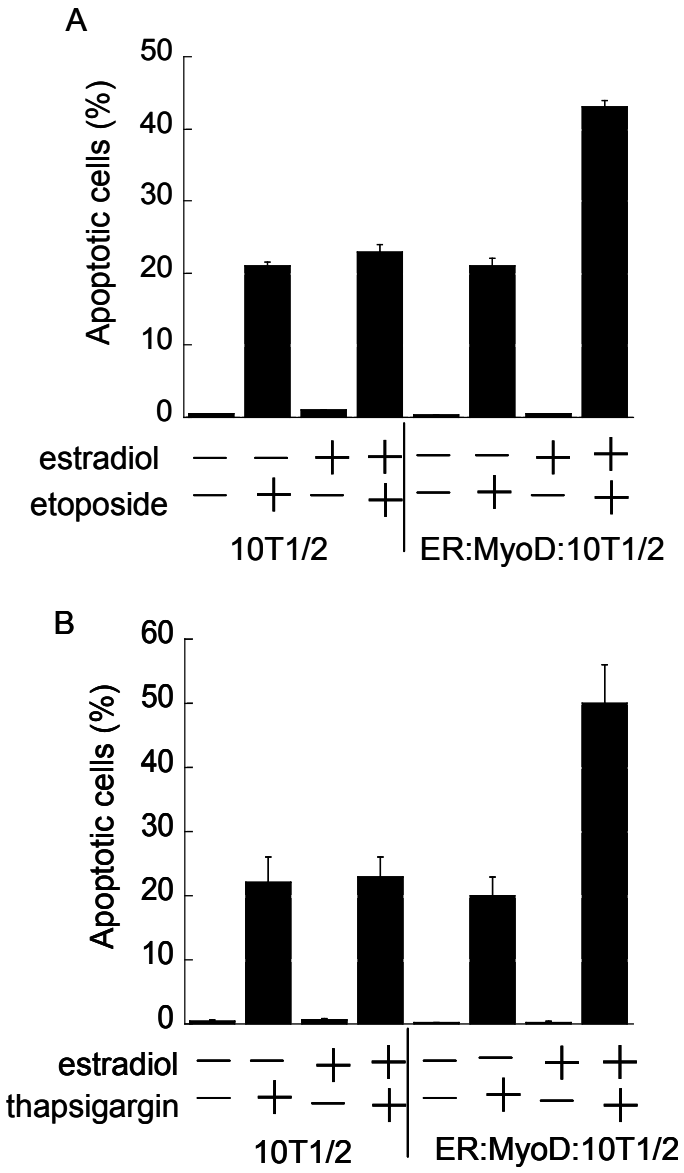


Figure 19. **MyoD activation in 10T1/2 fibroblasts expressing an estrogen receptor MyoD fusion protein leads enhanced apoptosis induced by agents which cause DNA damage or ER-Stress.** Parental 10T1/2 fibroblasts or ER-MyoD:10T1/2 fibroblasts were cultured in GM in (A) with either vehicle or 200 μM etoposide or in (B) 3 μM thapsigargin and +/- estradiol 24 hours as indicated. Next day, detached cells and adherent cells were collected then counted. Percent apoptosis was calculated (number of detached cells/ detached cells plus adherent cells). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments.

3.3 MyoD mediated regulation of the apoptotic response correlates with the level of induction of the pro-apoptotic Bcl-2 family member PUMA

We have previously reported that the proapoptotic Bcl-2 member PUMA (p53 up regulated modulator of apoptosis) expression increases when cells are switched to DM for three hours (Shaltouki et al. 2007). We have also previously shown that this induction of PUMA is p53 independent. Additionally, when myoblasts are untreated, basal levels of PUMA expression are difficult to detect. Our lab has also shown that PUMA plays a critical role in the apoptosis associated with differentiation of skeletal myoblasts (Shaltouki et al. 2007). Based on these data, we hypothesized that MyoD may play a role in the induction of PUMA expression as a consequence of culture in DM. First, we assayed for the induction of PUMA in response to culture in DM in parental and control transduced 23A2 or C2C12 myoblasts and compared these levels to their respective shMyoD clones. After switching the cells to DM for three hours, we collected total cellular RNA utilizing standard Trizol protocol followed by RT-PCR. We found that PUMA mRNA induction was impaired in 23A2:shMyoD and C2C12:shMyoD clones as compared to the parental and vector transduced control myoblasts (Figure 20). Additionally, after 3 hours in DM, we found PUMA protein induction was also impaired in 23A2:shMyoD and C2C12:shMyoD clones as compared to the parental and vector transduced control myoblasts (Figure 21).

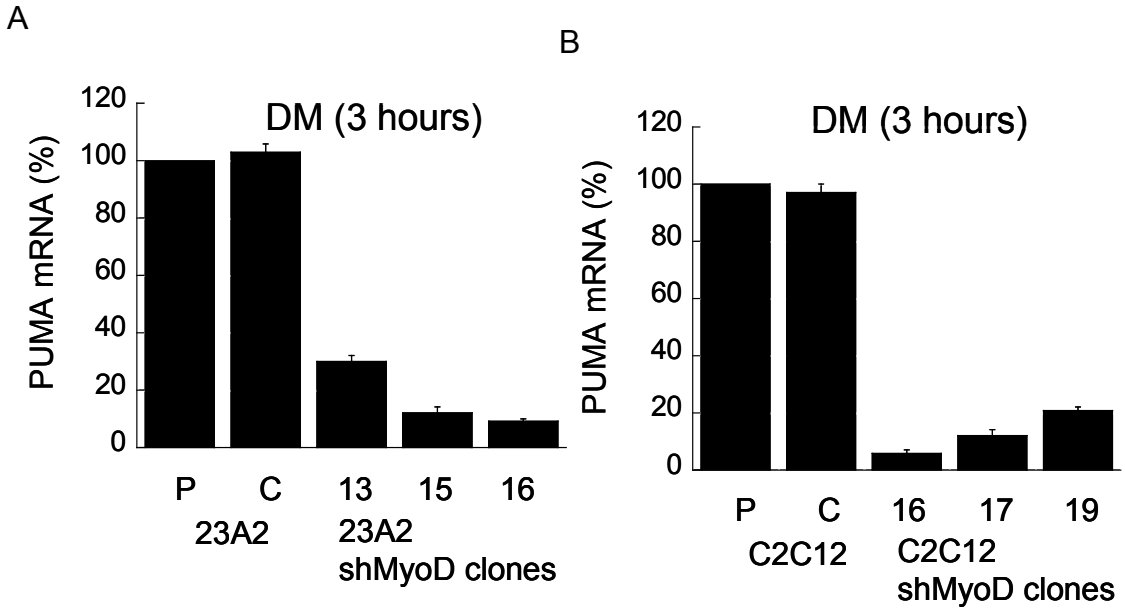


Figure 20. Elevated PUMA mRNA expression levels as a consequence of serum withdraw correlates with MyoD expression levels in myoblasts. In (A) 23A2 myoblast parental, vector and shMyoD clones and in (B) C2C12 myoblast parental, vector and shMyoD clones were screened for PUMA induction. Equal number of cells was plated and the next day given either fresh GM or DM for 3 hours. Total cellular RNA was isolated using standard TRIZOL method. Quantitative RT-PCR for PUMA was performed using the Quantitech Sybr Green PCR kit (Qiagen). Fluorescence was detected using an Opticon Monitor (MJ Research) Opticon software was used to calculate cycle threshold (Ct) values. The Ct value for PUMA PCR product was normalized to the Ct value for Actin PCR product run parallel. Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments.

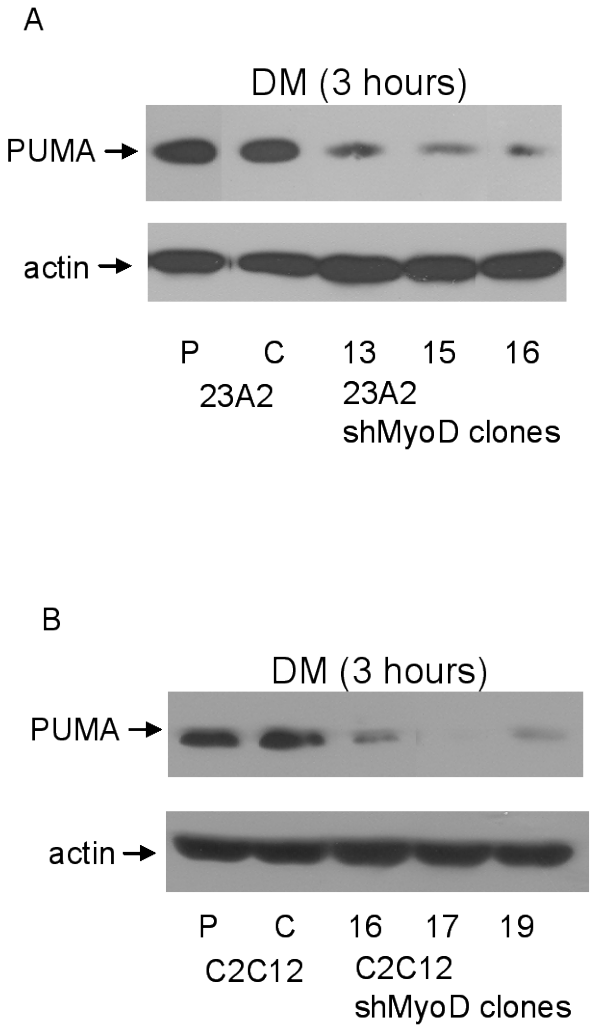


Figure 21. Elevated PUMA protein expression levels as a consequence of serum withdraw correlates with MyoD expression levels in myoblasts. In (A) 23A2 myoblast parental, vector and shMyoD clones and in (B) C2C12 myoblast parental, vector and shMyoD clones, equal numbers of cells were plated and the next day given either fresh GM or DM for 3 hours. In (A) whole cell lysates were collected then separated by SDS-Page prior to electrophoretic transfer. Immunoblot analysis for PUMA expression was preformed. Actin was monitored for equal loading. Shown are results from one experiment that are representative of 2 independent experiments.

We next compared the expression of PUMA in 10T1/2 fibroblasts and ER-MyoD:10T1/2 fibroblasts in response to culture in DM in the absence or presence of estradiol. Estradiol addition in DM resulted in approximately a four fold increase in PUMA mRNA and protein in the ER-MyoD:10T1/2 fibroblasts, yet had no effect on the level of PUMA mRNA or protein in the 10T1/2 fibroblasts (Figure 22).

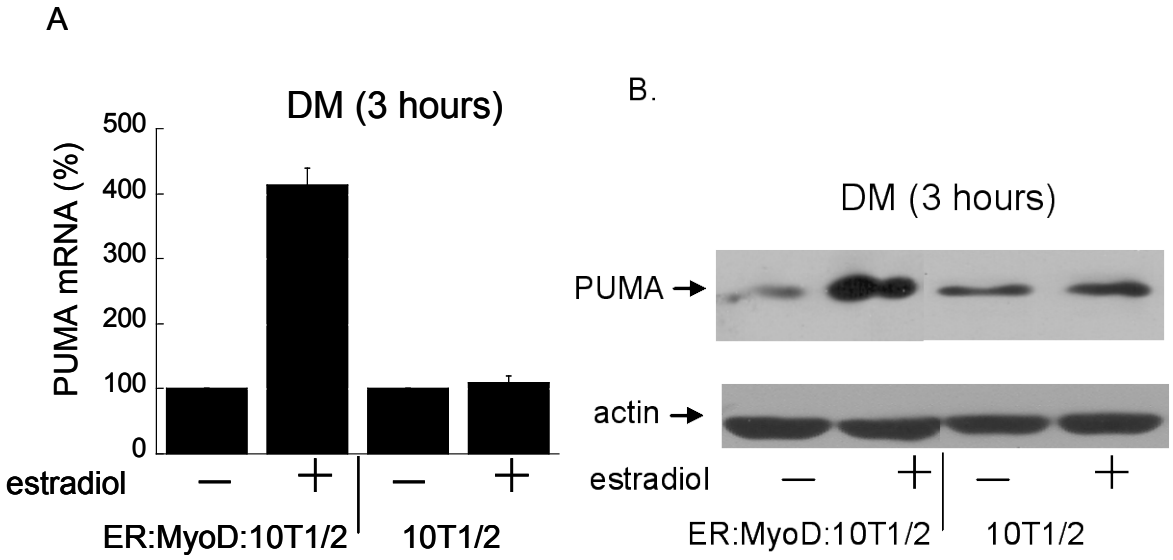


Figure 22. MyoD activation in 10T1/2 fibroblasts expressing an estrogen receptor MyoD fusion protein leads to PUMA induction in response to serum withdrawal. Parental 10T1/2 fibroblasts or 10T1/2 ER: MyoD expressing fibroblasts were cultured in DM 3 hours. In (A) total RNA was isolated using the standard Trizol method. RT-PCR for PUMA was performed and PCR product was separated on an ethidium bromide stained agarose gel. Images were analyzed using the GE Healthcare Life Sciences Typhoon 9410 Imager and band volume quantified using ImageQuant software (v5.2). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments. In (B) whole cell lysates were collected prior to separation by SDS-PAGE and electrophoretic transfer to PVDF. Immunoblot analysis for PUMA expression was performed. Actin was monitored for equal loading. Shown are results from one experiment that are representative of 2 independent experiments.

Some labs have reported the p53–dependent induction of PUMA in response to etoposide (Michalak et al. 2008) or thapsigargin (Li et al. 2006) in a variety of non-muscle cell types. Since we have determined that MyoD contributes to the apoptosis induced by these agents (Figures 18-20), we examined the contribution to the induction of PUMA in response to either etoposide or thapsigargin. First we performed a time course and concentration course analysis for treatment with etoposide or thapsigargin and determined that the minimum time and concentration required to increase the level of PUMA expression was 200 μM and eight hours for etoposide and 3 μM and 12 hours for thapsigargin. We then monitored the induction of PUMA as a consequence of culture with either etoposide or thapsigargin in GM. When comparing parental and control transduced 23A2 and C2C12 myoblasts to their respective cones, we found both PUMA mRNA as well as protein increased as expected in the parental and control transduced myoblasts; however, PUMA induction was impaired in the 23A2:shMyoD and C2C12:shMyoD clones in response to culture with etoposide or thapsigargin (Figures 23-26). This indicates the presence of MyoD is required for the induction of PUMA expression in response to inducers of DNA damage or ER-stress.

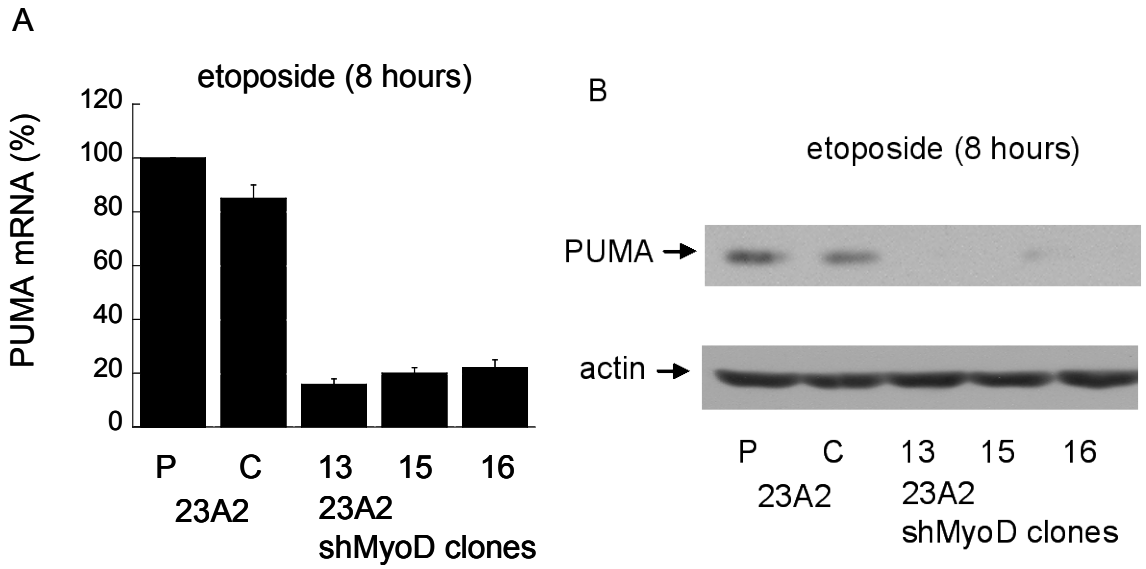


Figure 23. Reduced MyoD expression in 23A2 myoblasts abrogates increased PUMA expression in response to etoposide. Equal numbers of cells were plated and the next day treated +/- 200 μM etoposide for 8 hours. In (A) total RNA was isolated using standard Trizol method. RT-PCR for PUMA was performed and PCR products were separated on an ethidium bromide stained agarose gel. Images were analyzed using GE Healthcare Life Sciences Typhoon 9410 Imager and band volume quantified using ImageQuant software (v5.2). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments. In (B) whole cell lysates were collected prior to separation by SDS-PAGE and electrophoretic transfer to PVDF. Immunoblot analysis for PUMA expression was performed. Actin was monitored for equal loading. Shown are results from one experiment that are representative of 2 independent experiments.

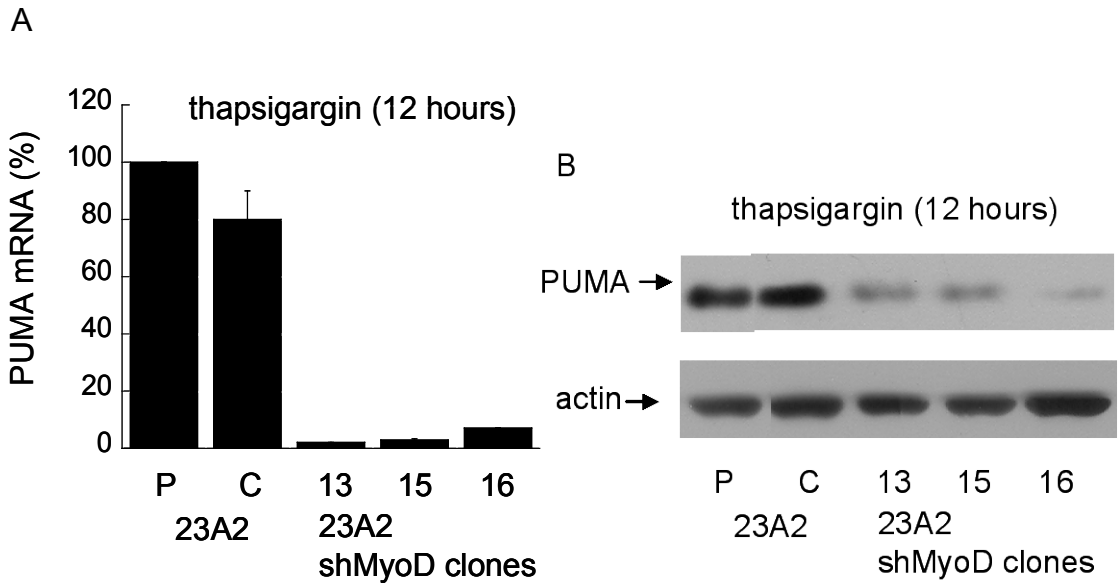


Figure 24. Reduced MyoD expression in 23A2 myoblasts abrogates increased PUMA expression in response to thapsigargin. Equal numbers of cells were plated and the next day treated +/- 3 μM thapsigargin for 12 hours. In (A) total RNA was isolated using standard Trizol method. RT-PCR for PUMA was performed and PCR products were separated on an ethidium bromide stained agarose gel. Images were analyzed using GE Healthcare Life Sciences Typhoon 9410 Imager and band volume quantified using ImageQuant software (v5.2). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments. In (B) whole cell lysates were collected prior to separation by SDS-PAGE and electrophoretic transfer to PVDF. Immunoblot analysis for PUMA expression was performed. Actin was monitored for equal loading. Shown are results from one experiment that are representative of 2 independent experiments.

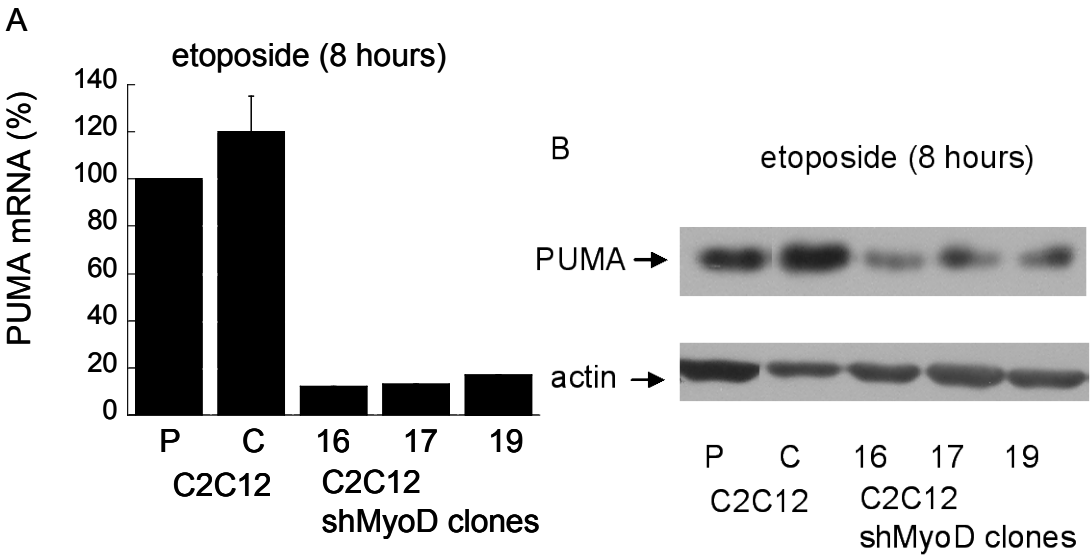


Figure 25. Reduced MyoD expression in C2C12 myoblasts abrogates increased PUMA expression in response to etoposide. Equal number of cells was plated and the next day treated +/- 200 μM etoposide for 8 hours. In (A) total RNA was isolated using standard Trizol method. RT-PCR for PUMA was performed and PCR product was separated on an ethidium bromide stained agarose gel. Images were analyzed using GE Healthcare Life Sciences Typhoon 9410 Imager and band volume was quantified using ImageQuant software (v5.2). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments. In (B) whole cell lysates were collected prior to separation by SDS-PAGE and electrophoretic transfer to PVDF membrane. Immunoblot analysis for PUMA expression was performed. Actin was monitored for equal loading. Shown are results from one experiment that are representative of 2 independent experiments.

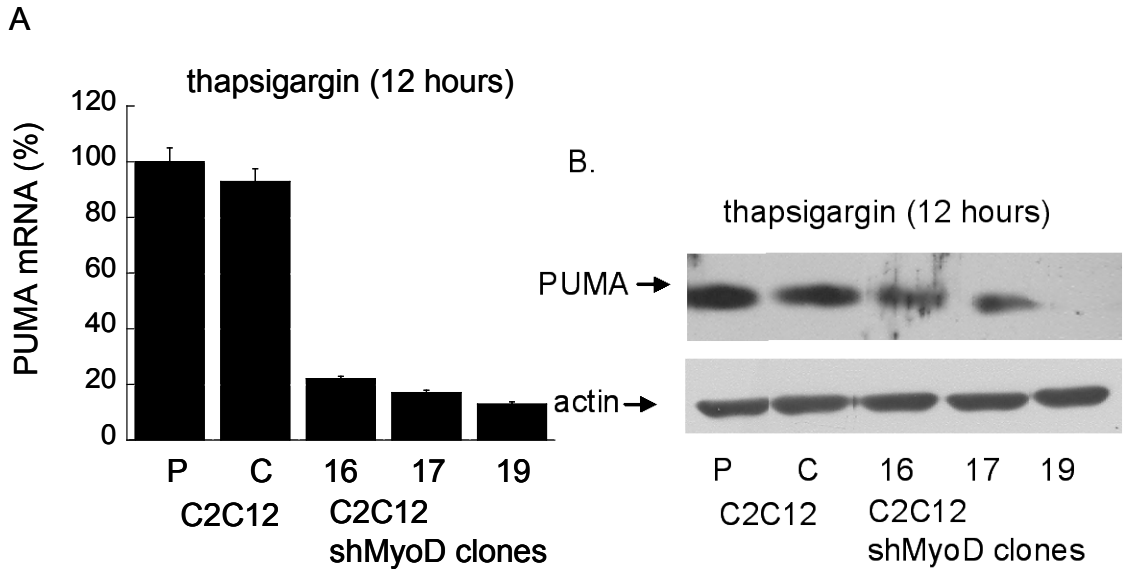


Figure 26. Reduced MyoD expression in C2C12 myoblasts abrogates increased PUMA expression in response to thapsigargin. Equal numbers of cells were plated and the next day treated +/- 3 μM thapsigargin for 12 hours. In (A) total RNA was isolated using standard Trizol method. RT-PCR for PUMA mRNA expression was performed and PCR product was separated on an ethidium bromide stained agarose gel. Images were analyzed using the GE Healthcare Life Sciences Typhoon 9410 Imager and band volume quantified using ImageQuant software (v5.2). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments. In (B) whole cell lysates were collected prior to separation by SDS-PAGE and electrophoretic transfer to PVDF membrane. Immunoblot analysis for PUMA expression was performed. Actin was monitored for equal loading. Shown are results from one experiment that are representative of 2 independent experiments.

Finally, we compared the expression of PUMA in ER-MyoD:10T1/2 fibroblasts and parental 10T1/2 fibroblasts cultured with either etoposide or thapsigargin in the absence or presence of estradiol in GM. Estradiol treatment combined with etoposide resulted in approximately a 3.5 fold induction in PUMA mRNA and protein in the ER-MyoD:10T1/2 fibroblasts, yet had no effect on the level of PUMA mRNA or protein on parental 10T1/2 fibroblasts treated with etoposide (Figure 27). Likewise, estradiol treatment combined with thapsigargin in GM resulted in over a three fold increase in PUMA mRNA and protein in the ER-MyoD:10T1/2 fibroblasts, yet had no effect on the level of PUMA mRNA or protein induction in the parental 10T1/2 fibroblasts treated in the same manner (Figure 28).

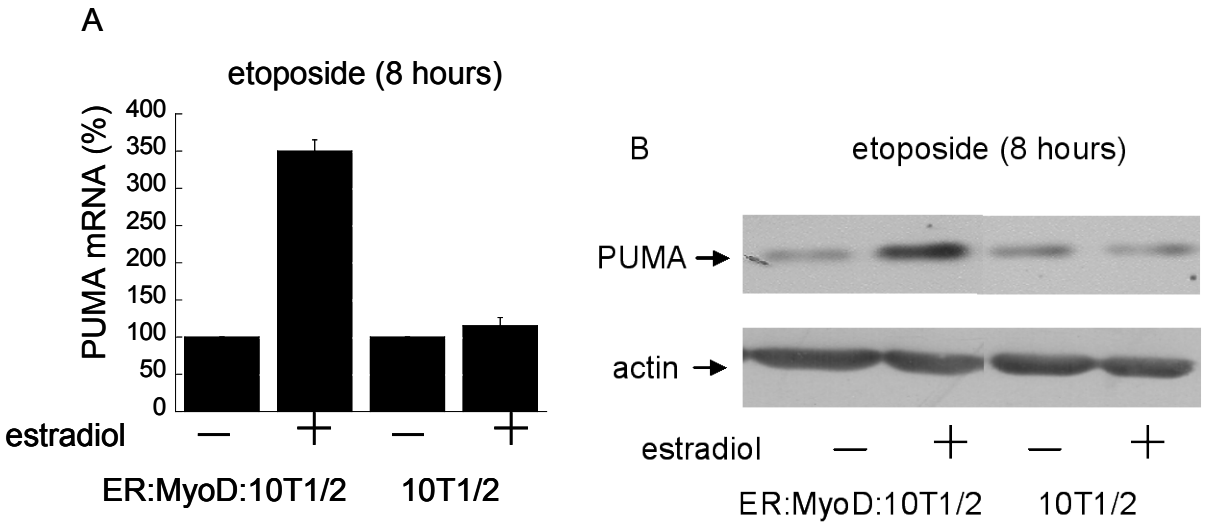


Figure 27. MyoD activation in 10T1/2 fibroblasts expressing an estrogen receptor MyoD fusion protein leads to PUMA induction in response to etoposide. A. Parental 10T1/2 fibroblasts or 10T1/2 ER: MyoD expressing fibroblasts were cultured in GM + 200 μM etoposide +/- estradiol for 8 hours. In (A) total RNA was isolated using standard Trizol method. RT-PCR for PUMA was performed and PCR products were separated on an ethidium bromide stained agarose gel. Images were analyzed using the GE Healthcare Life Sciences Typhoon 9410 Imager and band volume was quantified using ImageQuant software (v5.2). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments. In (B) whole cell lysates were collected prior to separation by SDS-PAGE and electrophoretic transfer to PVDF membrane. Immunoblot analysis for PUMA expression was performed. Actin was monitored for equal loading. Shown are results from one experiment that are representative of 2 independent experiments.

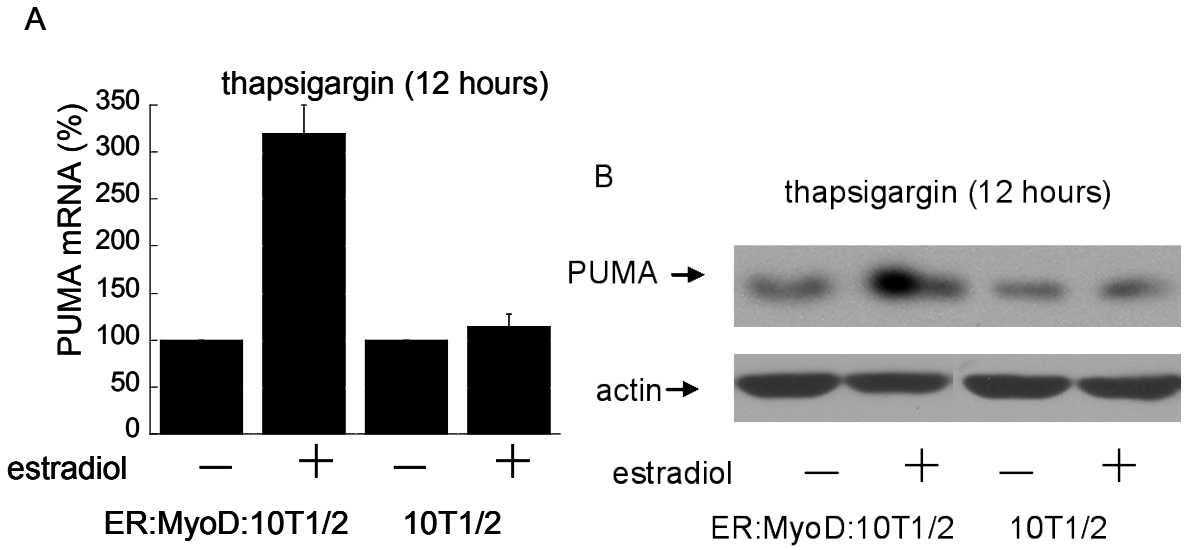


Figure 28. MyoD activation in 10T1/2 fibroblasts expressing an estrogen receptor MyoD fusion protein leads to PUMA induction in response to thapsigargin. A. Parental 10T1/2 fibroblasts or 10T1/2 ER: MyoD expressing fibroblasts were cultured in GM + 3 μM thapsigargin +/- estradiol for 12 hours. In (A) total RNA was isolated using standard Trizol method. RT-PCR for PUMA was performed and PCR product was separated on an ethidium bromide stained agarose gel. Images were analyzed using GE Healthcare Life Sciences Typhoon 9410 Imager and band volume quantified using ImageQuant software (v5.2). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments. In (B) whole cell lysates were collected prior to separation by SDS-PAGE and electrophoretic transfer to PVDF membrane. Immunoblot analysis for PUMA expression was performed. Actin was monitored for equal loading. Shown are results from one experiment that are representative of 2 independent experiments.

Our lab has previously shown that PUMA induction in skeletal myoblasts when cultured in DM is p53 independent. This experiment utilized the pharmacological p53 inhibitor pifithrinα (Komarov et al. 1999). We saw PUMA was still induced in the presence of pifithrinα (Shaltouki et al. 2007). Additionally, other labs (Han et al. 2001) have shown that PUMA induction in response to culture in DM is p53-independent in human tumor cells including HT29 cells which express a mutant form of p53, Jurkat cells which are p53 negative, and p53 null HL60. Since our data has lead us to believe that MyoD may be acting in place of p53 in skeletal myoblasts with regard to the induction of PUMA in response to serum withdrawal, in response to etoposide as well as thapsigargin, we needed to verify that basal levels of p53 were not altered during the transduction of these myoblasts with the shRNA against MyoD. We performed an immunoblot analysis for the expression of p53 and found that when comparing parental and control transduced 23A2 and C2C12 myoblasts to their respective clones, basal p53 levels remained unchanged in the shMyoD clones with respect to the parental and control myoblasts in both 23A2 and C2C12 cell lines (Figure 29).

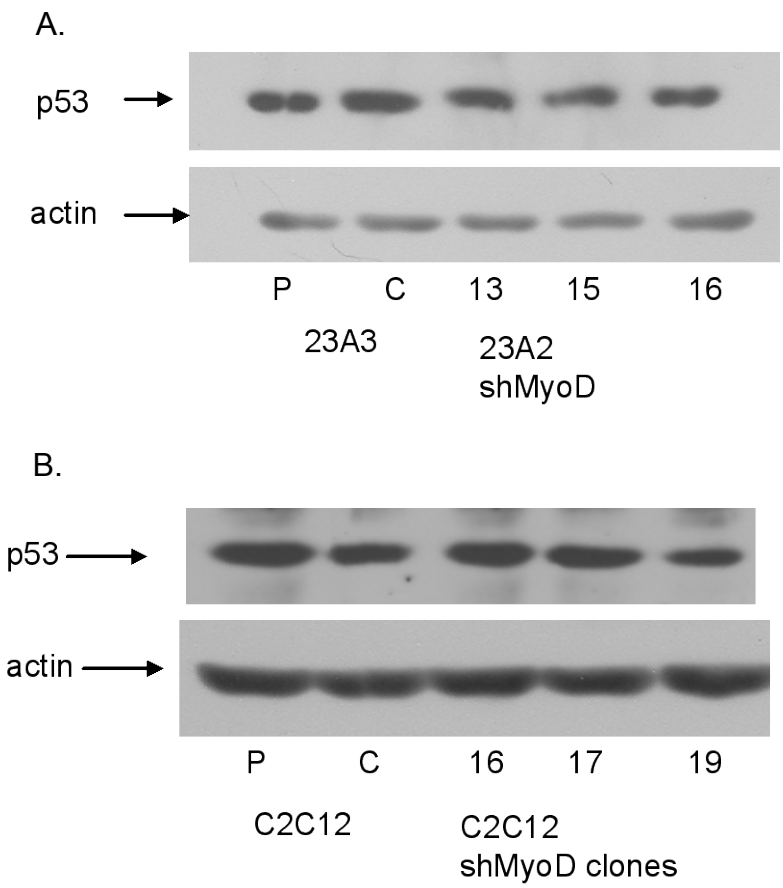


Figure 29. Basal expression levels of p53 in myoblasts transduced with shRNA against MyoD is not affected. In (A), 23A2 myoblast parental, vector and shMyoD clones and in (B) C2C12 myoblast parental, vector and shMyoD clones,p53 expression was detected by immunoblot analysis. Actin was monitored to ensure equal loading. Shown are results from one experiment that are representative of 2 independent experiments.

Finally, in an effort to determine if PUMA induction in response to growth factor withdrawal required de novo protein synthesis, we cultured 23A2 and C2C12 myoblasts in GM or DM for three hours in the presence or absence of cyclohexamide. Cyclohexamide inhibits protein synthesis by blocking translational elongation. PUMA mRNA is induced in response to culture in DM in both 23A2 and C2C2 myoblasts. When cyclohexamide is added to DM, PUMA mRNA induction is still induced (Figure 30). We also found that in ER-MyoD:10T1/2 fibroblasts that PUMA mRNA is induced in DM containing estradiol. This induction is still occurs in the presence of cyclohexamide, however in the absence of estradiol, this induction is abrogated (Figure 31). This finding indicates that de novo protein synthesis is not required for the induction of PUMA mRNA and suggests that MyoD could be directly affecting the increase of PUMA mRNA levels.

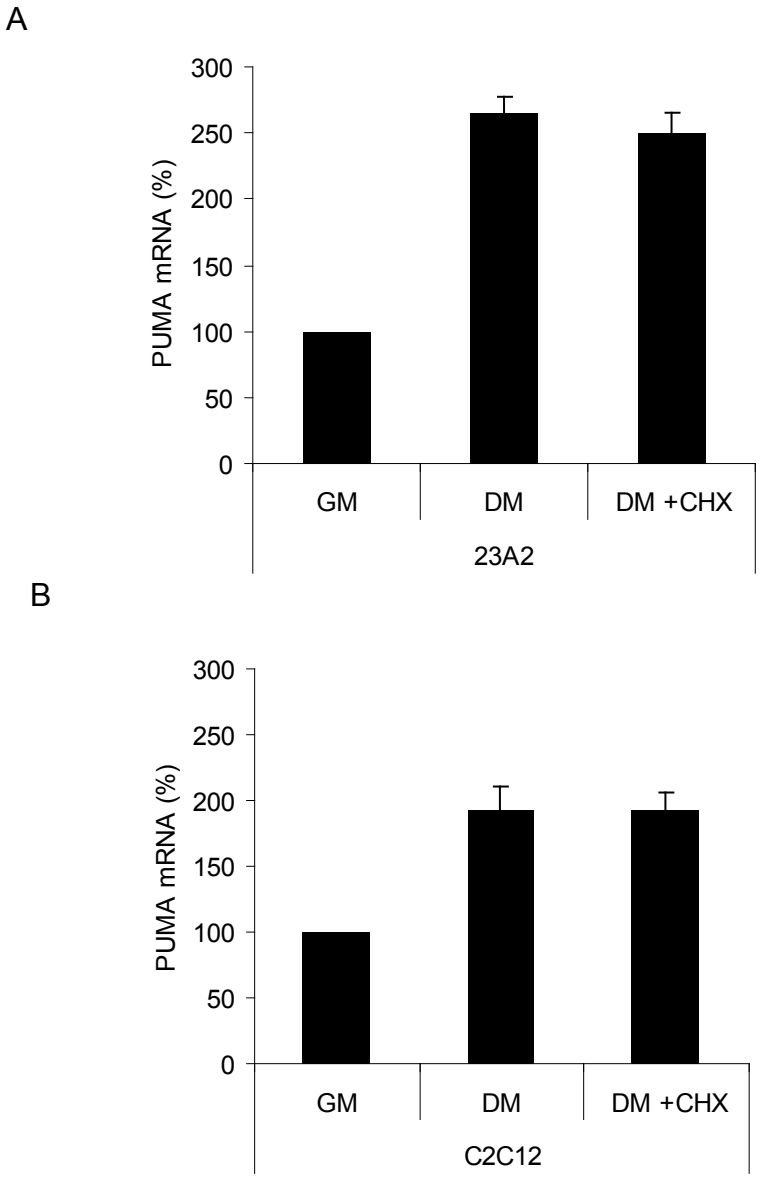


Figure 30. PUMA mRNA induction does not require de novo translation in myoblasts. In (A) 23A2 and in (B) C2C12 myoblasts were cultured in GM, DM or DM + cyclohexamide (CHX) for 3 hours. Total RNA was isolated using standard the Trizol method. RT-PCR for PUMA was performed and PCR products were separated on an ethidium bromide stained agarose gel. Images were analyzed using the GE Healthcare Life Sciences Typhoon 9410 Imager and band volume was quantified using ImageQuant software (v5.2). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments.

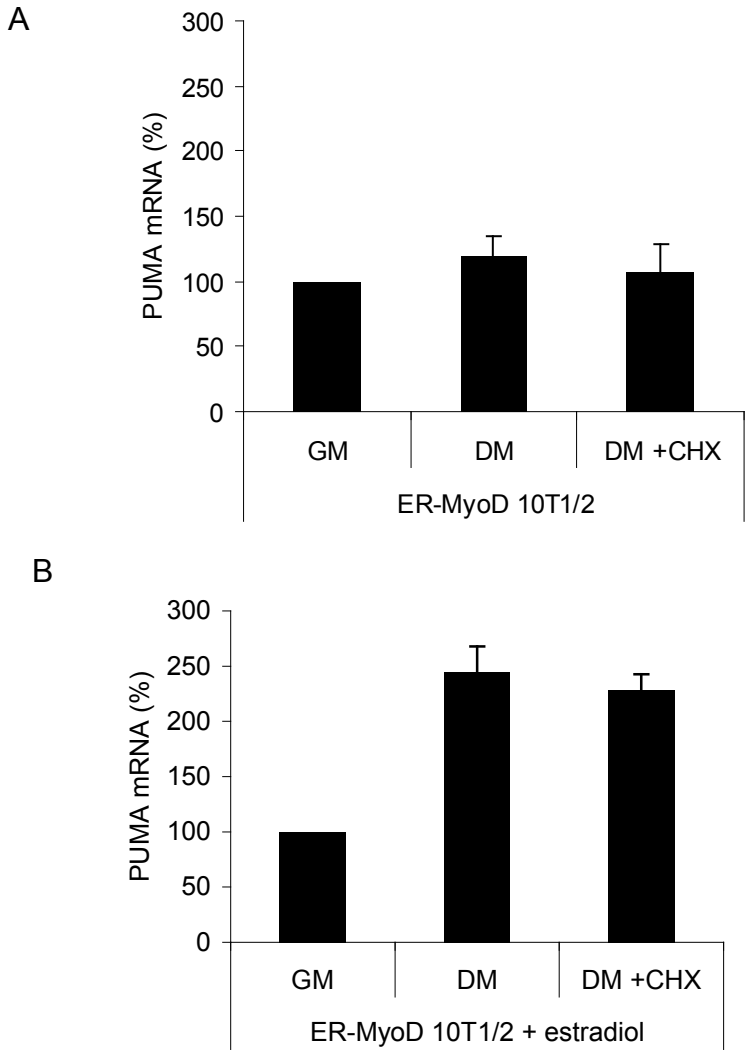


Figure 31. PUMA mRNA induction does not require de novo translation in 10T1/2 fibroblasts expressing estrogen receptor MyoD fusion protein. 10T1/2 ER: MyoD expressing fibroblasts were cultured in GM, DM or DM + cyclohexamide (CHX) in the absence (A) or in the presence (B) of estradiol for 3 hours. Total RNA was isolated using the standard Trizol method. RT-PCR for PUMA was performed and PCR products were separated on an ethidium bromide stained agarose gel. Images were analyzed using the GE Healthcare Life Sciences Typhoon 9410 Imager and band volume was quantified using ImageQuant software (v5.2). Shown is an average of triplicates (mean +/- standard deviation) from one experiment that is representative of three independent experiments.

CHAPTER IV

DISCUSSION

4.1 Overview

The discovery of the myogenic transcription factor MyoD by Lassar et al. in 1986, followed by the cloning of the other myogenic factors Myf5 (Braun et al. 1989), myogenin (Wright et al. 1989)and MRF4 (Rhodes and Konieczny 1989) soon after, lead to an entirely new area of muscle research. These initial studies showed expression of any of these myogenic factors could convert multipotent stem cells to the myogenic cell lineage. However, later studies revealed these factors all are temporally expressed during the differentiation process and that MyoD and Myf5 are responsible for determination while myogenin and MRF-4 are required for differentiation. Thus, skeletal myoblasts serve as an excellent model system to study determination and differentiation. With our findings, we can now state that skeletal myoblasts can also be a model system for the study of differentiation and the associated apoptosis.

MyoD has long been recognized for its role as the master regulatory transcription factor responsible for specification and for driving the differentiation process following cell cycle exit. With our findings, we conclude that MyoD also

serves in a pro-apoptotic capacity. The processes of differentiation and the associated apoptosis are mutually exclusive biological endpoints. For this to be true there must be a point of bifurcation in the signaling events leading to the decision to differentiate or undergo apoptosis.

It has been the focus of the Weyman lab to determine the molecular mechanisms responsible for the fact that a subpopulation of myoblasts will undergo apoptosis rather than differentiation when cultured in the absence of serum. We have previously shown the pro-apoptotic Bcl-2 family member PUMA is required for apoptosis in skeletal myoblasts upon serum withdrawal. Additionally, we have demonstrated that PUMA is not required for differentiation. Specifically, our lab has previously shown that myoblasts transduced with lentivirus expressing shRNA against PUMA mRNA, when cultured in DM no longer underwent apoptosis but can still differentiate as evidenced by the expression of MHC. Interestingly, increased PUMA expression in skeletal myoblasts in response to serum withdrawal is not a p53 mediated event. When 23A2 myoblasts were cultured in DM with the addition of the p53 inhibitor pifithrin, PUMA mRNA and protein expression still increased (Shaltouki et al. 2007). Non-p53 mediated increase in PUMA expression occurs in other cells in response to serum withdrawal, including the human cancer line HT29 cells (p53 mutant) , Jurkat cells, and HL60 cells (both p53 null) (Han et al. 2001). Sp1 and p73 were later found to regulate PUMA induction under serum withdrawal conditions in HT29 cells (Ming, et al. 2008). PUMA expression levels in skeletal myoblasts in response to culture in DM correlates to the expression levels of

MyoD (Karasarides, et al. 2006). We have shown herein that MyoD is necessary for both differentiation as well as the associated apoptosis. Since MyoD is common to both differentiation and apoptosis, whereas PUMA is only necessary for apoptosis, this appears to be a point of bifurcation in the signaling pathways required to decide between undergoing differentiation or apoptosis. (Figure 32).

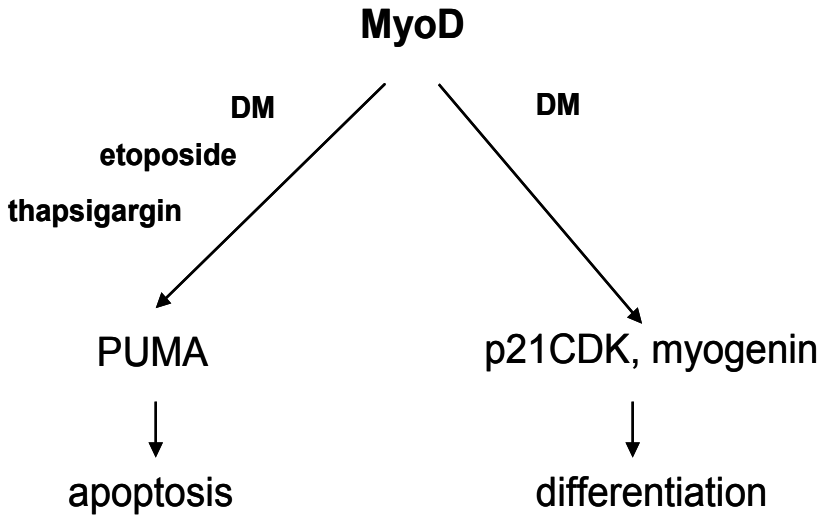


Figure 32. A Role for MyoD in the regulation of PUMA and apoptosis. The transcription factor MyoD has long been recognized for its direct role in the regulation of myogenin expression and the expression of other genes controlling muscle differentiation. We provide data to support a novel pro-apoptotic role in the regulation of PUMA expression and apoptosis. Direct regulation of PUMA expression by MyoD, as well as the molecular mechanisms responsible for determining whether MyoD induces p21 CDKI, myogenin and differentiation or MyoD's induction of PUMA and apoptosis awaits further investigation.

MyoD is acknowledged as the master regulatory transcription factor that controls the expression of many genes in a temporal fashion. MyoD can directly induce the expression of the cell cycle inhibitor p21 CDKI and Rb to induce cell cycle withdrawal. MyoD can also induce the expression of cyclin D3 during differentiation which appears contradictory to the typical functions of cyclins (Cenciarelli et al. 1999). Additionally, MyoD can directly induce the expression of myogenin, but not until the cell has exited the cell cycle. Interestingly, MyoD is bound to the myogenin promoter even in a proliferative state but remains transcriptionally silent (Mal and Harter 2003). Myogenin is expressed only upon chromatin remodeling in response to cues to differentiate (de la Serna et al. 2001). Mef2d is yet another gene MyoD induces. MyoD along with Mef2d co-operatively regulate the expression of late differentiation genes such as MHC

Despite the abundance of understanding of skeletal myoblast differentiation and the molecular mechanisms responsible, there is relatively very little known in regards to the molecular mechanisms that drive skeletal myoblast apoptosis. Our challenge now is to elucidate the molecular mechanisms which allow MyoD to induce PUMA and apoptosis in a sub population of myoblasts instead of muscle specific genes and differentiation. We can speculate that the decision for MyoD to induce PUMA and apoptosis or myogenin and differentiation is a consequence of the cells position in the cell cycle. Previous reports have shown that manipulating the expression of cell cycle regulatory proteins can either block or induce apoptosis in myoblasts or in MyoD-expressing

fibroblasts (Wang and Walsh 1996). Expression of the CDK inhibitor p21 promotes survival and additionally, forced expression of p21 leads to resistance to differentiation associated apoptosis and required Rb. Rb-/- myogenic cells are more susceptible to apoptosis even with normal increased p21 expression and reduced CDK expression (Wang and Walsh 1996, and Wang et al. 1997). Reduced levels of Rb result in massive apoptosis (Zacksenhaus et al. 1996). We have an asynchronous population of myoblasts which are neither Rb nor p21 deficient. Based on this information, we believe the difference in cells which undergo apoptosis rather than differentiation is their position near the G1/S checkpoint where p21 no longer blocks Rb activation by cyclin dependent kinases. Inhibition of Rb activity leads to an increase in E2F. E2F leads to the increase of the apoptosis stimulating protein of p53-2 (ASSP). Increased ASSP then stimulates p53 which will, in turn, up-regulate pro-apoptotic genes (Chen et al. 2005). During neurogenesis, neuronal apoptosis occurs at the G1/S restriction point. E2F is repressed by Rb, which is required for survival of neurons (Liu and Greene 2001). We believe the mechanism might be similar which allows MyoD to either induce PUMA and apoptosis or myogenin and differentiation.

Initial studies of apoptosis involved *C. elegans* as the model system (Review, Horvitz 1999). During the development of *C. elegans*, 1090 cells are formed but 131 of these cells die via apoptosis. The study of the molecular mechanisms involved in the death of these cells identified a set of four genes which co-ordinate the apoptotic process. Egl-1 (egg-laying defective), ced-9 (cell death defective), ced-4 and ced-3 where found, when mutational analyses were conducted, to interfere with cell death. Egl-1 is a pro-apoptotic molecule and acts to inhibit ced-9, an anti-apoptotic molecule. Ced-4, an adapter molecule, activates ced-3, a protease. Ced-4 can be inhibited by ced-9, thereby blocking the activation of ced-3. These factors have been found to have homologues in vertebrate systems. Ced-3 is akin to caspase 3, ced-4 is most similar to the adapter molecule Apaf-1, which makes up part of the apoptosome, ced-9 is homologous to the anti-apoptotic Bcl-2 protein and Egl-1 shares similarity to pro-apoptotic BH-3 only Bcl-2 family members. (Figure 33).

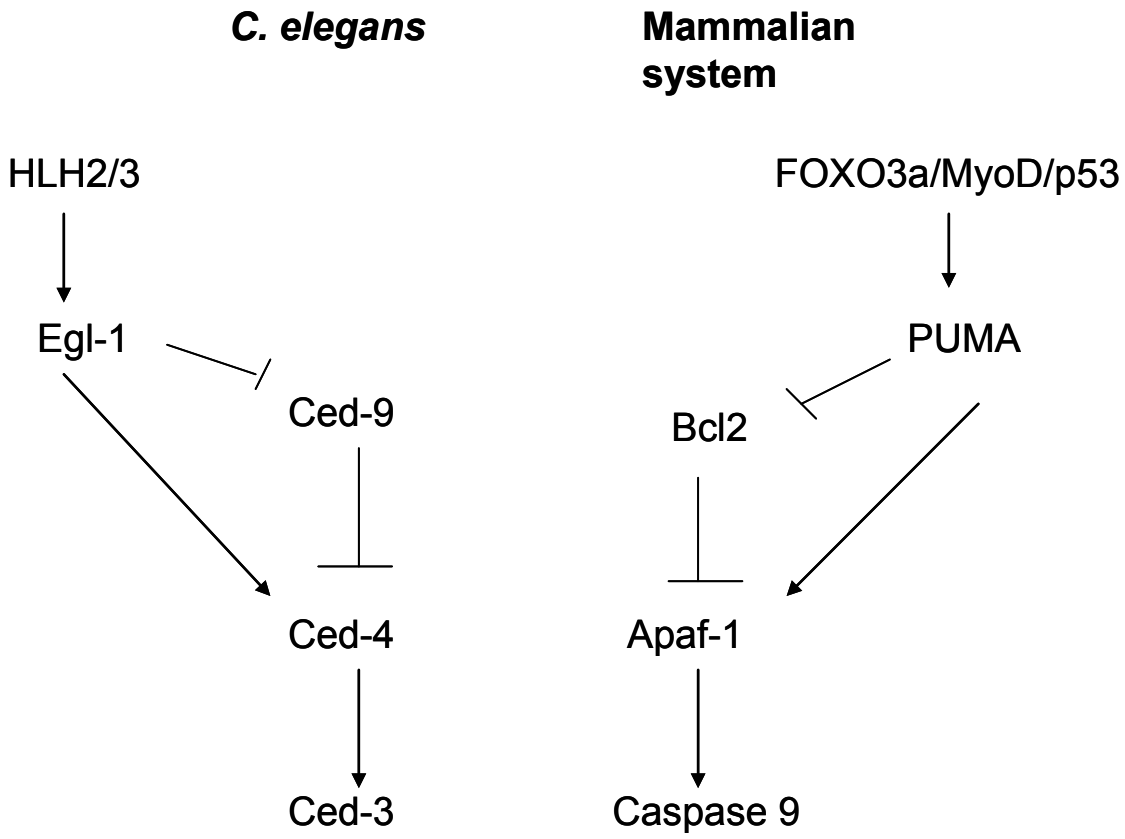


Figure 33. Apoptotic factors discovered in C. elegans and mammalian homologs. Factors involved in apoptosis during development were first discovered in C. elegans. Later, homologs of these factors were discovered in mammalian systems. Egl-1, a BH3 only protein, was found to inhibit Ced-9, whereas Ced-9 inhibited Ced-4. By Egl-1 blocking the activity of Ced-9, Ced-4 was activated, leading to the activation of Ced-3. In mammalian cells, Egl-1 is most like PUMA, also a BH3 only protein. The mammalian homolog of Ced-9 is the antiapoptotic Bcl-2. Ced-4 most closely relates to the mammalian adapter molecule APAF-1, which makes up part of the apoptosome, and Ced-3 homolog in the mammalian system is Caspase 9, an initiator caspase.

MyoD is a basic helix-loop-helix (bHLH) transcription factor and, to our knowledge, the only other bHLH transcription factors that are known to play a role in cell specification, differentiation and apoptosis are the Daughterless-like (HLH-2) and Achaete-scute-like bHLH (HLH-3) transcription factors expressed in *C. elegans*, and is responsible for neuronal development. HLH-2 and HLH-3 has been shown to form heterodimers and bind to E box consensus sequences (5'-CANNTG-3') much like MyoD:E protein heterodimers in mammalian systems. HLH-2:HLH-3 heterodimers are responsible for the transcription of Egl-1 by binding to E-boxes found in a cis- regulatory region of the Egl-1 promoter. Egl-1 is a pro-apoptotic BH3 only Bcl-2 protein as is PUMA. Egl-1 expression is regulated by HLH-2:HLH3 heterodimer, which resemble MyoD:E protein heterodimers, both of which bind E-boxes in promoter regions. Based on this information and our data presented herein, we believe MyoD is an excellent candidate transcription factor in skeletal myoblasts responsible for the expression of PUMA (Figure 33).

The idea that a single transcription factor can regulate genes responsible for mutually exclusive physiological endpoints is not without precedent in mammalian systems. The Myc transcription factor has the dual capacity to induce either proliferation or apoptosis. Regulation of proliferation by Myc is through transcriptional targets such as cdc25 and CDK4. Myc also functions in apoptosis by indirectly inducing the release of cytochrome c from the mitochondria and may involve the pro-apoptotic Bcl-2 family member Bax.

Additionally, Myc has been shown to activate p19 ARF, which in turn induces p53 dependent gene expression (Pelengaris et al. 2002).

The transcription factor p53 also performs several functions. p53 plays an important role in growth arrest by the transcription of p21 CDKI. Expression of p21 CDKI blocks progression through the cell cycle at the G_1/S phase checkpoint. p53 carries out pro-apoptotic functions by inducing the expression of PUMA, as well as other pro-apoptotic Bcl-2 family members, which leads to the activation of the intrinsic apoptotic pathway. The ability of MyoD to induce growth arrest and differentiation or apoptosis is analogous to the ability of p53 to induce growth arrest or apoptosis in response to DNA damage in other cell types. Interestingly, p53 has been shown to be critical in myoblast differentiation. p53 has been shown to induce differentiation of mouse embryonic stem cells (ESCs) through the suppression of the protein Nanog, which is required to block differentiation of ESCs (Lin et al. 2005). Conversely, p53 has been shown to block differentiation of MEF to adipocytes. However, this too, is likely achieved by transcriptional inhibition of PPARγ and/or CEBPα (Molchadsky 2008). Utilizing C2C12 myoblasts, a dominant negative form of p53 was expressed which binds to endogenous p53, resulting in transcription inhibition. These myoblasts failed to upregulate Rb under differentiation conditions (Porrello et al. 2000). Rb has dual functions contributing to cell cycle arrest and expression of late myogenic factors. In myoblasts with defective p53, cell cycle gene expression was not altered and myogenin was still expressed, however, MHC failed to be expressed (Huh et al.

2004). Even more fascinating is that p53 in skeletal myoblasts does not affect differentiation associated apoptosis. Utilizing a p53 reporter construct upstream of GFP gene, C2C12 myoblasts were transfected followed by induction of DNA damage by adriamycin treatment while culturing in differentiation media. The authors found no fluorescence in apoptotic population at anytime within 48 hours however fluorescence was visualized in all differentiated cells after 24 hours after culture in DM (Cerone et al. 2000). This data shows p53 is not required for apoptosis in skeletal myoblasts. The future challenge previously mentioned, to understand the molecular mechanism that allows MyoD to induce PUMA and apoptosis, instead of p21 CDKI, cell cycle exit and muscle specific genes as described in Figure 32, resembles the long standing challenge to understand the same features of p53 in the choice to induce p21CDKI or PUMA (Vousden et al. 2009).

We, and others, have previously suggested an apoptotic role for MyoD. We reported that the level of MyoD expression correlated with the apoptotic potential as well as the differentiation potential in response to culture in DM (Karasarides et al. 2005). Another group has reported that over-expression of ectopic MyoD in cells lacking functional retinoblastoma protein (Rb) induces apoptosis (Peschiaroli et al. 2002 and Gottifredi et al. 1999). This MyoD induced apoptosis was also shown to be p53 independent (Gottifredi et al. 1999). As 23A2 myoblasts, C2C12 myoblasts and 10T1/2:ER-MyoD fibroblasts, are not Rb deficient (data not shown), the data we have presented in this manuscript

indicates that endogenous levels of MyoD can confer the ability to undergo apoptosis in cells with functional Rb. In support of our findings, a recent report using primary MyoD -/- satellite derived myoblasts, upon transplantation into mice after cardiotoxin induced muscle injury, showed enhanced survival upon engraftment. It is important to note here that these myoblasts are differentiation delayed but not deficient and this can be attributed to a five fold increase in the expression of Myf5, which has been shown to compensate for MyoD during skeletal myoblast differentiation during embryonic development (Rudnicki et al. 1992). In agreement with our previous findings that PUMA is required for myoblast apoptosis (Shaltouki et al. 2007), ectopic expression of PUMA in these MyoD -/- satellite cells restored their apoptotic potential (Asakura et al. 2007).

We also demonstrate here that MyoD plays a role in responding to etoposide-induced apoptosis. MyoD-/- myoblasts were also resistant to UV-induced apoptosis showing a four fold reduction in the number of apoptotic cells after treatment in comparison to MyoD +/+ myoblasts (Asakura et al. 2007). Taken together, these results suggest that MyoD plays a role in responding to DNA damage. Typically, tumor suppressor activity has been attributed to molecules like p53, which mediate apoptosis in response to DNA damage (Vousden and Prives 2009). The significance of a pro-apoptotic role for MyoD with respect to the development of Rhabdomyosarcoma has not yet been explored. Further, we show that MyoD also plays a role in responding to ER-stress induced apoptosis. Since ischemia elicits an ER-stress induced up-regulation of PUMA expression

(Toth et al. 2006 and Nickson et al. 2007), the importance of a pro-apoptotic role for MyoD in ischemia induced muscle damage should also be explored.

Finally, we show that the role of MyoD in responding to either DNA damage (as induced by etoposide treatment) or ER-stress (as induced by thapsigargin), similar to MyoD's role in responding to culture in DM (serum withdrawal), involves PUMA (Figure 31). In the absence of an apoptotic stimulus, such as culture in DM or treatment with etoposide or thapsigargin in GM, reduced levels of MyoD has no detectable effect on basal PUMA expression (data not shown). What remains to be explored is whether the mechanism of MyoD's induction of PUMA in response to DM is similar to the mechanisms initiated by culture with agents that cause DNA damage or ER-stress.

What we mean by this is MyoD may have different transcriptional partners at different promoter locations. MyoD binds E proteins at E boxes of myogenic genes, however, the binding partner or other transcriptional enhancers may be involved in the expression of non-myogenic genes. As an example, under hypoxic conditions, MyoD drives the transcription of UCP3 in differentiated C2C12 myoblasts and UCP3 expression is enhanced by the transcription factor ATF-1 (Lu et al. 2008). The expression of UCP3 by MyoD is achieved by binding to a non-canonical E box in the UCP promoter. Additionally, under normal metabolic situations, UCP3 transcription is achieved by MyoD and PPARα rather than ATF-1 (Solanes et al. 2003).

The report demonstrating the increased survival of MyoD-/- myoblasts correctly suggests that targeting MyoD could be beneficial to myoblast transfer strategies to aid in the expansion of muscle stem cells (Asakura et al. 2007). Again, we must reiterate the fact that MyoD-/- satellite cell-derived myoblasts display delayed differentiation (Sabourin et al. 1999) and are also deficient in muscle regeneration (Megeney et al. 1996). Our findings show that PUMA plays a critical role in the apoptotic process, but plays no role in the differentiation process. This suggests that PUMA may also be a good candidate for therapeutic manipulation to enhance the regenerative potential of adult muscle stem cells and to increase the efficacy of myoblast transfer therapies (Shaltouki et al. 2007).

4.2 Future Directions

Now we have provided data to show that MyoD is sufficient and necessary for the induction of apoptosis in response culture in DM (serum withdrawal), etoposide treatment (DNA damage) or thapsigargin treatment (ER-stress) in skeletal myoblasts. Our next step is to identify the molecular mechanisms by which induction of PUMA expression occurs. To this end, we have proposed a set of experiments to test whether MyoD directly controls PUMA expression. Additionally, since MyoD is necessary for apoptosis in skeletal myoblasts, we must look at other molecules involved in the apoptotic process and determine what effects can be attributed to MyoD.

Our first hypothesis is that MyoD directly regulates PUMA expression. In support of this hypothesis, we have performed *in silico* analysis of a 5kb region of the murine PUMA promoter utilizing the online promoter analysis program MatInspector. We have found at least 5 potential canonical E box MyoD:E12/47 heterodimer binding sites within this region. This program selects only canonical binding sites for MyoD. As MyoD can bind non-canonical E boxes within promoters, it is very possible there are more potential binding sites within this region. In order to test our hypothesis, we propose the following experiments. We will begin by creating a pGLC3 basic luciferase reporter construct containing a portion of the PUMA promoter cloned upstream of the luciferase gene. To create this construct, we will PCR amplify approximately a 2kb 5' region of the PUMA gene and clone into the pGLC basic luciferase reporter construct. This or control

vector will be transfected into 23A2 and C2C12 myoblasts and next day, luciferase activity will be measured following culture in GM as compared to DM. We will also use this same method utilizing the ER-MyoD 10T1/2 fibroblasts +/- estradiol treatment cultured in GM or DM. Once we determine MyoD responsiveness, we will then generate a series of truncations to the 5' promoter region to identify the minimal MyoD responsive cis regulatory region. We will then transfect the vector containing the minimal region into 23A2 and C2C12 myoblasts silenced for MyoD, culture in GM or DM and assess for luciferase activity. The luciferase activity will be normalized to Renilla luciferase for transfection efficiency. Renilla luciferase activity is not affected by culture in GM versus DM.

In order to determine if this interaction is through direct binding of the PUMA promoter, we will next perform electrophoretic mobility shift assay (EMSA) analysis using the minimal MyoD responsive cis-regulatory region previously determined. We will prepare nuclear extracts of 23A2 and C2C2 myoblasts cultured in GM or DM and from ER-MyoD 10T1/2 fibroblasts cultured in GM or DM in the absence or presence of estradiol. Extracts will be incubated with a radiolabelled minimal cis-regulatory region as described previously then subjected to EMSA. To ensure specificity, EMSA will also be performed using a mutant version of this regulatory region and competition experiments will be carried out using unlabelled sequences. The identity of the protein in the shifted complex will be examined using an anti-MyoD antibody. To serve as a negative

control, supershift analysis will be performed using anti-IgG. AS a positive control, EMSA and supershift assays will be performed using the known MyoD cis regulatory sequence from the myogenin gene. We will duplicate these experiments utilizing the 23A2 and C2C12 myoblasts silenced for MyoD expression.

In order to test if the previous association of MyoD and the PUMA promoter occur specifically *in vivo*, we will perform chromatin immunoprecipitation (CHiP) analysis using chromatin isolated from 23A2 and C2C12 myoblasts cultured in GM or DM and ER-MyoD10T1/2 fibroblasts cultured in GM or DM in the absence or presence of estradiol. Chromatin will be cross-linked using 1% formaldehyde then digested with MNase to achieve a length between 200-100bp fragments. Immunoprecipitation will be performed using EZ CHiP chromatin Immunoprecipitation kit per manufacturers' instructions. Chromatin will be pre-cleared using A/G sepharose after blocking using salmon sperm DNA and BSA. A portion will be set aside as an input control, while the remainder of the sample will be immunoprecipitated using anti-MyoD antibody. Samples will be treated with proteinase K, cross-linking reversal then quantitative RT-PCR will be performed to detect the putative MyoD cis-regulatory sequence identified previously. Negative control will be immunoprecipitation using appropriate IgG and positive control will include PCR for the myogenin gene.

Another part of this project is to determine what role serine proteases play in skeletal myoblast apoptosis. In rat fibroblasts, DNA damage and ER stress induced apoptosis resulted in activation of serine proteases. Preliminary data collected by our lab has shown that serine proteases are activated within three hours of culture in DM in 23A2 skeletal myoblasts. We will continue to investigate serine protease activation in response to various apoptotic inducing factors. Using FLISP (Fluorescently labeled inhibitor of serine protease) to selectively and covalently label in situ active serine proteases, we have found many serine proteases are activated upon culture in DM. When myoblasts cultured in DM were treated with pharmacological inhibitors of serine proteases, we found cytochrome C release and DNA fragmentation was abrogated. This was also observed in 10T1/2 fibroblasts expressing ectopic MyoD. To this end we hypothesize MyoD is both sufficient and necessary to induce mitochondrial disruption and apoptosis through the increased activation of serine proteases. To investigate this hypothesis, we will assess the activation of serine proteases in myoblasts cultured in GM or DM and ER-MyoD:10T1/2 fibroblasts cultured in GM or DM in the absence or presence of estradiol. We will then assess activation of serine proteases in myoblasts silenced for MyoD. We will then immunoprecipitate the activated serine proteases labeled with FLISP using an anti-FLISP antibody. The immunoprecipitated activated serine proteases will be subjected to separation by SDS-PAGE, followed by visualization of bands, which will be then sent for sequence analysis. If samples subjected to immunoprecipitation cannot be analyzed, we will separate samples by 2D gel electrophoresis and visualize

spots using GE Healthcare Life Sciences Typhoon 9410 Imager which will detect the FLISP labeled activated serine proteases. Spots can then be sent for sequence analysis.

4.3 Conclusions

Differentiation and apoptosis are coordinately regulated in many cell types, and to date, this coordinate regulation is not well understood. We have identified in this body of work that the muscle regulatory transcription factor MyoD is a molecule common to both the induction of differentiation as well as apoptosis. Further, we have determined that MyoD is both necessary and sufficient to contribute to the increased expression of the pro-apoptotic Bcl-2 family member PUMA, a molecule critical to only the apoptotic process. Finally, we have determined that MyoD and the corresponding induction of PUMA contribute to the apoptotic process in response to treatments with etoposide or thapsigargin. Thus, our data suggests that MyoD is critical to the coordination of both differentiation and apoptosis. Additionally, and most exciting is that MyoD also serves as a modulator of apoptosis in response to more traditional and widely applicable apoptotic agents.

While this body of work has offered novel and exciting new roles for MyoD in skeletal myoblasts, it is in no way the end to the important question, what is the mechanism responsible for the decision to undergo either differentiation or

apoptosis. We look forward to the future in continuing these studies as outlined in future directions, and delving in further to elucidate the mechanisms MyoD is responsible in contributing to apoptosis.

REFERENCES

Acehan, D., Jiang, X., Morgan, D. G., Heuser, J. E., Wang, X., and Akey, C. W. 2002. Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell *9*, 423-432.

Asakura, A., Hirai, H., Kablar, B., Morita, S., Ishibashi, J., Piras, B. A., Christ, A. J., Verma, M., Vineretsky, K. A., and Rudnicki, M. A. 2007 Increased survival of muscle stem cells lacking the MyoD gene after transplantation into regenerating skeletal muscle. Proc Natl Acad Sci U S A *104*, 16552-16557.

Bader, D., Masaki, T., and Fischman, D. A. 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. J Cell Biol *95*, 763-770.

Bergstrom, D. A., Penn, B. H., Strand, A., Perry, R. L., Rudnicki, M. A., and Tapscott, S. J. 2002. Promoter-specific regulation of MyoD binding and signal transduction cooperate to pattern gene expression. Mol Cell *9*, 587-600.

Blackwell, T. K., and Weintraub, H. 1990. Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science *250*, 1104-1110.

Bouillet, P., and Strasser, A. 2002. BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. J Cell Sci *115*, 1567-1574.

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., and Arnold, H. H. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. Embo J *8*, 701-709.

Campbell SL, Khosravi-far R, Rossman K L, Clark G J, and Der C J 1998. Increasing complexity of Ras signaling. *Oncogene* 17:1395-1413

Cenciarelli, C., De Santa, F., Puri, P. L., Mattei, E., Ricci, L., Bucci, F., Felsani, A., and Caruso, M. 1999. Critical role played by cyclin D3 in the MyoD-mediated arrest of cell cycle during myoblast differentiation. Mol Cell Biol *19*, 5203-5217.

Cerone MA, Marchetti A, Bossi G, Blandino G, Sacchi A, Soddu S 2000 p53 is involved in the differentiation but not in the differentiation-associated apoptosis of myoblasts. Cell Death Differ 7:506-8

Chao DT, Korsmeyer SJ 1998 BCL-2 family: regulators of cell death. Annu Rev Immunol 16:395-419

Chen, D., Padiernos, E., Ding, F., Lossos, I. S., and Lopez, C. D. 2005. Apoptosis-stimulating protein of p53-2 (ASPP2/53BP2L) is an E2F target gene. Cell Death Differ *12*, 358-368.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

Davis, R. L., Cheng, P. F., Lassar, A. B., and Weintraub, H. 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell *60*, 733-746.

de Bruin EC, Meersma D, de Wilde J, et al. 2003 A serine protease is involved in the initiation of DNA damage-induced apoptosis. Cell Death Differ 10:1204-12

Dee K, Freer M, Mei Y, Weyman CM 2002 Apoptosis coincident with the differentiation of skeletal myoblasts is delayed by caspase 3 inhibition and abrogated by MEK-independent constitutive Ras signaling. Cell Death Differ 9:209-18

de la Serna, I. L., Roy, K., Carlson, K. A., and Imbalzano, A. N. 2001. MyoD can induce cell cycle arrest but not muscle differentiation in the presence of dominant negative SWI/SNF chromatin remodeling enzymes. J Biol Chem *276*, 41486-41491.

Denault, J. B., and Salvesen, G. S. 2002. Caspases: keys in the ignition of cell death. Chem Rev *102*, 4489-4500.

Dilworth FJ, Seaver KJ, Fishburn AL, Htet SL, Tapscott SJ. 2004 *In vitro* transcription system delineates the distinct roles of the coactivators pCAF and p300 during MyoD/E47-dependent transactivation. Proc Natl Acad Sci USA 101:11593–11598.

Dona M, Sandri M, Rossini K, Dell'Aica I, Podhorska-Okolow M, Carraro U 2003 Functional in vivo gene transfer into the myofibers of adult skeletal muscle. Biochem Biophys Res Commun 312:1132-8

Egger L, Schneider J, Rheme C, Tapernoux M, Hacki J, Borner C 2003 Serine proteases mediate apoptosis-like cell death and phagocytosis under caspase-inhibiting conditions. Cell Death Differ 10:1188-203

el-Deiry, W. S. 1998. Regulation of p53 downstream genes. Semin Cancer Biol *8*, 345-357.

Ellis, R. E., and Horvitz, H. R. 1991. Two *C. elegans* genes control the programmed deaths of specific cells in the pharynx. Development *112*, 591-603.

Fidzianska A, Goebel HH 1991 Human ontogenesis. 3. Cell death in fetal muscle. Acta Neuropathol (Berl) 81:572-7

Florini, J. R., Ewton, D. Z., and Coolican, S. A. 1996. Growth hormone and the insulin-like growth factor system in myogenesis. Endocr Rev *17*, 481-517.

Friday, B. B., Mitchell, P. O., Kegley, K. M., and Pavlath, G. K. 2003. Calcineurin initiates skeletal muscle differentiation by activating MEF2 and MyoD. Differentiation *71*, 217-227.

Gatz, S. A., and Wiesmuller, L. 2006. p53 in recombination and repair. Cell Death Differ *13*, 1003-1016.

Gilley, J., Coffer, P. J., and Ham, J. 2003. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. J Cell Biol *162*, 613-622.

Gossett, L. A., Kelvin, D. J., Sternberg, E. A., and Olson, E. N. 1989. A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol Cell Biol *9*, 5022-5033.

Gottifredi, V., Peschiaroli, A., Fimia, G. M., and Maione, R. 1999. p53-independent apoptosis induced by muscle differentiation stimuli in polyomavirus large T-expressing myoblasts. J Cell Sci *112 (Pt 14)*, 2397-2407.

Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. 1993. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell *72*, 309-324.

Han J, Flemington C, Houghton AB, et al. 2001 Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc Natl Acad Sci U S A 98:11318-23

Hegde, R., Srinivasula, S. M., Zhang, Z., Wassell, R., Mukattash, R., Cilenti, L., DuBois, G., Lazebnik, Y., Zervos, A. S., Fernandes-Alnemri, T., and Alnemri, E. S. 2002. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. J Biol Chem *277*, 432-438.

Hollenberg, S. M., Cheng, P. F., and Weintraub, H. 1993. Use of a conditional MyoD transcription factor in studies of MyoD trans-activation and muscle determination. Proc Natl Acad Sci U S A *90*, 8028-8032.

Horvitz, H. R. 1999. Genetic control of programmed cell death in the nematode Caenorhabditis elegans. Cancer Res *59*, 1701s-1706s.

Huang, D. C., O'Reilly, L. A., Strasser, A., and Cory, S. 1997. The anti-apoptosis function of Bcl-2 can be genetically separated from its inhibitory effect on cell cycle entry. Embo J *16*, 4628-4638.

Huh, M. S., Parker, M. H., Scime, A., Parks, R., and Rudnicki, M. A. 2004. Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. J Cell Biol *166*, 865-876.

Kandasamy K, Srinivasula SM, Alnemri ES, et al. 2003 Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome c andSmac/DIABLO release. Cancer Res 63:1712-21

Karasarides M, Dee K, Schulman D, Wolfman A, Weyman CM 2006 Active Ras-induced effects on skeletal myoblast differentiation and apoptosis are independent of constitutive PI3- kinase activity. Cell Biol Int 30:308-318

Kerr, J. F., Wyllie, A. H., and Currie, A. R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer *26*, 239-257.

Komarov PG, Komarova EA, Kondratov RV, et al. 1999 A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 85:1733-7

Konieczny SF, Emerson CP, Jr. 1984 5-Azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. Cell 38:791-800

Konieczny SF, Drobes BL, Menke SL, Taparowski EJ 1989 Inhibition of myogenic differentiation by the H-Ras oncogene is associated with the downregulation of the MyoD1 gene. Oncogene 4:473-81

Kroemer G 1999 Mitochondrial control of apoptosis: an overview. Biochem Soc Symp 66:1-15

Kumar A, Commane M, Flickinger TW, Horvath CM, Stark GR 1997 Defective TNF-alph ainduced apoptosis in STAT1-null cells due to low constitutive levels of caspases. Science 278:1630-2

Laemmli U. 1970. Cleavage of structural proteins during assembly of the head bacteriophage T4. Nature 227, 680-685.

Letai, A., Bassik, M. C., Walensky, L. D., Sorcinelli, M. D., Weiler, S., and Korsmeyer, S. J. 2002. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell *2*, 183-192.

Li J, Lee B, Lee AS 2006 Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. J Biol Chem 281:7260-70

Lin, T., Chao, C., Saito, S., Mazur, S. J., Murphy, M. E., Appella, E., and Xu, Y. 2005. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat Cell Biol *7*, 165-171.

Lindsten, T., Ross, A. J., King, A., Zong, W. X., Rathmell, J. C., Shiels, H. A., Ulrich, E., Waymire, K. G., Mahar, P., Frauwirth, K.*, et al.* 2000. The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. Mol Cell *6*, 1389-1399.

Liu, D. X., and Greene, L. A. 2001. Neuronal apoptosis at the G1/S cell cycle checkpoint. Cell Tissue Res *305*, 217-228.

Locksley, R. M., Killeen, N., and Lenardo, M. J. 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell *104*, 487-501.

Lu, Z., and Sack, M. N. 2008. ATF-1 is a hypoxia-responsive transcriptional activator of skeletal muscle mitochondrial-uncoupling protein 3. J Biol Chem *283*, 23410-23418.

Ludolph, D. C., and Konieczny, S. F. 1995. Transcription factor families: muscling in on the myogenic program. Faseb J *9*, 1595-1604.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. 1998. Bid, a Bcl-2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell *94*, 481-490.

Michalak, E. M., Villunger, A., Adams, J. M., and Strasser, A. 2008. In several cell types tumour suppressor p53 induces apoptosis largely via Puma but Noxa can contribute. Cell Death Differ *15*, 1019-1029.

Mal, A. K. 2006. Histone methyltransferase Suv39h1 represses MyoD-stimulated myogenic differentiation. Embo J *25*, 3323-3334.

Mal, A., and Harter, M. L. 2003. MyoD is functionally linked to the silencing of a muscle-specific regulatory gene prior to skeletal myogenesis. Proc Natl Acad Sci U S A *100*, 1735-1739.

Martin JF, Schwarz JJ, Olson EN 1993 Myocyte enhancer factor (MEF) 2C: a tissue restricted member of the MEF-2 family of transcription factors. Proc Natl Acad Sci U S A 90:5282-6

Megeney LA, Kablar B, Garrett K, Anderson JE, Rudnicki MA 1996 MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev 10:1173-83

Megeney LA, Rudnicki MA 1995 Determination versus differentiation and the MyoD family of transcription factors. Biochem Cell Biol 73:723-32

Menasche P 2004 Skeletal myoblast transplantation for cardiac repair. Expert Rev Cardiovasc Ther 2:21-8

Miller JB, Stockdale FE 1986 Developmental regulation of the multiple myogenic cell lineages of the avian embryo. J Cell Biol 103:2197-208

Ming, L., Sakaida, T., Yue, W., Jha, A., Zhang, L., and Yu, J. 2008. Sp1 and p73 activate PUMA following serum starvation. Carcinogenesis *29*, 1878-1884.

Molchadsky, A., Shats, I., Goldfinger, N., Pevsner-Fischer, M., Olson, M., Rinon, A., Tzahor, E., Lozano, G., Zipori, D., Sarig, R., and Rotter, V. 2008. p53 plays a role in mesenchymal differentiation programs, in a cell fate dependent manner. PLoS One *3*, e3707.

Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., and et al. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell *58*, 537-544.

Nakanishi K, Sudo T, Morishima N 2005 Endoplasmic reticulum stress signaling transmitted by ATF6 mediates apoptosis during muscle development. J Cell Biol 169:555-60

Nakano K, Vousden KH 2001 PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell 7:683-94

Newmeyer DD, Ferguson-Miller S 2003 Mitochondria: releasing power for life and unleashing the machineries of death. Cell 112:481-90

Nickson, P., Toth, A., and Erhardt, P. 2007. PUMA is critical for neonatal cardiomyocyte apoptosis induced by endoplasmic reticulum stress. Cardiovasc Res *73*, 48-56.

Obexer, P., Geiger, K., Ambros, P. F., Meister, B., and Ausserlechner, M. J. 2007. FKHRL1-mediated expression of Noxa and Bim induces apoptosis via the mitochondria in neuroblastoma cells. Cell Death Differ *14*, 534-547.

O'Flaherty, J., Mei, Y., Freer, M., and Weyman, C. M. 2006. Signaling through the TRAIL receptor DR5/FADD pathway plays a role in the apoptosis associated with skeletal myoblast differentiation. Apoptosis *11*, 2103-2113.

Olson EN 1992 Interplay between proliferation and differentiation within the myogenic lineage. Dev Biol 154:261-72

Peden, E., Killian, D. J., and Xue, D. 2008. Cell death specification in C. elegans. Cell Cycle *7*, 2479-2484.

Pelengaris, S., Khan, M., and Evan, G. 2002. c-MYC: more than just a matter of life and death. Nat Rev Cancer *2*, 764-776.

Perry, R. L., and Rudnick, M. A. 2000. Molecular mechanisms regulating myogenic determination and differentiation. Front Biosci *5*, D750-767.

Peschiaroli A, Figliola R, Coltella L, et al. 2002 MyoD induces apoptosis in the absence of RB function through a p21(WAF1)-dependent re-localization of cyclin/cdk complexes to the nucleus. Oncogene 21:8114-27

Pinney DF, Pearson-White SH, Konieczny SF, Latham KE, Emerson CP, Jr. 1988 Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. Cell 53:781-93

Porrello, A., Cerone, M. A., Coen, S., Gurtner, A., Fontemaggi, G., Cimino, L., Piaggio, G., Sacchi, A., and Soddu, S. 2000. p53 regulates myogenesis by triggering the differentiation activity of pRb. J Cell Biol *151*, 1295-1304.

Rhodes, S. J., and Konieczny, S. F. 1989. Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev *3*, 2050-2061.

Robertson JD, Orrenius S and Zhivotozsky B. 2000 Review: Nuclear events in apoptosis. Journal of Structural Biology 129:346-58

Rudnicki, M. A., and Jaenisch, R. 1995. The MyoD family of transcription factors and skeletal myogenesis. Bioessays *17*, 203-209.

Sabourin LA, Girgis-Gabardo A, Seale P, Asakura A, Rudnicki MA 1999 Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. J Cell Biol 144:631-43

Salvesen GS, Dixit VM 1997 Caspases: intracellular signaling by proteolysis. Cell 91:443-6

Sandri MC, M. Massimino, M.L. Geromel, V., Arslan, P. 1996 Myoblasts and myotubes in primary cultures deprived of growth factors undergo apoptosis. Basic Appl. Myol. 6:257-260

Sandri M, El Meslemani AH, Sandri C, et al. 2001 Caspase 3 expression correlates with skeletal muscle apoptosis in Duchenne and facioscapulo human muscular dystrophy. A potential target for pharmacological treatment? J Neuropathol Exp Neurol 60:302-12

Sartorelli, V., and Caretti, G. 2005. Mechanisms underlying the transcriptional regulation of skeletal myogenesis. Curr Opin Genet Dev *15*, 528-535.

Sartorius, U., Schmitz, I., and Krammer, P. H. 2001. Molecular mechanisms of death-receptor-mediated apoptosis. Chembiochem *2*, 20-29.

Savill, J., and Fadok, V. 2000. Corpse clearance defines the meaning of cell death. Nature *407*, 784-788.

Scorrano L, Korsmeyer SJ 2003 Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun 304:437-44

Scorsin M, Hagege A, Vilquin JT, et al. 2000 Comparison of the effects of fetal cardiomyocyte and skeletal myoblast transplantation on postinfarction left ventricular function. J Thorac Cardiovasc Surg 119:1169-75

Shaltouki A, Freer M, Mei Y, Weyman CM 2007 Increased expression of the pro-apoptotic Bcl (2) family member PUMA is required for mitochondrial release of cytochrome C and the apoptosis associated with skeletal myoblast differentiation. Apoptosis 12:2143-54

Skuk D, Tremblay JP 2003 Cell therapies for inherited myopathies. Curr Opin Rheumatol 15:723-9

Skuk D, Tremblay JP 2003 Myoblast transplantation: the current status of a potential therapeutic tool for myopathies. J Muscle Res Cell Motil 24:285-300

Skurk C, Maatz H, Kim HS, et al. 2004 The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability through modulation of the caspase-8 inhibitor FLIP. J Biol Chem 279:1513-25

Slee, E. A., Harte, M. T., Kluck, R. M., Wolf, B. B., Casiano, C. A., Newmeyer, D. D., Wang, H. G., Reed, J. C., Nicholson, D. W., Alnemri, E. S.*, et al.* 1999. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of

caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol *144*, 281-292.

Solanes G, Pedraza N, Iglesias R, Giralt M, and Villarroya F. 2003 "Functional relationship between MyoD and peroxisome proliferator-activated receptor-dependent regulatory pathways in the control of the human uncoupling protein-3 gene transcription," *Molecular Endocrinology*, vol. 17, no. 10, pp. 1944–1958,.

Suzuki K, Murtuza B, Smolenski RT, et al. 2001 Cell transplantation for the treatment of acute myocardial infarction using vascular endothelial growth factor-expressing skeletal myoblasts. Circulation 104:I207-12

Tapscott, S. J. 2005. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. Development *132*, 2685-2695.

Talanian, R. V., Yang, X., Turbov, J., Seth, P., Ghayur, T., Casiano, C. A., Orth, K., and Froelich, C. J. 1997. Granule-mediated killing: pathways for granzyme B-initiated apoptosis. J Exp Med *186*, 1323-1331.

Taylor DA, Atkins BZ, Hungspreugs P, et al. 1998 Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. Nat Med 4:929-33

Thellmann M, Hatzold J, Conradt B 2003 The Snail-like CES-1 protein of C. elegans can block the expression of the BH3-only cell-death activator gene egl-1 by antagonizing the function of bHLH proteins. Development 130:4057-71

Thornberry NA, Lazebnik Y 1998 Caspases: enemies within. Science 281:1312-6

Toth, A., Jeffers, J. R., Nickson, P., Min, J. Y., Morgan, J. P., Zambetti, G. P., and Erhardt, P. 2006. Targeted deletion of Puma attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion. Am J Physiol Heart Circ Physiol *291*, H52-60.

Tran H, Brunet A, Grenier JM, Datta SR, Fornace AJ, Jr, DiStefano PS, Chiang LW, Greenberg ME. 2002 DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science.* 296:530–4.

Verhagen, A. M., Silke, J., Ekert, P. G., Pakusch, M., Kaufmann, H., Connolly, L. M., Day, C. L., Tikoo, A., Burke, R., Wrobel, C.*, et al.* 2002. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. J Biol Chem *277*, 445-454.

Voronova, A., and Baltimore, D. 1990. Mutations that disrupt DNA binding and dimer formation in the E47 helix-loop-helix protein map to distinct domains. Proc Natl Acad Sci U S A *87*, 4722-4726.

Vousden, K. H., and Prives, C. 2009. Blinded by the Light: The Growing Complexity of p53. Cell *137*, 413-431.

Wang J, Walsh K 1996 Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation. Science 273:359-61

Wang J, Guo K, Wills KN, Walsh K 1997 Rb functions to inhibit apoptosis during myocyte differentiation. Cancer Res 57:351-4

Weyman, C. M., Taparowsky, E. J., Wolfson, M., and Ashendel, C. L. 1988. Partial down-regulation of protein kinase C in C3H 10T 1/2 mouse fibroblasts transfected with the human Ha-ras oncogene. Cancer Res *48*, 6535-6541.

Weyman, C. M., and Wolfman, A. 1997. Oncogenic Ras-induced secretion of a novel inhibitor of skeletal myoblast differentiation. Oncogene *15*, 2521-2528.

Weyman, C. M., and Wolfman, A. 1998. Mitogen-activated protein kinase kinase (MEK) activity is required for inhibition of skeletal muscle differentiation by insulin-like growth factor 1 or fibroblast growth factor 2. Endocrinology *139*, 1794-1800.

Wildey, G. M., and Howe, P. H. 2009. Runx1 is a co-activator with FOXO3 to mediate transforming growth factor beta (TGFbeta)-induced Bim transcription in hepatic cells. J Biol Chem *284*, 20227-20239.

Wildey GM, Patil S, Howe PH 2003 Smad3 potentiates transforming growth factor beta (TGFbeta)-induced apoptosis and expression of the BH3-only protein Bim in WEHI 231 B lymphocytes. J Biol Chem 278:18069-77

Wright, W. E., Sassoon, D. A., and Lin, V. K. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell *56*, 607-617.

Wu GS, Burns TF, McDonald ER, 3rd, et al. 1997 KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. Nat Genet 17:141-3

You H, Pellegrini M, Tsuchihara K, et al. 2006 FOXO3a-dependent regulation of Puma in response to cytokine/growth factor withdrawal. J Exp Med 203:1657-63

Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B 2001 PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell 7:673-82

Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J. D., Phillips, R. A., and Gallie, B. L. 1996. pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. Genes Dev *10*, 3051-3064.

Zhang P, Wong C, Liu D, Finegold M, Harper JW, and Elledge SJ 1999. $p21^{CIP1}$ and $p57^{KIP2}$ control muscle differentiation at the myogenin step. *Genes and Development* 13(2):213-224.