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Novel Translational Regulation of the Proapoptotic bcl2 Member Puma and Its Role During Skeletal Myoblast Apoptosis

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NOVEL TRANSLATIONAL REGULATION OF THE PROAPOPTOTIC BCL2 MEMBER PUMA AND ITS ROLE DURING SKELETAL MYOBLAST APOPTOSIS

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DEDICATION

To my parents,
for their unending support, love and believing in me

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NOVEL TRANSLATIONAL REGULATION OF THE PROAPOPTOTIC BCL2 MEMBER PUMA AND ITS ROLE DURING SKELETAL MYOBLAST APOPTOSIS

ATOSSA SHALTOUKI

ABSTRACT

Differentiation and apoptosis are coordinately regulated in skeletal myoblasts. During the process of skeletal myoblast differentiation, our lab has determined that roughly 30% of myoblasts undergo apoptosis rather than differentiation. Further, we have reported that cytochrome C is released, and that the expression level of the pro-apoptotic Bcl2 family member PUMA is elevated, when 23A2 myoblasts are cultured in DM. Our long-term goal is to identify targets for manipulation that would abrogate this apoptosis without affecting differentiation. These findings could be significant to the study of myoblast transfer as a therapeutic approach.

The goals of this dissertation are to:

1- determine the significance of PUMA in apoptosis associated with skeletal myoblast differentaion
2- determine the mechanism responsible for the increased expression of PUMA

In chapter II, we provide the data documenting that the release of cytochrome C in 30% of 23A2 cells correlates with the percentage of the cells undergoing apoptosis. We also showed that suppression of PUMA expression using short hairpin interfering RNA-mediated silencing of PUMA decreased the release of cytochrome C and apoptosis in 23A2 cells, thus demonstrating that the increased expression of PUMA is critical for the apoptotic process. Abrogation of PUMA expression did not affect that ability of myoblasts to undergo differentiation. Using a p53 pharmacological inhibitor Pifithrin, we showed that signaling through p53 is not responsible for the increased expression of PUMA in DM. Finally, using a fibroblast cell line expressing an estrogen receptor:MYoD fusion protein, we have determined that addition of estradiol sensitizes these fibroblasts to apoptosis and results in increased PUMA expression.

In chapter III, we have determined that culturing of myoblasts in DM with actinomyocin D is sufficient to block the increase in PUMA mRNA levels, but does not prevent the increase in PUMA protein levels. Further, metabolic labeling of newly synthesized proteins in myoblasts cultured in either growth media (GM) or DM supplemented with actinomycin D followed by immunoprecipitation of PUMA has allowed us to conclude that this increase in PUMA protein is a consequence of increased translation. Polyribosome analysis of PUMA mRNA indicated that translational regulation most probably occurs at the initiation step. We have also found that the increase in PUMA protein levels occurs under conditions of eIF2 alpha phosphorylation and subsequent global

decrease in total protein synthesis. Analysis of the 5' UTR of PUMA mRNA by Mfold program predicts stable secondary structures incompatible with efficient ribosome scanning. Further, we have determined that impairment of cap-dependent translation *in vitro* and *in vivo* did not affect the translation of PUMA mRNA. Finally, we have generated data indicating that the increased translation of PUMA in DM is mediated by an internal ribosome entry site (IRES). Using mono- and bi-cistronic reporters, we have identified a fragment of PUMA mRNA that allows for cap-independent translation in vitro and in vivo in response to culture in DM. This fragment encompasses regions upstream and downstream of the initiation AUG codon. Thus, our data indicate that increased expression of PUMA in skeletal myoblasts relies on IRES-meditated translation.

In summary, we identified PUMA as the BH3-only proapoptotic molecule that plays an essential role in the apoptosis but not the differentiation of skeletal myoblasts. Thus, we have identified PUMA as a potential target for therapeutic manipulation to abrogate apoptosis without affecting differentiation. Finally we identified that IRES-mediated translation of PUMA as the mechanism responsible for the increase in PUMA expression in response to different stress stimuli.

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LIST OF ABBREVIATIONS

A-1	Bcl2 related protein A1
AIF	Apoptosis inducing factor
ANT	Adenine nucleotide translocator
Apaf-1	Apoptotic Protease Activating Factor 1
ATM	Ataxia telangiectasia mutated)
ATP	Adenosine Triphosphate
ATR	Ataxia telangiectasia and Rad3
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	Bcl2 homology domain
bHLH	Basic helix loop helix
Bid	Bcl-2 homology interacting domain death agonist
Bim	BCL-2-interacting mediator of cell death
BIR	Baculovirus IAP repeat
Bik	Bcl2-interacting killer
Blk	B lymphocyte kinase,
BME	Beta mercaptoethanol/Basal Medium Eagle
BMEM	Basal modified Eagle’s medium

Bmf	Bcl2-modifying factor
BNIP3	Bcl2/adenovirus E1B 19 kDa protein-interacting protein 3
Bok	Bcl2-related overian killer
BSA	Bovine Serum Albumin
CARD	Caspase recruitment domain
Caspases	Cysteine-aspartic acid specific proteases
CDK4	Cyclin dependent kinase 4
cDNA	Copy deoxyribonucleic acid
CHAPS	3-[3-(chloramidopropyl)dimethylammonio]-1-propanesulfonic acid
CHX	Cyclohexamide
cIAPs	Cellular inhibitor of apoptosis
C_t	Coefficient time C_t Coefficient time
DED	Death effector domain
DEPC	Diethylpyrocarbonate
Diablo	**D**irect IAP-binding protein with low pI
DISC	Death inducing signaling complex
DM	Differentiation medium
DMEM	Dulbecco modified eagle medium
DMSO.	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PKc	DNA-activated protein kinase catalytic subunit
dNTPs	Deoxyribonucleo triphosphates
DR4	Death receptor 4
DR5	Death receptor 5

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
ELISA	Enzyme linked immunosorbant assay
EndoG	Endonuclease G
FADD	Fas-associated death domain
Fas	Fatty acid synthase
FasL	Fas ligand
FBS	Fetal bovine serum
FLIP	FLICE-inhibitory protein
GAPDH	Glyceraldehyde phosphodehydrogenase
GCN2	General control nondepressible 2
GM	Growth medium
GTP	Guanosine triphosphate
HLH	Helix loop helix
Hrk	Harakiri
HRI	Heme regulated eIF2 kinase inhibitor
HRP	Horse radish peroxidase
Hsp70	Heat shock protein 70
HtrA2	High temperature requirement protein A2
IAPs	Inhibitor of apoptosis
Id	Inhibitor of differentiation protein
IGF	Insulin-like growth factor

IRES	Internal Ribosome Entry Site
ITAFs	IRES Transacting Factors (ITAFs)
Luc	Luciferase
Met/Cys	Methionine/Cystine
Mcl-1	Myeloid leukemia cell differentiation protein1
MHC	Myosin heavy chain
MOMP	Mitochondrial outer membrane permeabilization
MOMP	Mitochondrial outer membrane potential
MOPS	3-(N-Morpholino)propanesulfonic acid; n-(3-sulfopropylorpholine
MRFs	Muscle regulatory factors
MRF4	Muscle Regulatory factor-4
mRNA	Messenger Ribonucleic acid
Mtd	Matador
mTOR	Mammalian target of rapamycin
MTP	Mitochondrial permeability transition
Myf5	Myogenic factor-5
MyoD	Myogenic differentiation
OD405	Optical density at 405 nm wavelength
P	Parental
PBS	Phosphate Buffered Saline
PCBP2	Poly(rC) binding protein 2
PERK	Protein kinase-like endoplasmic reticulum kinase
PI3K	Phosphatidylinositol 3-kinases

PIKK	Phosphoinositide kinase-related kinase
PKR	Protein Kinase R
PMF	Phenyl methylsufonylfluoride
P/S	Penicillin/streptomycin
PTB	Polypyrimidine –tract-binding protein
PTP	Mitochondrial permeability transition pore
PUMA	p53 upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
Raf	Ras associated factor
Ras	Rat sarcoma
Rb	Retinoblastoma
RNAi	Ribonucleic acid interference
RT-PCR	Reverse-transcription-polymerase chain reaction
S6K1	S6 kinase 1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	Short hairpin ribonucleic acid
Smac	Second mitochondria-derived activator of caspase
Spike	*S*mall *p*rotein with *i*nherent *k*illing *e*ffect
tBid	Truncated Bid
TCA	Trichloroacetic acid
TNF	Tumor necrosis factor

TNFR	Tumor necrosis factor receptor
TRAIL	TNF related apoptosis inducing ligand
TBS-T	Tris buffered saline with Tween 20ER endoplasmic reticulum
tRNA	Transfer RNA
TRADD	Tumor necrosis factor receptor type 1 associated death domain
Unr	Upstream of N ras
UTR	Untranslated region
VDAC	Voltage dependent anion channel
Xiaps	X linked inhibitor of apoptosis

CHAPTER I

INTRODUCTION

The skeletal myoblast model system

Skeletal muscle is a valuable model for studying the mechanisms and signaling pathways that regulate cell lineage specification and differentiation. The myogenic program requires a group of transcription factors belonging to the muscle regulatory transcription factor (MRF) family consisting of Myf-5, MyoD, myogenin and MRF4 (1-6). Each member of the family shares a region of homology consisting of a basic region followed by a Helix loop Helix motif important for its protein oligomerization and DNA binding properties (7-9). Further (8-10) each factor is capable of inducing muscle lineage upon its expression in nonmuscle cells (2-4, 11). Examination of MRF expression patterns in the embryo as well as in muscle cell lines has revealed a temporal and spatial expression pattern for each MRF member with MyoD and Myf-5 being expressed in proliferating myoblasts and Myogenin and MRF4 being expressed upon differentiation (12-14). MyoD was the first member of MRFs identified and has been the most studied (8).

Detailed understanding of the mechanistic regulation of myogenesis has come from *in vitro* studies using murine myoblast cell lines. The myoblast cell lines most widely used are either isolated from C3H mice (C2C12 and BC3H-1) or derived from non-specified 10T1/2 fibroblasts also from C3H mice (23A2 myblasts) (15, 16). *In vivo*, circulating mitogens maintain myoblasts in an actively dividing and undifferentiated state. Appropriate migration results in a decrease in available mitogens, activation of MyoD, withdrawal from cell cycle and subsequent differentiation.

In proliferating myoblasts, MyoD is constitutively expressed but is transcriptionally inactive (2, 11, 17, 18). Transcriptional activation by MyoD requires dimerization with E-protein members through basic helix-loop-helix (bHLH) domain to form a heterodimeric complex capable of binding a DNA consensus sequence: CANNTG which is known as E-box elements within the regulatory regions of most muscle-specific genes (7, 19, 20). In the presence of serum growth factors expression levels of Id remain high. Id is a HLH protein that lacks the basic region. Id heterodimerization with E-proteins prevents their binding to MyoD. In the absence of serum, Id proteins are degraded and differentiation is achieved upon MyoD transcriptional activation (21-23). The *in vitro* differentiation of skeletal myoblasts can thus be induced by mitogen withdrawal. Our lab (24) and others (25, 26) have previously reported that, in response to mitogen withdrawal, approximately 70% of myoblasts undergo differentiation while 30% will undergo apoptosis. The apoptotic process in other

cell systems has been studied extensively. The goal of the Weyman's lab has been to understand the functional apoptotic pathways in skeletal myoblasts.

Overview of Apoptosis

Apoptosis is a programmed cell death that occurs during normal development and is also important for maintaining tissue homeostasis (27). During development, maintaining the proper number of cells within a given tissue is achieved by a balance between proliferation and cell death. This ability of the cell to "commit suicide" in any tissue when no longer necessary, damaged or when lacking a survival factor is an active process and requires activation of certain conserved apoptotic genes (28, 29). Apoptosis has been implicated in the deletion of excess cells during myogenesis (30, 31), in the central nervous system (32), and in the removal of self-reactive T cells in the immune response (33) as well as during limb formation (34) and metamorphosis (29). Cells with damaged genetic content are also known to undergo the apoptotic process. Programmed cell death is a highly regulated process and therefore its disregulation is associated with a variety of diseases such as cancer (which is the case of too little apoptosis) or autoimmune disease (excessive apoptosis) (35). Cells undergoing programmed cell death exhibit a characteristic of a condensed cytoplasm and nucleus in addition to chromatin aggregation. Apoptosis is characterized by the production of apoptotic bodies, plasma membrane blebbing and the generation of nuclear DNA fragments of

internucleosomal size (36, 37). These morphological changes associated with apoptosis are achieved by a family of proteases known as caspases.

Caspases

Caspases are cysteine-aspartic acid specific proteases that catalyze the cleavage of their substrate after Aspartic Acid residues by their cysteine-rich active sites (28, 38). In every cell, caspases are made as inactive proenzymes consisting of a small and a large subunit and an N-terminus prodomain of variable length important for their recruitment and activation. Signaling events that lead to proteolysis between the subunits and removal of the prodomain, result in formation of active caspases which are a heterotetramer of 2 small and 2 large subunits of two procaspase molecules. Caspases are divided into two groups based on the size of their prodomains; the “initiator caspases” (caspase 2, 8, 9 and 10) have a long N-terminal prodomain while the “executioner caspases” (caspase 3, 6 and 7) have a short prodomain. Subclassification of initiator caspases based on their mode of activation divides this group of caspases further into two groups. Caspases 2 and 8 have a long prodomain containing a death effector domain (DED), while caspase 9 has a caspase recruitment domain (CARD). In general, activation of initiator caspases by internal or external cues initiates a caspase cascade reaction, activating the downstream executioner caspases and leading to destruction of the cell (28, 39, 40). Two signaling pathways have been implicated in the initiation of apoptosis: the extrinsic pathway and the intrinsic pathway (41, 42)

The intrinsic pathway

The internal pathway of apoptosis begins with the mitochondria in response to a wide range of stimuli such as DNA damage, ER stress, oxidative stress, serum deprivation and other death inducing signals and results in mitochondrial outer membrane permeabilization (MOMP). MOMP results in the release of pro-apoptotic proteins, such as cytochrome C (43), Smac/Diablo (second mitochondria-derived activator of caspase/ **d**irect IAP-binding protein with low pI) (44), omi/HtrA2 (high temperature requirement protein A2) (45, 46) and EndoG (Endonuclease G) (47, 48) from the intermembrane space of the mitochondria (49). After the release of cytochrome C from the mitochondria, a caspase cascade is initiated (50-52). In the cytosol, binding of cytochrome C with a WD-40 repeat of Apaf-1 (apoptotic protease activating factors) in an ATP dependent manner causes a conformational change and hepamerization of Apaf-1. A multiprotein complex known as apoptosome is then formed which participates in initiating the recruitment and activation of procaspase 9. Activation of caspase 9 within the apoptosome complex leads to cleavage and activation of downstream caspases 3 and 7 (53-55). In the cytosol, activity of caspases 3, 7 and 9 can be further inhibited by cytosolic cIAPs (cellular inhibitor of apoptosis), a family of proteins with antiapoptotic activity originally identified in the genome of baculovirus (56). The binding of caspases to the conserved baculovirus IAP repeat (BIR) domain of cIAPs inhibits apoptosis (57). However, this inhibition of caspase activity is relieved by the release of mitochondrial proteins such as

Smac/Diablo and Omi/HrtA, two mammalian IAP-binding proteins (58-61) (Fig. 1).

The extrinsic pathway

The external pathway of apoptosis involves the activation of death receptors belonging to the tumor necrosis factor receptor superfamily (TNFR) (62, 63) in response to external stimuli resulting directly in caspase activation. The cytoplasmic regions of these death receptors (TNFR-1, Fas/CD95, TRAIL DR4, DR5) consist of death domains while the extracellular region is cysteine rich and allows trimerization and activation of the receptor by its ligand (64, 65). Upon binding of a death ligand to its receptor, procaspase- 8 is recruited to the death receptor through binding to death effector domains of adaptor proteins like FADD or TRADD in the death inducing signaling complex (DISC). Once several procaspase 8 molecules are recruited to the DISC complex, then by virtue of close proximity they cleave and activate each other through a process known as autoproteolysis. Activation of caspase 8 molecules leads to a caspase cascade resulting in the activation of effector caspases 3, 6, and 7 (40). Activation of caspase 8 has also been implicated in mitochondrial mediated cell death. It has been shown that caspase 8 cleaves Bid, a BH3-only member of the Bcl2 family, and that truncated Bid can translocate to the mitochondia and induce the release of cytochrome C via activation of Bax and Bak (66) (Fig. 2).

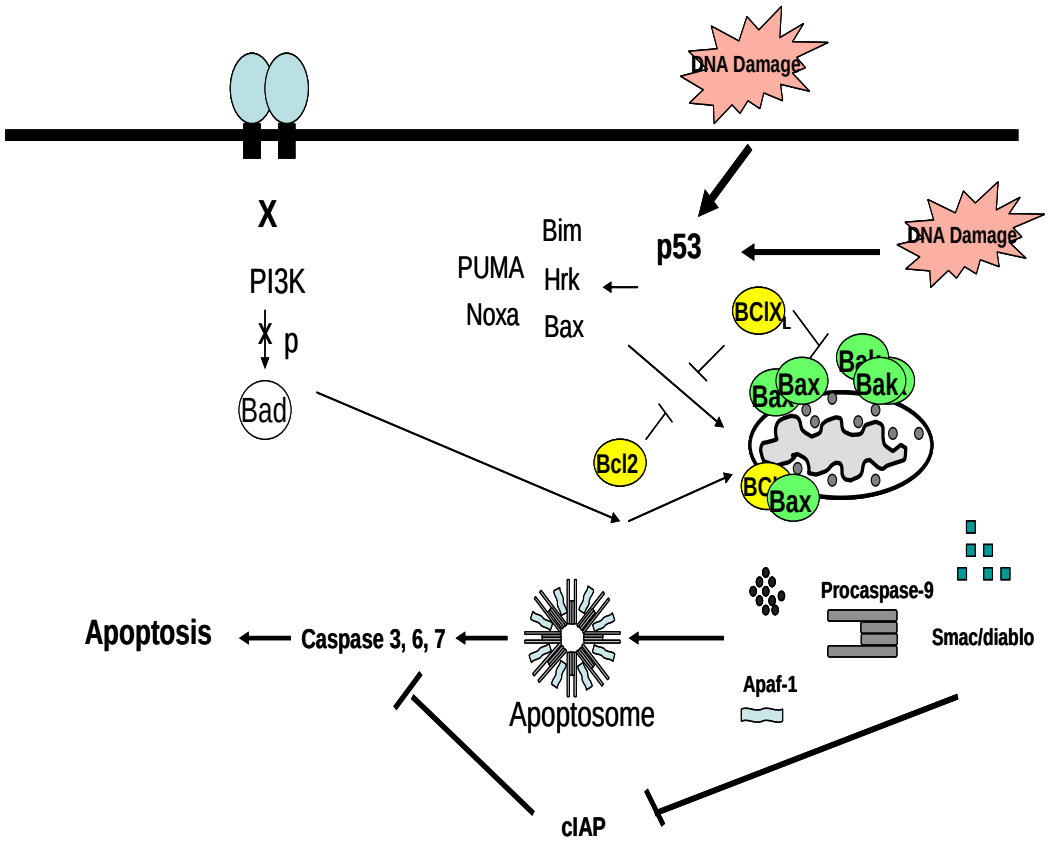

Figure 1.1 The Intrinsic Pathway of Apoptosis. In the presence of serum, PI3K phosphorylates the proapoptotic member Bad resulting in the cytosolic localization of Bad through its interaction with scaffold protein 14-3-3. In the absence of serum, the PI3K signaling pathway is inactive. Bad translocates to the mitochondria where it binds and blocks the activity of the antiapoptotic Bcl2 members. In response to DNA damage, the expression of BH3-only members like Bax and PUMA is upregulated in a p53-dependent manner. These proteins can then translocate to the mitochondria and cause permeabilization of the mitochondial membrane and the release of cytochrome C and other antiapoptotic molecules from the mitochondria. Cytochrome C binds Apaf-1 and caspase 9 resulting in apoptosome formation. Caspase 9 is active in the apoptosome and cleaves and activates caspases 3, 7 and downstream cellular targets.

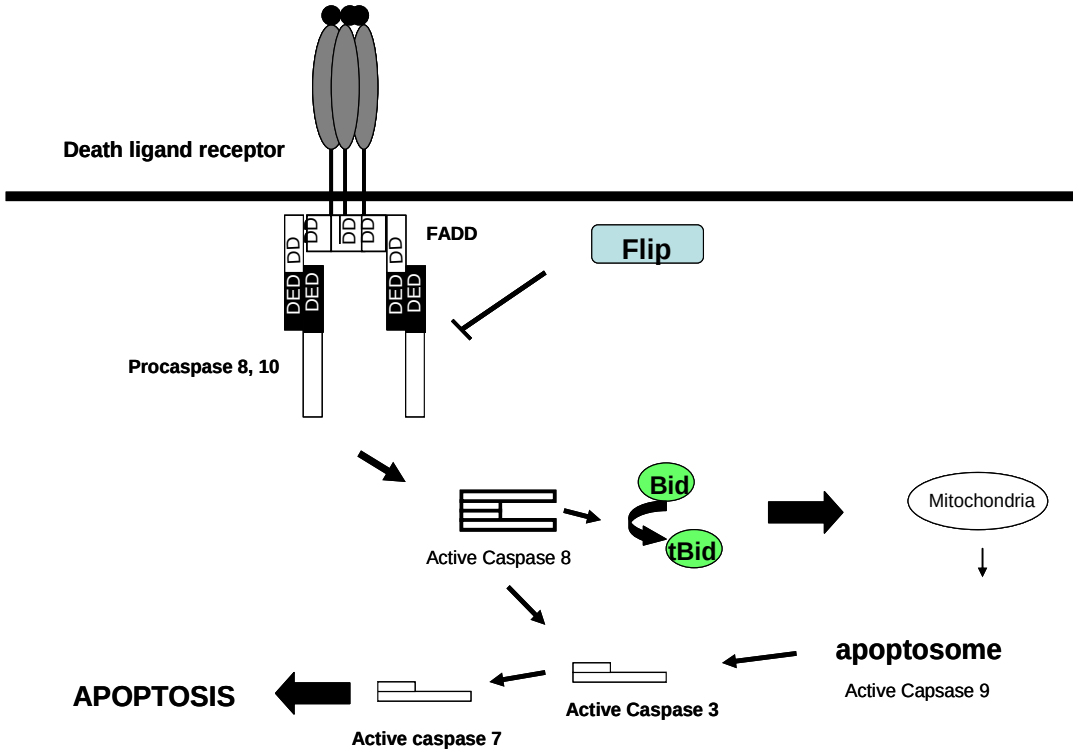

Figure 1.2 The Extrinsic Pathway of Apoptosis Activation of the extrinsic pathway begins with the binding of a death ligand to its receptor which causes oligomerization of the receptors. Adaptor proteins such as FADD (Fas-associated death domain) are recruited to intracellular domains of these receptors which are known as the “Death domains”, forming the death-inducing signaling complex (DISC). Initiator caspases 8 and 10 are recruited to DISC through their death effector domains and are self-cleaved and activated by virtue of close proximity. Activated caspases 8 and 10 can then cleave and activate downstream caspases 3, 6, and 7. The intrinsic arm of this pathway involves the cleavage of pro-apoptotic Bcl2 member Bid by caspase 8. Truncated Bid translocates to the mitochondria and dimerizes with Bax, converging on the intrinsic pathway.

Bcl2 Family

The release of cytochrome C is subject to regulation by the Bcl-2 family of proteins (67). Bcl2 (B cell lymphoma 2) was the first member of the family identified as a proto-oncogene in follicular lymphoma cells with the t(14:18) chromosome translocation involving heavy chain joining regions of the immunoglobulin heavy chain locus on chromosome 14 and the bcl-2 gene on chromosome 18 (68). Many members of this protein family have a C-terminal transmembrane domain important for membrane docking (69). The involvement of the Bcl2 protein family in the apoptotic process came from genetic studies in C. *elegans.* These studies showed that the product of the ced9 gene, a homolog of mammalian Bcl2, is essential in preventing programmed cell death during worm development (70). Anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-xL, Bcl-w, A-1/Bfl-1 and Mcl-1, E1B, BHRF1, CED9) prevent mitochondrial disruption while pro-apoptotic members of the Bcl-2 family initiate mitochondrial disruption (60, 71-73). Sequence homology among members of Bcl-2 family has identified regions of high homology (BH1 to BH 4) important for protein-protein interaction. All anti-apoptotic members of the Bcl-2 family contain all four BH domains (Bcl2 homology domain) (74). Deletion of the BH4 domain has shown to promote pro-apoptotic activity in anti-apoptotic members of Bcl-2 family, indicating that this BH4 domain is essential for prosurvival activity of these members (69, 75). All proapoptotic members of Bcl2 lack this BH4 domain but must contain at least one BH3 domain. Classification of the pro-apoptotic members are either multi-domain (Bax, Bak and Bok/Mtd) or BH3-only domain

(Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, PUMA, Blk, BNIP3 and Spike) (76, 77). The primary function of the BH3 only family members is to induce the activation of Bax or Bak (78, 79) or to inhibit the anti-apoptotic function of Bcl-2 members (80). Knockout studies have demonstrated that both Bax and Bak are important regulators of mitochondia-mediated cell death since inactivation of both Bax and Bak in mice resulted in decreased apoptosis in different tissues during development. In addition, cells derived from these double knockout mice have been shown to be resistant to apoptosis in response to many stress stimuli (81, 82). In the absence of death signals, Bax exists as a monomer loosely associated with the membrane in the cytosol while Bak is located at the mitochondria. Following death signals, Bax and Bad undergo conformational changes that allow them to insert into the outer mitochondrial membrane and oligomerize (83), resulting in permeablization of the outer mitochondial membrane and release of proapoptotic proteins. It has been postulated that interaction with BH3 only members of the Bcl-2 family contributes to the oligomerization and channel forming ability of multidomain Bax and Bak while antiapoptotic members prevent this (83, 84). Alternatively, the increase in mitochondrial outer membrane permeability (MOMP) associated with the intrinsic apoptotic pathway can involve a sudden increase in permeability of the inner mitochondrial membrane to molecules up to 1.5 Kda, which result in mitochondrial swelling and rupture of outer mitochondrial membrane. The induction of mitochondrial permeability transition (MTP) through formation of the mitochondrial permeability transition pore (PTP) can be achieved by various

stimuli. PTP complexes consist of the ANT protein (adenine nucleotide translocator) in the inner mitochondrial membrane, the VDAC (voltage dependent anion channel) in the outer mitochondrial membrane, the benzodiazepine receptor and cyclophilin D (85-87). Although Bax and Bak are not essentional for MTP induction, interaction of Bax with VDAC and ANT has been reported to cause MOMP (88, 89).

Skeletal myoblast apoptosis

Apoptosis has been observed in cultures of primary myoblasts, established muscle cell lines, and in vertebrate models during the process of differentiation (24-26, 30, 31). Molecular mechanisms responsible for regulating apoptosis in skeletal muscle are still emerging. In skeletal myoblasts, a correlation between CDK inhibitor p21 induction and an apoptosis-resistance phenotype has been established (26). Our lab has previously reported that a delay of apoptosis can be achieved by the pharmacological inhibition of caspase 3 activity (24) and that the apoptosis associated with differentiation is abrogated by activated Ras or Raf signaling (90, 91). Over-expression of MyoD has been reported to be pro-apoptotic in cell lines lacking functional retinoblastoma protein (Rb) through expression of SV40 large T (92). Our lab has determined that activated Ras-mediated increased survival of myoblasts correlates with decreased levels of MyoD expression (93). Taken together, these data suggest a novel pro-apoptotic role for MyoD. Specifically, our data suggest that endogenous levels of MyoD can confer the ability to undergo apoptosis in cells

with functional Rb. We have further determined that culture in DM results in increased levels of the pro-apoptotic Bcl2 family member PUMA and the release of cytochrome C from the mitochondria (94). This increase in PUMA expression can still be detected in the absence of increased levels of PUMA mRNA (Shaltouki, manuscript in prepration). Thus, the Aims of my dissertation are to

1- determine the ability of MyoD to induce the expression of PUMA and apoptosis

2- determine the significance of PUMA to the release of cytochrome C from the mitochondria and apoptosis

3- investigate general translational regulation during myogenesis and explore translational regulation as the mechanism responsible for the increased expression of PUMA

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CHAPTER II

INCREASED EXPRESSION OF THE PRO-APOPTOTIC BCL2 FAMILY MEMBER [1]PUMA IS REQUIRED FOR MITOCHONDRIAL RELEASE OF CYTOCHROME C AND THE APOPTOSIS ASSOCIATED WITH SKELETAL MYOBLAST DIFFERENTIATION

The data in this chapter is included in two separate manuscripts entitled " Increased expression of the pro-apoptotic BCL2 family member PUMA is required for mitochondrial release of cytochrome C and the apoptosis associated with skeletal myoblast differentiation." Shaltouki, A., Freer, M., Mei. Y., Weyman, C.M., Apoptosis.Dec. 2007, and " Increased expression of the pro-apoptotic Bcl2 family member PUMA and apoptosis by the muscle regulatory transcription factor MyoD in response to a variety of stimuli" Harford, T. J., Shaltouki, A. Weyman, C.M., Apoptosis 2009 in press.

ABSTRACT

We have previously shown that when skeletal myoblasts are cultured in differentiation medium (DM), roughly 30% undergo caspase 3-dependent apoptosis rather than differentiation. Herein, we demonstrate that culturing 23A2 myoblasts in DM results in a time dependent release of mitochondrial cytochrome C into the cytosol in roughly 30% of myoblasts. Additionally, it has been shown previously in our lab that inclusion of cycloheximide inhibits the release of cytochrome C, the activation of caspase 9 and apoptosis. These data indicate that the mitochondrial pathway plays a role in this apoptotic process and that engagement of this pathway relies on *de novo* protein synthesis. Through RT-PCR and immunoblot analysis, we have determined that the expression level of the pro-apoptotic Bcl2 family member PUMA is elevated when 23A2 myoblasts are cultured in DM. Using shRNA against PUMA, we show that the silencing of PUMA inhibits the release of cytochrome C and apoptosis. Furthermore, signaling by the transcription factor p53 is not responsible for the increased level of PUMA. We also provide evidence that myoblasts rescued from apoptosis by either inhibition of elevated caspase 9 activity or silencing of PUMA are competent for differentiation. These results indicate a critical role for PUMA in the apoptosis associated with skeletal myoblast differentiation and that a p53-independent mechanism is responsible for the increased expression of PUMA in these cells. Finally, we demonstrate that MyoD activation can induce the expression of PUMA, cytochrome C release and apoptosis, supporting our previous finding that MyoD expression levels correlate with apoptosis.

INTRODUCTION

Differentiation and apoptosis are coordinately regulated in many cell types throughout development and to maintain homeostasis. During the process of skeletal myoblast differentiation, roughly 30% of myoblasts undergo apoptosis rather than differentiation (1, 2). The apoptosis of myoblasts is a physiological process that likely serves the necessary function of removing excess myoblasts during muscle regeneration (3) and myogenesis (4). This coordinate regulation of differentiation and apoptosis could decrease the efficacy of myoblast transfer to treat a variety of diseases. Skeletal myoblast apoptosis also contributes pathologically to a variety of disease states (1, 5-12). Elucidating the functional apoptotic pathways in skeletal myoblasts, therefore, will identify targets for therapeutic manipulation that could increase the efficacy of myoblast transfer and could alleviate disease states associated with muscle degeneration.

Despite the vast knowledge detailing skeletal myoblast determination and differentiation, our understanding of the molecular mechanisms controlling the apoptosis associated with skeletal myoblast differentiation is limited (2, 13-21).

The molecular mechanisms responsible for apoptosis in other systems, however, have been extensively studied. In most systems, mitochondrial disruption and subsequent release of pro-apoptotic proteins like cytochrome C is a critical event in the process of apoptosis (22). The release of these factors

from the mitochondria is antagonized by anti-apoptotic members of the Bcl2 family and initiated by pro-apoptotic members. Pro-apoptotic members can be activated by post-translational modification, subcellular translocation and/or by increased expression (22-24).

PUMA has been identified as a transcriptional target of p53 by SAGE analysis (25, 26) and as a Bcl2 interacting protein by a yeast two-hybrid (27). PUMA is a member of the BH3-only pro-apoptotic Bcl2 family sharing only a conserved Bcl2 homology (BH3) domain with other members of this family. Like other BH3-only members, PUMA localizes at the mitochondria in response to death signals. At the mitochondria, PUMA heterodimerizes with multidomain Bcl2 family members such as Bcl-xl or Bcl-2, antagonizing their function by disassociating them from Bax, allowing Bax oligomerization, depolarization of the mitochondrial membrane potential and release of cytochrome C (28). Released cytochrome C interacts with the adaptor protein Apaf-1 (adaptor protease-activating factor 1). This interaction results in a conformational change in Apaf-1 and induces oligomerization and activation of the initiator caspase 9 in an dATP dependent manner and subsequent activation of caspase 3 (29).

PUMA expression is regulated at the transcriptional level. Analysis of the PUMA promoter has identified two p53-responsive elements upstream of the 5'-untranslated region of PUMA. PUMA induced apoptosis in response to DNA damaging agents has been shown to be mediated by p53; however, serum

starvation, glucocorticoids and cytokine withdrawal have been reported to induce PUMA expression in a p53-independent manner (25, 30, 31). The mechanism of increased expression of PUMA in the absence of p53 is unknown. Recently, a consensus sequence corresponding to Foxo3a binding sites within the first intronic region of PUMA has been identified to be responsible for the increased expression of PUMA in response to cytokine or growth factor deprivation (32).

Recently, a tumor suppressive role for PUMA has been implicated. PUMA-/- mice and P53-/- mice exhibit similar phenotypes and are resistant to stress-induced cell death stimuli such as DNA damaging agents, oncogenic activation and ionizing irriadiation (31). These reports, along with recently published data showing the silencing or deregulated expression of PUMA in number of primary cancer cell lines, highlights the importance of PUMA as an emerging therapeutic target (33).

Previous published data suggest a pro-apoptotic role for the muscle regulatory transcription factor MyoD. Over-expressed ectopic MyoD through culture in differentiation media (DM) has been reported to be pro-apoptotic in cell lines lacking functional retinoblastoma protein (34). Others in our laboratory have previously shown a correlation between MyoD expression levels and apoptosis in 23A2 cells. It has been demonstrated that activated Ras-mediated increased survival of myoblasts correlates with decreased levels of MyoD expression and that re-expression of endogenous levels of MyoD in these active

Ras-expressing cell lines restores their ability to undergo both differentiation and apoptosis (35). These findings suggest that endogenous levels of MyoD might confer the ability to undergo apoptosis in cells with functional Rb. Therefore, we hypothesized that Myo D activation might be sufficient to induce the expression of PUMA, cytochrome C release and apoptosis.

Our group has previously reported that caspase 3 plays a role in the apoptosis associated with differentiation in skeletal myoblasts(2). Others in our laboratory have shown that culturing the 23A2 myoblasts in DM results in the activation of caspases 9 and 3 downstream molecules of the mitochondria. Activation of caspase 9 requires apoptosome formation and therefore the release of cytochrome C from the mitochondria. In agreement with this, subcellular fractionation studies performed in our lab confirmed the release of cytochrome C into the cytosol as 23A2 myoblasts are switched from GM to DM. Reported data suggested that the release of cytochrome C and apoptosis in 23A2 myoblasts require new transcription and translation. This led to identification of the pro-apoptotic family member PUMA whose expression increases as a consequence of culture in DM. Herein, we investigate the mitochondrial disruption as a consequence of culture in differentiation media and identify the pro-apoptotic family member PUMA (p53 up-regulated modulator of apoptosis) as an additional contributor to this apoptotic process in skeletal myoblasts. Finally, we suggest that PUMA expression may be controlled by MyoD.

MATERIALS AND METHODS

Cells and cell culture

All 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 and monitored after switching cells from growth medium to differentiation medium (DM), which consists of BME, 1% P/S and 10% FBS (2, 17-20, 35-38). Cells were incubated at 37°C in 5% CO2. 23A2 myoblasts are a clonal line of determined myoblasts derived by 5-azacytidine conversion (39). The caspase 9 inhibitor, z-LEHD-fmk, and the p53 inhibitor, pifithrin, were each dissolved in DMSO. Cycloheximide (CHX) was dissolved in methanol. Appropriate volumes of DMSO or methanol alone were added to control cultures and did not exceed 0.15% v/v.

Total cellular, cytosolic and mitochondrial lysate preparation

Myoblasts were plated at equal density and the next day switched to fresh GM or DM. For total cell lysates, cells were lysed in 1x p21 buffer (20 mM MOPS pH 7.4, 200 mM Sucrose, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT) containing 1% CHAPS, protease inhibitors (aprotinin, leupeptin, pepstatin, PMSF) and phosphatase inhibitors. For cytosolic (S100) and mitochondrial (P10) lysates, cells were harvested by trypsinization and subjected to centrifugation at 1000xg for 10 minutes. The pellet was washed twice with phosphate buffered saline and suspended in 5 volumes of buffer A (20 mM Hepes-KOH pH 7.5, 10 mM KCL,

1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) containing 250 mM sucrose and protease inhibitors (aprotinin, leupeptin, pepstatin, PMSF). Cells were homogenized by 10 strokes in a 2 ml Kontes glass Dounce homogenizer. Lysates were subjected to centrifugation at 750xg for 5 minutes and the resulting supernatant was subjected to centrifugation at 10,000xg for 15 minutes. The resulting pellet was designated as the P10 fraction while the supernatant was subjected to centrifugation at 100,000xg for 45 minutes. The resulting supernatant was designated as the S100 fraction. All centrifugation was performed at 40°C. The protein concentrations of all lysates were determined using Coomassie Protein Assay Reagent from Pierce as per the manufacturer's instructions.

Analysis of cytosolic cytochrome C

ELISA: Cytosolic cytochrome C was measured using the Quantikine M cytochrome C ELISA kit per manufacturer's instructions. Briefly, 10 μg (50 μl) of each S100 fraction was added to a well of a flat-bottomed microtiter plate pre-coated with monoclonal anti-cytochrome C antibody. To this, 75 μl of anti-cytochrome C antibody conjugated to horse-radish peroxidase (HRP) was added. After two hours rocking at room temperature, wells were washed three times prior to the addition of HRP substrate. After 30 minutes, an equal volume of STOP solution was added and product was measured on a spectrophotometer at 450nm. Experiments were performed within the linear range of the assay.

Immunostaining

23A2 cells were plated at 105 cells per chamber slide and the next day switched to fresh GM or DM for 3 hours. Cells were then washed with PBS and fixed in freshly prepared formaldehyde at room temperature for 25 minutes. After washing three times in PBS, the fixed cells were permeabilized in 0.1% Triton X-100 in PBS for 15 minutes prior to incubation for 30 minutes in blocking buffer (5% bovine serum albumin in PBS). Cells were then incubated for 4 hours either with a mouse monoclonal antibody against cytochrome C at 1:200 dilution (Pharmingen) or no antibody. After washing three times for 10 minutes each in blocking buffer, the cells were incubated with a fluorescein-conjugated donky anti-mouse IgG (Jackson ImmunoResearch) at 1:200 for one hour. The cells were then washed in PBS three times at 10 minutes each followed by staining with 1 μg/ml DAPI (Molecular Probe). Images were acquired using a Leica TCS SP2 laser scanning spectral confocal microscope and analyzed using Image Pro Plus 5.0. Over 250 cells from different fields were analyzed by two investigators without prior knowledge of sample identity.

Immunostain for MHC

23A2 cells were plated at 105 cells per chamber slide and the next day switched to fresh GM or DM for 48 hours. Cells were processed and counted as above except that the primary antibody was MF20. Images were visualized using an Olympus IX71 microscope.

Cytosolic nucleosome ELISA

The presence of cytosolic nucleosomes indicative of DNA fragmentation and nuclear membrane disruption was used as a marker for apoptosis. Myoblasts were plated at equal density and the next day switched to fresh GM or DM. We have carefully correlated cell density and time in DM with the level of cytosolic nucleosomes and the percentage of myoblasts undergoing apoptosis. Through this analysis we determined that, when myoblasts are plated at a density of $4x10^5$ cells per 100 mm dish, the level of cytosolic nucleosomes present after eight hours of culture in DM is representative of the 30% of myoblasts that finally undergo apoptosis (4, 23). Thus, analysis of cytosolic nucleosomes is generally performed under these conditions. Cytosolic nucleosomes were measured using the Cell Death Detection ELISA Plus Kit (Roche Diagnostics) as per the manufacturer's instructions. Briefly, attached cells were rinsed in PBS and then lysed by incubation in 300 μL cell lysis buffer from the kit on a rocker for 30 minutes at room temperature. Twenty microliters of each sample was transferred to a 96-well, flat-bottomed, streptavidin-coated microtiter plate. Seventy-two microliters of 1x incubation buffer from the kit and 4 μL each of biotin conjugated anti-histone antibody and peroxidase conjugated anti-DNA antibody was added to the lysate in the microtiter plate and incubated at 4°C overnight. Following three washes with 300 μL of incubation buffer, the presence of cytosolic nucleosomes was measured by adding 100 μL of the substrate, ABTS. Absorbance at 405 nm was measured at 60 second intervals using a

spectrophotometer. Values were taken from the time point where all samples were within the linear range of the assay for each individual experiment.

Quantitative RT-PCR

Myoblasts were plated at equal density and the next day switched to fresh GM or DM with or without additional treatment. Total RNA was prepared using 1 mL of Trizol (Invitrogen) reagent per plate for lysis and following the manufacturer's instructions. Five hundred μg of RNA was then used for a 20 μL SuperScript II RT (Invitrogen) reverse transcription reaction. Two μL of this reaction was used for quantitative PCR, with 0.3 μM each primer (for PUMA, forward: 5'GTCCGCGCCCCTTCCCGCTC and Reverse: 5'GGTGGGGCCTCCTGCCAGGG for myogenin, forward: 5'ATGCACTGGAGTTCGGTCC and reverse: 5'GACAGACAATCTCAGTTGGGC) and SYBR green reagent (DyNAmo, MJ Research) in a total of 20 μL. Fluorescence was detected using an Opticon Monitor (MJ Research). The cycle number at which the amplification of product began linearly increasing (cycle threshold (Ct) value) was taken from the Opticon software and normalized as described (18).

Immunoblot analysis

Following protein determination, lysates (150 μg of the S100 and P10 lysate for cytochrome C oxidase (COX) and 100 μg of total cellular lysate for Bax, Bim, PUMA, MHC and myogenin) were denatured in 5x sample buffer (10% SDS,

50% glycerol, 10% 2-mercaptoethanol, pH 6.8) and electrophoresed through denaturing polyacrylamide gels (12% for COX, Bax, Bim and PUMA and 10% for MHC and myogenin). Following SDS polyacrylamide gel electrophoresis (SDS-PAGE), samples were transferred electrophoretically for four amp hours to Hybond-P polyvinylidene difluoride membranes in transfer buffer containing 80% methanol and 1 g/L SDS. Membranes were blocked for one hour in 1xTBS/0.1% NP40 with 10% newborn calf serum and 5% dry milk. The following primary antibodies were incubated with the appropriate membranes: anti-COX antibody (A-6403, Molecular Probes) diluted 1:500, anti-PUMA antibody (Abcam) diluted 1:500, anti-Bax, anti-Bim and anti-myogenin antibodies (each from BDPharmingen) each diluted 1:1000, anti-MHC MF20 monoclonal antibody and anti-actin (Sigma) diluted 1:30,000. Appropriate HRP-conjugated secondary antibodies, each diluted 1:1000, were incubated with the membranes for one hour. After each incubation with antibody and prior to the addition of chemiluminescent substrate, membranes were washed five times in 1xTBS (tris-buffered saline pH 7.4) with 1% NP-40. Membranes were then incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 60 seconds and bands were visualized using Kodak Scientific Imaging Film.

Silencing

Lentiviral shRNA Silencing Construct - The murine PUMA-specific shRNA expressing lentiviral vector was purchased from OPEN Biosystems. The pLKO.1 puro vector is ligated with a 59-mer oligonucleotide containing a hairpin of 21

base-pair sense and antisense stem and a 6 base-pair loop. The sequence of oligonucleotide was as follows (sense and antisense stem in bold, loop in underlined letters, restriction enzyme sites in italics and the Pol III termination signal in lowercase letter):

5′-
*CCGG***GAGGGTCATGTACAATCTCTT**CTCGAG**AAGAGATTGTACATGGACC CT***C*****ttttt**-3

Lentiviral production and infection - The recombinant lentiviruses were produced by co-transfection of 293-T cells in Dulbecco's modified Eagle's medium containing 10% FBS and 1% penicillin/streptomycin with pLKO.1-PUMA shRNA (or pLKO.1 puro), pCMV-VSV-G and pHR′ CMV-dR8.2 plasmids at a ratio of 2:1:2 using lipofectamine 2000 (Invitrogen). Second generation packaging plasmids and assistance were kindly provided by Dr. Gudkov, Lerner Research Institute, Cleveland, Ohio. Lentiviral-containing supernatant was harvested at 24, 48 and 72 hours post-transfection, 0.22 μM-filtered and snap-frozen at -70 °C. Transduction of 23A2 cells with recombinant lentivirus was conducted in the presence of 10 μg/ml polybrene for 12 hours. Following lentiviral infection, the cells were selected in 2.5 μg/ml puromycin for 4 days to eliminate uninfected cells. Individual puromycin resistant colonies were then isolated and propagated for further analysis. PUMA levels in parental cells and in each clone were assessed by Western analysis. PUMA levels did not change within each of the cell treatment groups through serial cell passages.

RESULTS

There is a correlation between the release of cytochrome C and the apoptosis associated with skeletal myoblast differentiation

We have previously reported that caspase 3 is maximally activated after three hours of culture in DM and that caspase 3 activity plays a role in the apoptosis associated with differentiation in skeletal myoblasts (2). Caspase 9 is an initiator caspase capable of activating caspase 3 in other systems (40). Others in our laboratory have shown that culturing of 23A2 myoblasts in DM results in activation of caspases 9 and 3, downstream molecules of mitochondria. It was also observed that a dose dependent, and nearly complete, inhibition of caspase 9 activity by LEHD-fmk corresponds to a dose dependent, albeit not complete, inhibition of caspase 3 activity and the ensuing apoptosis. The activation of caspase 9 suggested that cytochrome C had been released from the mitochondria (41). Subcellular fractionation studies performed in our lab confirmed the release of cytochrome C from mitochondria into the cytosol as 23A2 myoblasts are switched from GM to DM. In addition, it was shown that the release of cytochrome C and apoptosis required new protein synthesis. We have previously determined that 30%+/-4 of myoblasts undergo apoptosis (2). To determine if there is any correlation between the percentage of cells displaying mitochondrial release of cytochrome C and the percentage of cells undergoing apoptosis, we performed immunostaining analysis using confocal microscopy to assess the mitochondrial release of cytochrome C. Using this approach, we determined that 29%+/-3 of myoblasts have released mitochondrial cytochrome

C into the cytosol (Fig. 2.1A). We also assessed the percentage of cells with altered mitochondrial membrane potential using the MitoCapture Apoptosis detection kit (CalBiochem) that employs a cationic dye that fluoresces red when aggregated in the mitochondria. In apoptotic cells, this dye fails to aggregate in the mitochondria due to the altered mitochondrial membrane potential and fluoresces green. Using this approach, we determined that 30%+/-3 of myoblasts display altered mitochondrial membrane potential. To determine if this disruption of mitochondrial membrane potential requires *de novo* protein synthesis, cultures were switched to GM or DM supplemented with cycloheximide (CHX) sufficient to inhibit new protein synthesis (data not shown). The inclusion of CHX was sufficient to prevent the disruption in mitochondrial membrane potential (Fig. 2.1B).

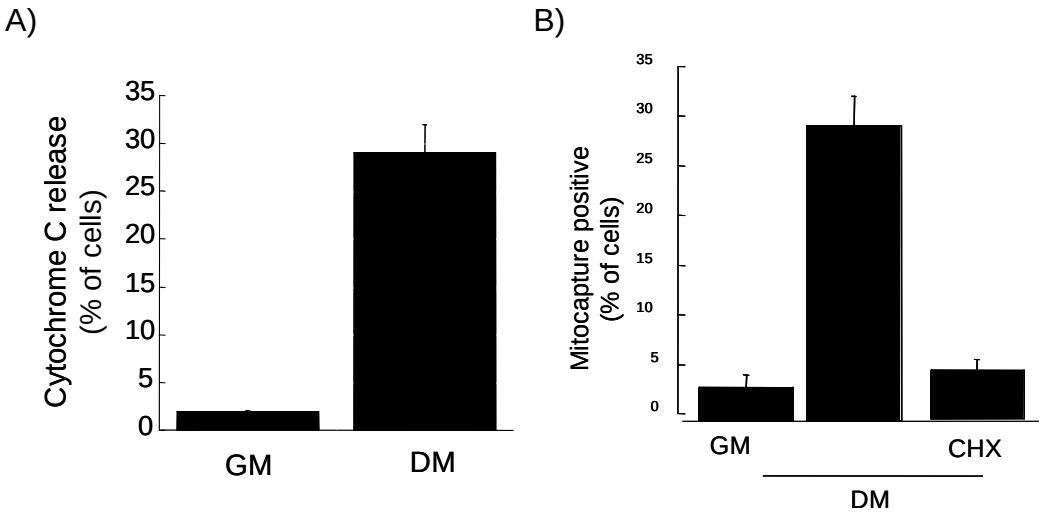


Figure 2.1 Culture in differentiation medium induces the mitochondrial release of cytochrome C and disruption of mitochondrial membrane. Equal cell numbers were plated and the next day cultured in DM for the indicated time in (A) or 3 h in (B). In (A), the mitochondrial release of cytochrome C was visualized by immunostaining for cytochrome C as described in Materials and methods. In (B), the disruption of mitochondrial membrane potential was determined in the presence or absence of cycloheximide (CHX) by mitocapture detection kit according to the manufacturer's instructions. Error bars represent the standard deviation of the mean from three values from two independent experiments.

Inhibition of elevated PUMA levels blocks the release of cytochrome C and apoptosis

The pro-apoptotic members of the Bcl2 family are regulated by increased expression and/or sub-cellular redistribution to the mitochondria (22-24). Since the release of cytochrome C and apoptosis relies on new protein synthesis, and because our lab previously focused on discerning which of the pro-apoptotic Bcl2 family members were regulated at the level of expression, we found that PUMA is the only member of the Bcl2 family whose expression is increased both at the level of mRNA and protein in 23A2 cells as a consequence of culture in DM. We therefore focused our attention on PUMA. In order to determine if this increase in expression of PUMA is required for the release of cytochrome C and apoptosis, we utilized RNAi. Recombinant lentiviruses were produced by co-transfection of 293-T cells with the murine PUMA-specific shRNA expressing lentiviral vector, pLKO.1-PUMA shRNA (or control vector pLKO.1 puro), pCMV-VSV-G and pHR′ CMV-dR8.2 plasmids. Transduced 23A2 myoblasts were then selected in puromycin. PUMA expression in untreated parental 23A2 myoblasts (P), a vector alone-transduced 23A2 myoblast clone (C) and PUMA shRNA-transduced myoblast clones (23A2:shPUMA clones), each cultured in DM for two hours, was monitored by Western blot analysis (Fig. 2.2A) and RT-qPCR (Fig. 2.2B). Clones 1 and 5 displayed reduced levels of PUMA protein and mRNA and were analyzed further. Clones 1 and 5, when compared to parental and vector control were each found to be repressed in the release of mitochondrial cytochrome C (Fig. 2.3A) and apoptosis (Fig. 2.3B) after culture in DM.

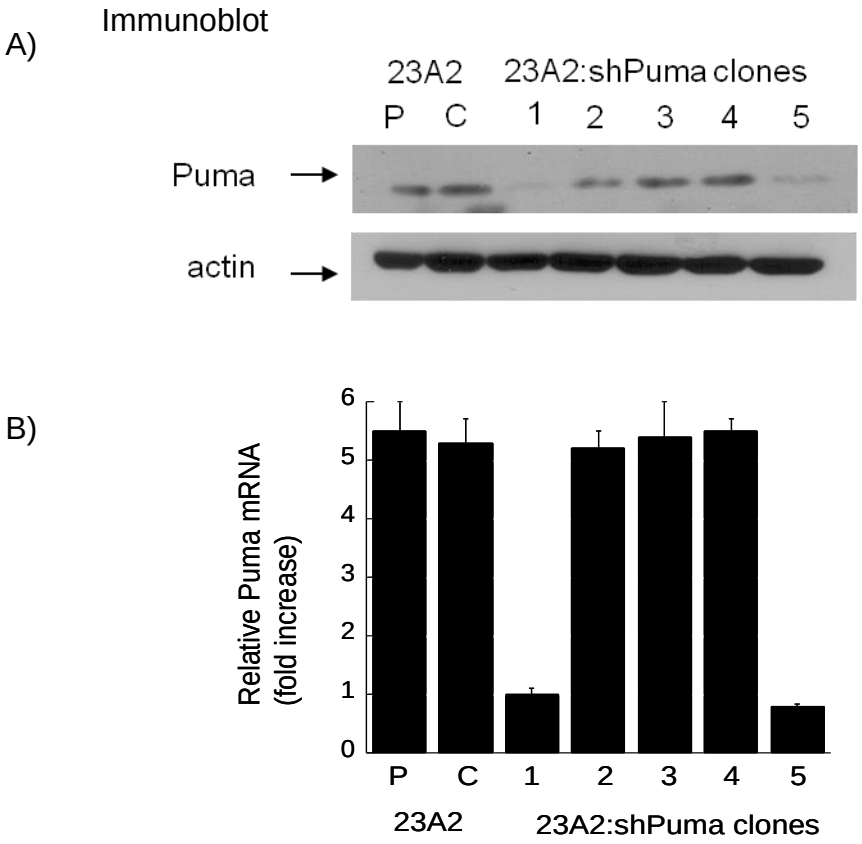
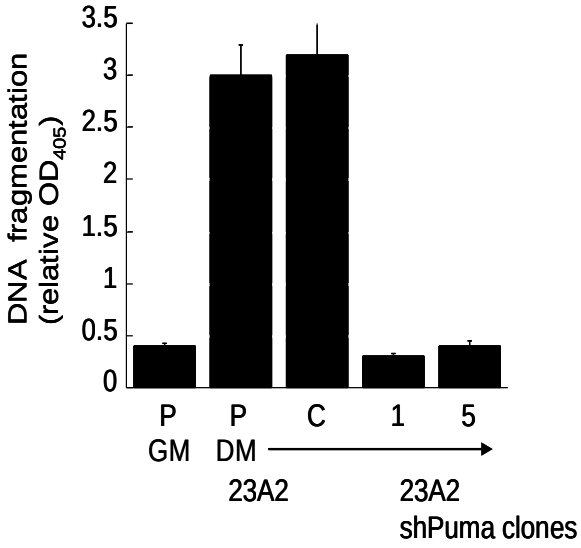


Figure 2.2 Lentiviral-mediated Puma suppression in skeletal myoblasts For stable and specific silencing of PUMA , a shRNA against PUMA RNA was used to induce silencing. Transduced cells were grown and analyzed. Equal cell numbers were plated and the next day cultured in fresh GM or DM. In (A), myoblasts were cultured in DM for 2 hours prior to analysis of Puma protein by Western analysis as described in Fig. 2. In (B), myoblasts were cultured in fresh GM or DM for 2 hours prior to analysis of Puma mRNA levels by RT-qPCR. Fold increase is relative to the result obtained in GM. Shown is an average of 3 experiments (mean +/- SEM).

A)



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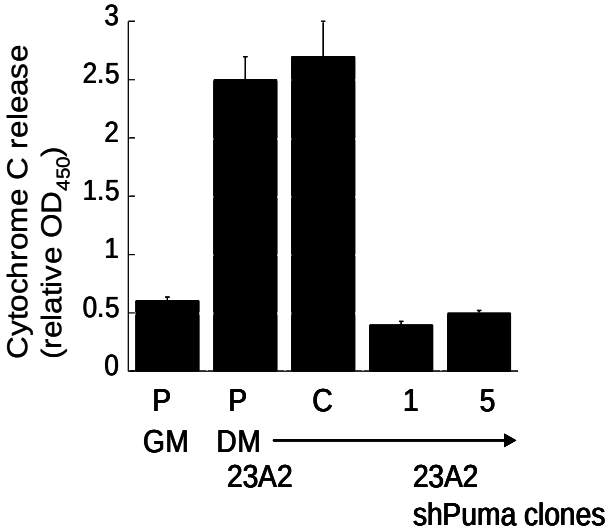

Figure 2.3 Lentiviral-mediated Puma suppression inhibits apoptosis and blocks cytochrome C release in skeletal myoblasts. In (A), myoblasts, 23A2 control and silenced clones for PUMA expression were cultured in fresh GM or DM for 3 hours prior to analysis of cytosolic cytochrome C as described in material and methods. In (B), myoblasts and silenced clones were cultured for six hours prior to the determination of DNA fragmentation using the Cell Death Detection ELISA Plus kit from Roche. Shown is an average of 3 experiments (mean +/- SEM).

Inhibition of elevated PUMA levels allows skeletal myoblast differentiation

Apoptosis and differentiation of skeletal myoblasts are mutually exclusive events. To elucidate the effect of PUMA suppression and inhibition of caspase 9 activity on differentiation, we used a pharmacological inhibitor against caspase 9 activity and PUMA suppressed clones. Using this approach we showed that either abrogation of caspase 9 activity or silencing of PUMA expression did not affect the ability of myoblasts to undergo differentiation as monitored by the expression of myosin heavy chain (Fig. 2.4A and B). Finally, the fact that the same percentage of cells stain positive for MHC in both the presence and absence of the caspase 9 inhibitor LEHD-fmk, and in both clones silenced and not silenced for PUMA expression, indicates that the myoblasts rescued from apoptosis can indeed differentiate. If they could not differentiate, one would expect that the percentage of cells staining positive for MHC would be reduced in the presence of LEHD-fmk and in the clones silenced for PUMA expression. Similarly, since equal protein is loaded, one would expect a reduction in the amount of MHC detected by Western analysis in the presence of LEHD-fmk, and in the clones silenced for PUMA expression, if the cells rescued from apoptosis could not differentiate.

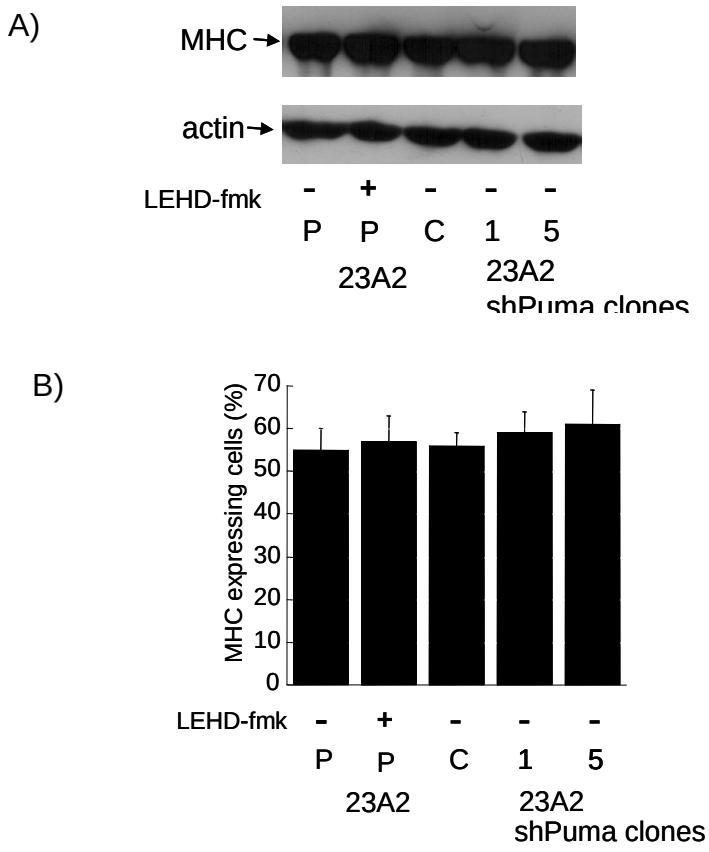

Figure 2.4 PUMA silencing or caspase 9 inhibition does not prevent myogenesis . Equal cell numbers were plated and the next day cultured in DM for 18 hours in the presence or absence of LEHD. In (A) Western blot analysis of 23A2 cell, control, silenced clones for PUMA using anti-MHC and anti-actin (control) antibodies. In (B) the percent of cells expressing MHC was assessed by immunostaining using anti-MHC and Texas Red conjugated anti-mouse IgG (Invitrogen). 10 different field of 100 cells were analyzed by two independent investigators. Error bars represent the standard deviation of the mean from three values from two independent experiments.

Increased expression of PUMA in 23A2 cells as a consequence of culture in DM is p53-independent

Since increased expression of PUMA was originally observed in response to activation of the transcription factor p53 (25-27), we used pifithrin, a selective pharmacological inhibitor of p53 (42), to examine a potential role for p53 in the induction of PUMA as a consequence of culture in DM. A role for p53 in the differentiation of skeletal myoblasts is well established (16, 43) so we first assessed the effect of pifithrin on differentiation as monitored by the expression of myogenin. As predicted, pifithrin inhibited the expression of myogenin mRNA and protein as a consequence of culture in DM (Fig. 2.5A,B). Pifithrin, however, had no detectable effect on the increased expression of PUMA mRNA or protein (Fig. 2.6 A,B) or the release of cytochrome C (Fig. 2.7A). Finally, Pifithrin had no detectable effect on the apoptosis of skeletal myoblasts as monitored by DNA fragmentation (Fig. 2.7B). This is consistent with previous reports employing dominant negative mutants of p53 as well as p53-/- cells which indicate that p53 is not involved in the apoptosis of skeletal myoblasts as a consequence of culture in DM (16).

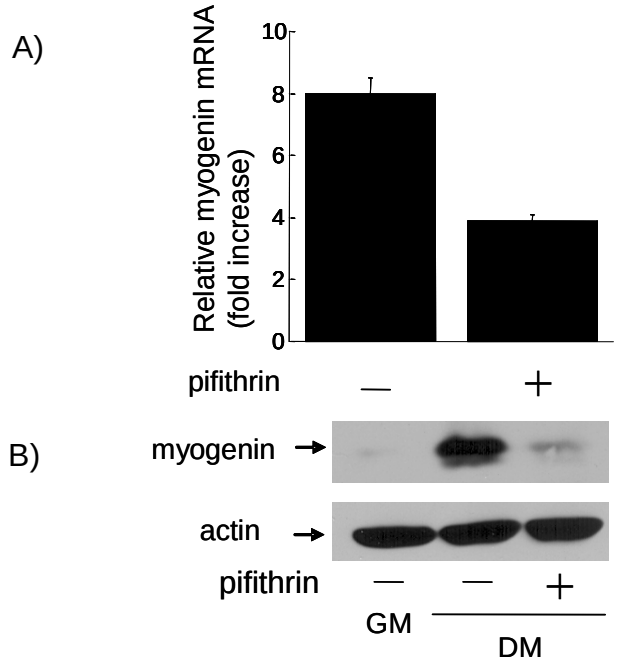

Figure 2.5 Pifithrin, a pharmacological inhibitor of P53 activity prevents differentiation of skeletal myoblasts as a consequence of culture in DM Equal cell numbers were plated and the next day cultured in fresh GM or DM for 18 h with or without the p53 inhibitor Pifithrin as indicated. In (A), quantitative RT-PCR was performed using the Quantitect SyBr Green PCR kit (Quiagen) as described in Material and Methods. Fold increase is relative to results obtained in GM. Shown is an average of 3 experiments (mean ± SEM). In (B), whole cell extracts were prepared 100 μg of total protein was separated by SDS-PAGE. Western analysis was performed using anti-myogenin or anti-actin (load control). Shown are results from one experiment that are representative of three independent experiments.

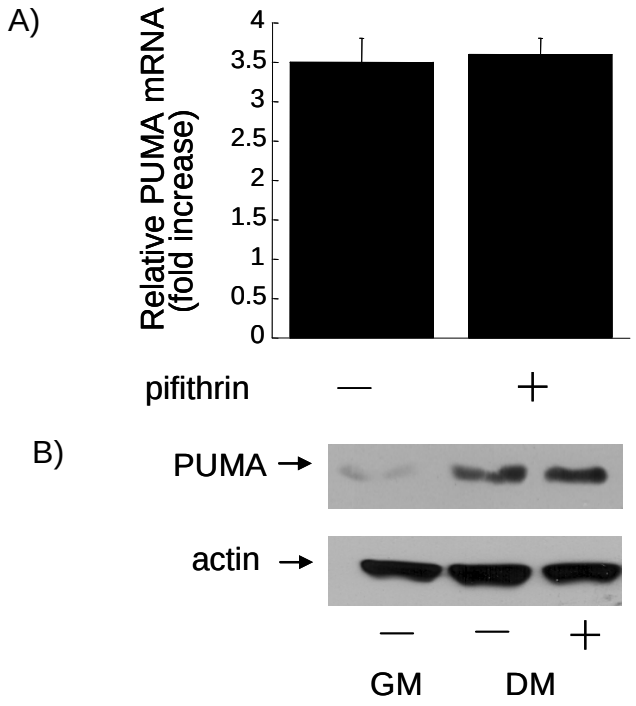

Figure 2.6 Inhibition of p53 activity by pifithrin does not prevent the increase in expression of PUMA mRNA and protein as a consequence of culture in DM Equal cell numbers were plated and the next day cultured in fresh GM or DM for 3 h with or without the p53 inhibitor Pifithrin as indicated. In (A), quantitative RT-PCR was performed using the Quantitect SyBr Green PCR kit (Quiagen) as described in Material and Methods. Fold increase is relative to results obtained in GM. Shown is an average of 3 experiments (mean ± SEM). In (B) whole cell extracts were prepared 70 (g of total protein was separated by SDS-PAGE. Western analysis was performed using anti-PUMA antibody or anti-actin (load control). Shown are results from one experiment that are representative of three independent experiments. Error bars represent mean + SEM.

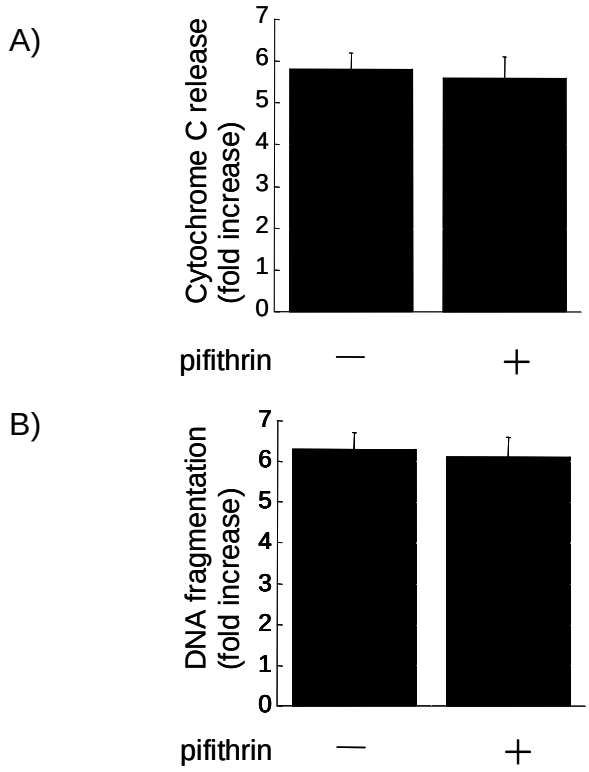

Figure 2.7 Inhibition of p53 activity does not affect the release of cytochrome C and apoptosis in 23A2 cells as a consequence of culture in DM Equal cell numbers were plated and the next day cultured in DM in the presence or absence of the inhibitor for the indicated time: 3 hrs in (A) and 6 hrs in (B). In (A), cytochrome C release was measured in the S100 fraction using Quantikine M cytochrome C ELISA kit from R&D. S100 fraction purity was det ermined by Western blot analysis using mitochondrial cytochrome C oxidase as described in Material and Methods. In (B), myoblasts were cultured in DM in the presence or absence of the inhibitor 6 hours prior to the determination of DNA fragmentation using the Cell Death Detection ELISA Plus kit from Roche. This is an average of three independent experiments (mean ± SEM).

Increased expression of PUMA, mitochondrial release of cytochrome C and apoptosis are induced by activation of MyoD

Others in our laboratory have previously shown a correlation between MyoD expression levels and apoptosis in 23A2 cells. Myoblasts expressing oncogenic Ras were reported to have decreased apoptosis, which correlated with decreased levels of MyoD expression (35). This finding prompted us to hypothesize that MyoD activation might be sufficient to induce the expression of PUMA, cytochrome C release and apoptosis. To test this hypothesis, we examined the ability of 10T1/2 fibroblasts stably expressing a conditionally active estrogen-receptor:MyoD fusion protein (ER:MyoD:10T1/2)(44) to undergo apoptosis in response to estrogen. Since the estrogen receptor is not expressed in these cells, ER:MyoD is the only estradiol target (data not shown). Treatment of ER:MyoD:10T1/2 cells with 1 μM of estradiol resulted in estradiol-dependent activation of MyoD which was verified by immunoblot analysis of myogenin and this activation of MyoD was sufficient to induce differentiation comparable to that observed in 23A2 myoblasts (Fig. 2.8A). Comparison of estradiol treated and not treated ER:MyoD:10T1/2 cells indicates that this MyoD activation is also sufficient to induce apoptosis (Fig. 2.8B) and the increased expression of PUMA (Fig. 2.9A & B). Finally, estradiol treatment of ER:MyoD 10T1/2 cells, induced the increased expression of PUMA (Fig. 2.9A &B) but not 10T1/2 fibroblasts (Fig. 2.9C).

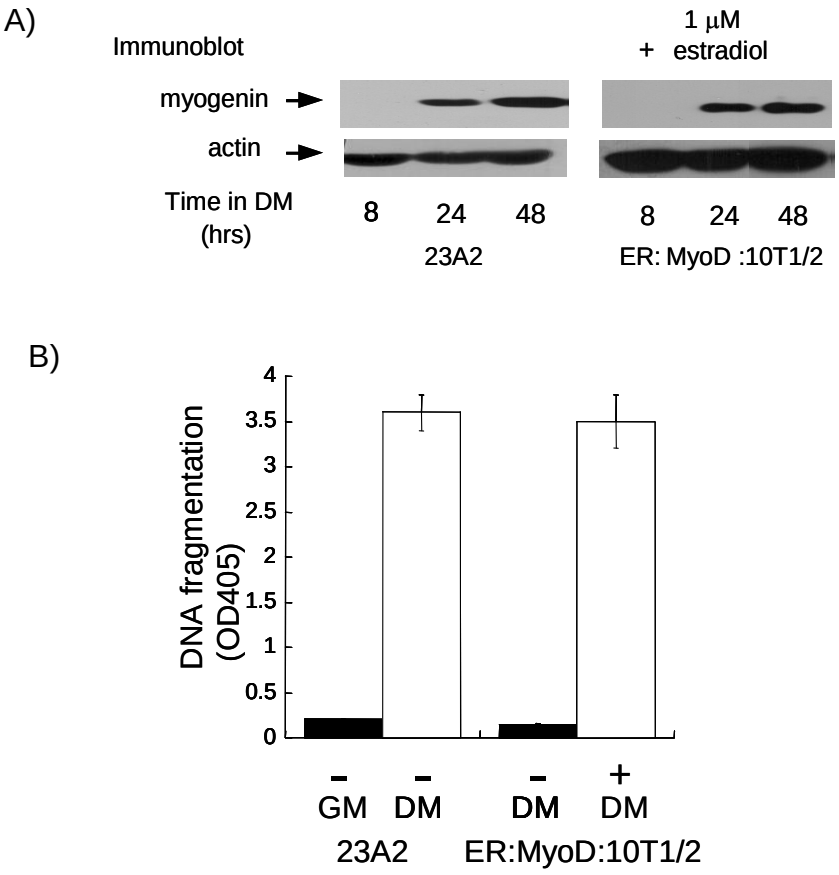

Figure 2.8 10T1/2 fibroblast cells stably expressing ER:MyoD fusion protein undergo apoptosis in response to estradiol
Equal cell numbers were plated and the next day cultured in fresh GM or DM with or without 1μM estradiol for 4 hours. In (A), Western blot analysis using ant-myogenin antibody (BD Bioscience) to confirm activation of MyoD. In (B) equal 23A2 cell numbers were plated. The next day, 23A2 cells were treated with one μM estradiol in GM. 23A2 cells then were switched to DM as indicated for six hours prior to the determination of DNA fragmentation using the Cell Death Detection ELISA Plus kit (Roche) according to the manufacturer's protocol. This result is an average of three independent experiments (mean ± SEM).

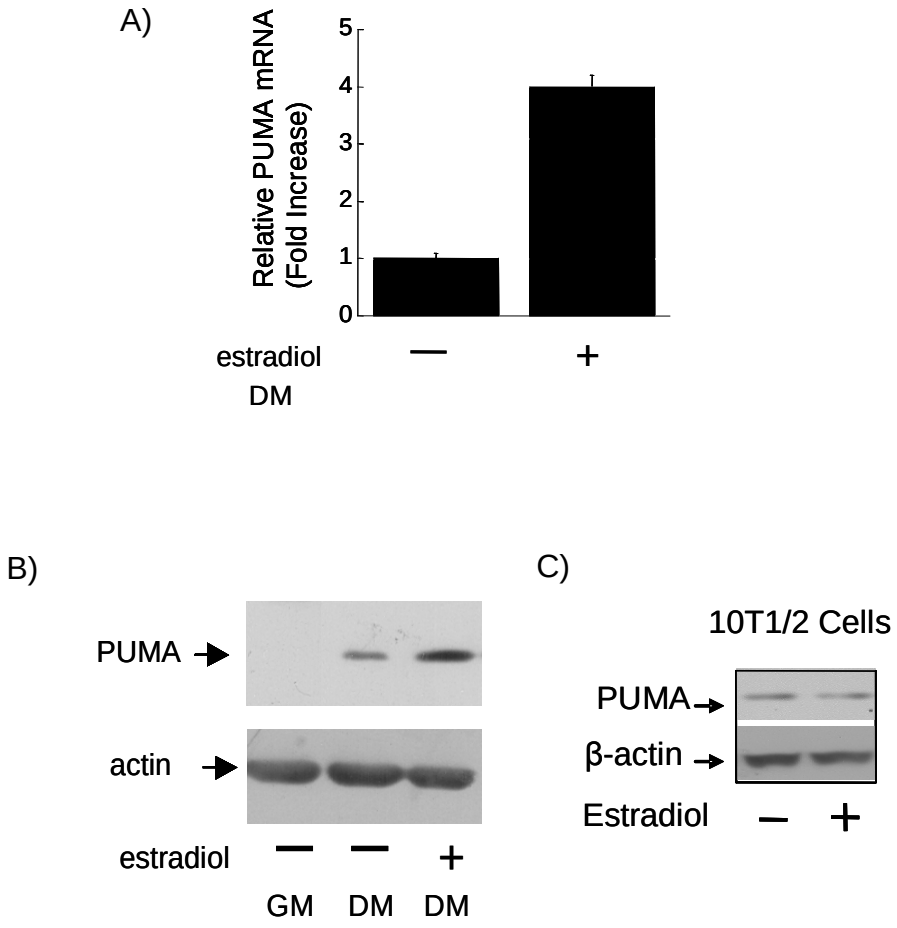

Figure 2.9 Increased level of PUMA as a consequence of activation of ER:MyoD in 10T1/2 fibroblasts Equal cell numbers were plated and the next day cultured in fresh GM or DM with or without 1μM estradiol for 4 hours. In (A), quantitative RT-PCR was performed as described in Materials and methods. Fold increase in DM is relative to results obtained in GM. Shown is an average of 3 experiments (mean +/- SEM). In (C), whole cell extracts were prepared after culture in DM for 4 hrs and 100 μg of total protein was separated by SDS-PAGE. Western analysis was performed using anti-Puma (Abcam) or anti-actin (load control). In (D), 10T1/2 treated or untreated with estrogen for 3 hours. This is an average of three independent experiments (mean ± SEM).

DISCUSSION

It is well established that differentiation and apoptosis are coordinately regulated in many systems which can be broadly divided into two groups. In some systems, apoptosis contributes to the differentiation process while in others apoptosis serves the purpose of eliminating excess cells (45, 46). The skeletal myoblast model system is a member of the latter group (46). Elucidating the molecules that mediate the apoptotic process in skeletal myoblasts has revealed a role for caspase 3 and the TRAIL/DR5/FADD/caspase 8/tBid pathway (2, 17) while expression of Bcl2 confers resistance (47). We report herein the novel observation that mitochondrial cytochrome C is released into the cytosol upon removal of serum and plays a role in the apoptosis associated with skeletal myoblast differentiation. Further, we report that the pro-apoptotic Bcl2-family member PUMA contributes to this release of cytochrome C and the ensuing apoptosis. While the involvement of PUMA in the release of mitochondrial cytochrome C has been established previously (26), this is the first report to document a role for the increased expression of PUMA in the release of mitochondrial cytochrome C and the ensuing apoptosis of skeletal myoblasts as a consequence of culture in differentiation media. Additionally, we have also shown that PUMA is not required for skeletal myoblast differentiation.

PUMA was originally identified through global profiling as a p53-inducible gene and through a yeast two-hybrid screen as a Bcl2 interacting protein (25-27). We have used the selective p53 pharmacological inhibitor pifithrin to rule out a role for p53 in the induction of PUMA, the mitochondrial release of cytochrome C

and apoptosis in this system. These findings are in agreement with previous reports indicating that the apoptosis associated with skeletal myoblast differentiation as a consequence of culture in DM is p53-independent (16). PUMA is also a transcriptional target of the p53 family member p73. The regulation of p73 is complex in that it is transcribed from two promoters, one permitting the expression of a transactivation competent (TA) protein and the other permitting the expression of a dominant negative (DN) protein (48). As a consequence of alternative splicing, each of these can be expressed as seven isoforms (αβγδεξ and η) with both overlapping and distinct functions. PUMA is among the transcriptional targets shared by TAp73 and p53 in that ectopically expressed TAp73 α, β and γ isoforms can increase the expression of PUMA and induce apoptosis in p53-null Saos cells (49). Recently, a role for p73 has been investigated in both the differentiation and the apoptosis of C2C12 skeletal myoblasts (50, 51). In C2C12 myoblasts, expression levels of TAp73β are rapidly decreased within one hour of culture in DM while expression levels of both TAp73α and DNp73α are simultaneously and similarly modestly increased with culture in DM (50). Forced expression of either isoform inhibited the differentiation of C2C12 myoblasts by roughly 50% suggesting that the timing of their expression is critical (50, 51). Further, a decrease in the apoptosis of C2C12 myoblasts was observed when TAp73α was silenced while an increase in the apoptosis of C2C12 myoblasts was observed when DNp73α was silenced. PUMA levels, however, were not assessed (50). We have detected an increase in PUMA expression as a consequence of culturing C2C12 myoblasts in DM (A.

Shaltouki, unpublished). We speculate, however, that the simultaneous and similar increase of both TAp73α and DNp73α would balance their opposing effects on apoptosis. Thus, while direct studies on p73 need to be performed, we suggest that p73 does not play a role in the increased expression of PUMA in skeletal myoblasts cultured in DM.

p53/p73 independent increased expression of PUMA has also been reported. Specifically, glucocorticoid treatment increases PUMA expression and apoptosis in primary thymocytes and serum deprivation increases PUMA expression and apoptosis in several tumor cell lines (27). The increase in PUMA expression in tumor cells in response to serum deprivation can be reversed by serum or specific growth factors and this reversal is prevented by inhibition of PI3-kinase (27). *In vivo*, myoblasts are maintained in a proliferative and undifferentiated state by certain mitogens. Differentiation is induced in response to decreasing gradients of these mitogens when myoblasts have appropriately migrated. To mimic this, the *in vitro* differentiation of skeletal myoblasts is initiated by serum deprivation (52). Thus, skeletal myoblasts are a non-transformed cell type in which serum deprivation as a consequence of culture in differentiation media correlates with increased expression of PUMA and apoptosis. While we report herein that p53 does not appear to play a role in the increased expression of PUMA in skeletal myoblasts, we have previously reported that PI3-kinase activity decreases during the time period in which PUMA induction is observed (18). Further, PI3K/Akt signaling does play a role in

myoblast survival during differentiation (53, 54). Decreased signaling through the PI3-kinase pathway allows nuclear localization of the FOXO3a transcription factor (55). Thus, the PI3-kinase/FOXO pathway may be responsible for the increased PUMA expression in skeletal myoblasts. However, this pathway is also responsible for increased expression of TRAIL and the pro-apoptotic Bcl2 family member Bim (56, 57) in other systems and the expression level of neither of these proteins is increased as a consequence of culture in differentiation media ((17) and herein). Interestingly, 10T1/2 fibroblasts, from which 23A2 myoblasts are derived (39), do not undergo apoptosis in response to culture in differentiation media. Further, when cultured in differentiation media, PI3-kinase activity decreases similarly in 10T1/2 fibroblasts and skeletal myoblasts (18) and C.M. Weyman, unpublished), yet PUMA induction in these fibroblasts is less than that observed in the skeletal myoblasts (A. Shaltouki, unpublished). Thus, we believe that the PI3-kinase/FOXO3a pathway cannot completely explain the increased PUMA expression in skeletal myoblasts and future experiments are focused on determining the mechanism responsible for this increase.

The irreversible nature of the apoptotic process necessitates that induction of such is tightly controlled and often requires the engagement of multiple pro-apoptotic molecules. For instance, increased expression of Hrk and Bim are required for neuronal apoptosis while many p53-regulated proteins such Fas, DR5, FLIP, Noxa, PUMA, Bax and Bad are associated with apoptosis in response to DNA damage (24, 58). Further, increased expression of PUMA in

response to DNA damage in fibroblasts induces quiescence rather than apoptosis. Speculation as to why thymocytes undergo apoptosis while fibroblasts become quiescent is the need for cooperation between PUMA and other pro-apoptotic molecules (24). Recently, both PUMA and Bim have been shown to contribute to lymphocyte development and to the death of activated T-cells (59, 60). While herein we report that the skeletal myoblast apoptosis as a consequence of culture in differentiation media requires increased expression of PUMA, we have previously reported that this apoptotic process also requires signaling through the TRAIL/DR5/FADD/caspase8 cleavage of Bid pathway. As is the case with some of the myoblasts in which PUMA has been silenced, some of the myoblasts expressing dominant negative mutants of FADD or DR5 will eventually undergo apoptosis (A. Shaltouki and J. O'Flaherty, unpublished). Thus, cooperation between PUMA and other pro-apoptotic molecules such as t-Bid is required for the efficient apoptosis that occurs in some skeletal myoblasts as a consequence of culture in differentiation media. Similarly, some of the myoblasts treated with the caspase 9 inhibitor will eventually undergo apoptosis (A. Shaltouki unpublished) suggesting a role for the permeabilization of the mitochondria and the ensuing release of factors capable of mediating caspase independent apoptosis. However, this delay in the apoptotic process caused by inhibiting caspase 9 or by preventing the elevation of PUMA is sufficient to reduce the number of apoptotic myoblasts. Further, those rescued are competent for differentiation. These findings could be useful in efforts to treat disease states associated with muscle degeneration and to the effectiveness of

any treatment utilizing skeletal myoblast transfer. Thus, it is noteworthy that silencing of PUMA does not inhibit skeletal myoblast differentiation.

While our studies performed on cultured myoblasts clearly demonstrate a role for PUMA in the apoptosis associated with skeletal myoblast differentiation, PUMA -/- mice develop normally with no obvious muscle related phenotype (31). A defective apoptotic phenotype in thymocytes and the developing nervous system of PUMA -/- mice is, however, observed in response to DNA damaging agents such as ionizing radiation. Further, a defective apoptotic phenotype can be detected in thymocytes derived from PUMA -/- mice in response to glucocorticoid, and in myeloid progenitors in response to Myc activation and IL-6 deprivation (31). Thus, although PUMA appears to play no role in the normal development of hematopoietic cells, PUMA clearly contributes to cell death in response to certain stress conditions. We, therefore, speculate that lack of a developmental muscle phenotype could indicate either that this pathway has no role during developmental myogenesis or that compensatory signaling negates detection of such a role. Further, a reduced apoptotic phenotype in PUMA -/- skeletal myoblasts might only be observed in response to specific stimuli such as conditions that lead to muscle wasting or regeneration, or in the context of myoblast transplantation (4, 61, 62). Finally, research on the molecules controlling the apoptotic process in human muscle has necessarily lagged behind studies performed in culture or in mice. These studies have suggested a role for caspase 3 (12) (63-65). The results presented herein suggest that assessing the

expression of PUMA, possibly as a molecule functioning prior to caspase 3 activation, is warranted. These future studies, combined with further analysis to understand the molecular mechanisms mediating the increased level of PUMA as a consequence of culture in differentiation media, might identify additional therapeutic targets for manipulation relevant to the use of myoblast transfer as a therapeutic approach or to modify skeletal myoblast apoptosis relevant to the amelioration of disease states with associated muscle degeneration.

We also report here a pro-apoptotic role for the muscle regulatory transcription factor MyoD. Our previous report suggested that the survival of Ras expressing myoblasts correlates with decreased levels of MyoD expression (35) 23A2 myoblasts are derived from 10T1/2 fibroblasts. 23A2 myoblasts express MyoD while 10T1/2 fibroblasts do not (39). 23A2 myoblasts undergo apoptosis in response to culture in DM while 10T1/2 fibroblasts do not (2). Increased expression of pro-apoptotic molecules by other transcription factors, notably p53 (23, 66) has been documented. p53 has the dual capacity to induce either growth arrest or apoptosis (67), while MyoD has the capacity to induce growth arrest and differentiation or apoptosis. p53, however, is ubiquitously expressed and responsive to DNA damage (67) while MyoD expression is restricted to skeletal muscle and responsive to differentiation cues (68). To our knowledge, the only other report of basic helix-loop-helix (bHLH) transcription factors shown to play a role in apoptosis are the Daughterless-like (HLH-2) and Achaete-scute-like (HLH-3) bHLH transcription factors responsible for inducing apoptosis during neuronal development in *C.elegans* (69). Here, we demonstrated that estradiol-

induced activation of MyoD:ER fusion protein in DM was sufficient to result in the increased expression of PUMA and apoptosis. In light of this information, the ability of MyoD to induce growth arrest and differentiation or apoptosis is redolent of p53 function. These data were consistent with other published reports suggesting that MyoD may play a role in apoptosis (70).

CONCLUSION

Mitochondrial release of cytochrome C, activation of caspase 9 and the apoptosis associated with skeletal myoblast differentiation require de novo protein synthesis. PUMA was identified in a search for increased expression of pro-apoptotic Bcl2 family members as a consequence of culturing skeletal myoblasts in differentiation media. Stable RNAi mediated silencing of this increase in PUMA expression decreases the mitochondrial release of cytochrome C and the ensuing apoptosis. Myoblasts rescued from apoptosis by either inhibition of elevated caspase 9 activity or silencing of PUMA are competent for differentiation. As p53 does not play a role in this apoptotic process, experiments are underway to understand the regulation of PUMA in this system. Finally, we showed that activation of MyoD is sufficient to induce apoptosis through the increased expression of PUMA.

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CHAPTER III

TRANSLATIONAL REGULATION OF THE PRO-APOPTOTIC BCL2 FAMILY MEMBER PUMA

ABSTRACT

We have previously reported that culture of 23A2 myoblasts in differentiation media (DM) induces an increase in both the mRNA and protein levels of the pro-apoptotic Bcl2 family member PUMA and that PUMA is critical for the apoptotic process that occurs in a subset of these myoblasts. Herein, we report a novel translational regulation mechanism of PUMA. Specifically, we have determined that culturing of myoblasts in DM with actinomyocin D is sufficient to block the increase in PUMA mRNA levels, but does not prevent the increase in PUMA protein levels. Further, metabolic labeling of newly synthesized proteins in myoblasts cultured in either growth media (GM) or DM supplemented with actinomycin D followed by immunoprecipitation of PUMA has allowed us to conclude that this increase in PUMA protein is a consequence of increased translation. Polyribosome analysis of PUMA mRNA indicated that translational regulation most probably occurs at the initiation step. We have also found that the increase in PUMA protein levels occurs under conditions of eIF2-

alpha phosphorylation and subsequent global decrease in total protein synthesis. Analysis of the 5' UTR of PUMA mRNA by Mfold program predicts stable secondary structures incompatible with efficient ribosome scanning. Further, we have determined that impairment of cap-dependent translation *in vitro* and *in vivo* did not affect the translation of PUMA mRNA. Finally, we have generated data indicating that the increased translation of PUMA in DM is mediated by an internal ribosome entry site (IRES). Using mono- and bi-cistronic reporters, we have identified a fragment of PUMA mRNA that allows for cap-independent translation in vitro and in vivo in response to culture in DM. This fragment encompasses regions upstream and downstream of the initiation AUG codon Thus, our data indicate that increased expression of PUMA in skeletal myoblasts relies on IRES-meditated translation.

INTRODUCTION

Translation Initiation (cap dependent translation)

The regulation of global translation is an important process that allows a cell to respond rapidly in response to environmental cues (1). Regulation of protein synthesis can occur at many steps during the process but in many cases the initiation step is subject to regulation. Initiation begins with binding of the 40S ribosomal subunit to the initiator Met-tRNA-^{Met} in a ternary complex with eukaryotic initiation factor 2α (eIF2α) and its bound GTP. Initiation of protein synthesis requires recruitment of this small ribosomal subunit to the mRNA (2, 3). This recruitment is mediated by a cap-binding protein complex known as

eukaryotic initiation factor-4F (eIF4F). The eIF4F complex is composed of 3 subunits. EIF4E(4), the smallest subunit of the eIF4F complex, is a cap-binding protein which recognizes the cap structures (m7GpppN) at the 5′-end of the mRNA. eIF4A, another subunit of the eIF4F complex, has RNA helicase activity (5), and eIF4G (6), the third subunit of eIF4F complex, acts as a scaffold protein connecting the small ribosomal subunit and the mRNA through eIF3 (7-10). Once the ribosome is recruited to the 5′ end of mRNA through its interaction with eIF4F, its binding to the mRNA is facilitated through the unwinding of mRNA secondary structures in the 5′ untranslated region (5′UTR). This unwinding is mediated by eIF4A and eIF4B (11). Initiation of translation is then achieved by a scanning process through which the ribosome and its associated factors begin to scan the mRNA in a 5′ to 3′ direction. After recognition of an initiation codon with the appropriate sequence context by the anticodon of Met-tRNA^{Met}, hydrolysis of the GTP bound to eIF2 is facilitated by eIF5, an GTPase activating protein. eIF1 then releases small ribosomal subunit bound initiation factors which are recycled for another round of translation initiation (12). Joining of the large ribosomal subunit 60s to the initiation complex is stimulated by eIF5B (13) and hydrolysis of a second molecule of GTP, leads to formation of the 80s initiation complex (14, 15).

4E-BP

Since the formation of the eIF4F complex is required for initiation, the regulation of translation initiation can be achieved by controlling the availability of

elF4E. 4E-binding proteins (4E-BPs) are a family of translational repressors that compete with eIF4G for binding to elF4E. Thus, binding of 4E-BPs to eIF4E inhibits cap-dependent translation (9, 16). Assembly of the eIF4F complex is regulated by the phosphorylation status of 4E-BP. While hypophosphorylated 4E-BP binds to eIF4E with high affinity, sequestering it from incorporation into the eIF4 complex, hyperphosphorylation of 4E-BP releases eIF4E (9, 16-19) Although inhibition of translation initiation by 4E-BP is reversible, the release of eIF4E from 4E-BP requires phosphorylation of a combination of multiple residues in a hierarchical manner (20) (Fig. 3.1).

Regulation of translation by insulin and other growth factors has been reported (9, 21). It has been shown that both serum and growth factors affect the phosphorylation status of 4E-BPs. Hyperphosphorylation of 4E-BP upon serum stimulation allows cap-dependent translation (16, 22-26). Recent studies have identified PI3K (phosphoinositide3-OH kinase) , its downstream effector Akt, and the AKT substrate mTOR (mammalian target of rapamycin), as the intracellular signaling pathway responsible for the increase in phosphorylation of 4E-BP in response to serum stimulation (4, 18, 27). Wortamine is a selective pharmacological inhibitor of PI3K while rapamycin is a selective pharmacological inhibitor of mTOR. It has been reported that 4E-BP phophorylation in response to serum is wortaminin and rapamycin sensitive, suggesting that signaling by PI3K through Akt to mTOR is responsible for 4E-BP phosphorylation (18, 27-29). The involvement of PI3K/Akt signaling pathway in regulating myogenesis is well

established. Differentiation of cultured myoblasts in response to serum depravation is believed to be mediated through autocrine/paracrine actions of IGFII (30-32). Our lab previously reported that, in response to culture in DM, signaling by PI3K to Akt initially decreases and then returns to levels comparable to that observed in GM (33).

TOR was originally identified through a genetic screen in *S. cerevisiae* to be responsible for rapamycin toxicity (34). mTOR and its yeast homolog belong to a larger protein family known as PIKK (phosphoinositide kinase-related kinase) which includes other members such as ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3) and DNA-PKc (DNA-activated protein kinase catalytic subunit) (35-41). It has been suggested that in yeast, TOR's control of cell proliferation is achieved through a translational regulation mechanism (22, 42). Regulation of translation initiation by mTOR and its yeast homologue is mediated by its ability to phosphorylate 4E-BP or S6K1. While the phosphorylation of 4E-BP contributes to cap-dependent translation initiation, S6K1 phosphorylation regulates oligopyrimidine tract-dependent translation initiation.

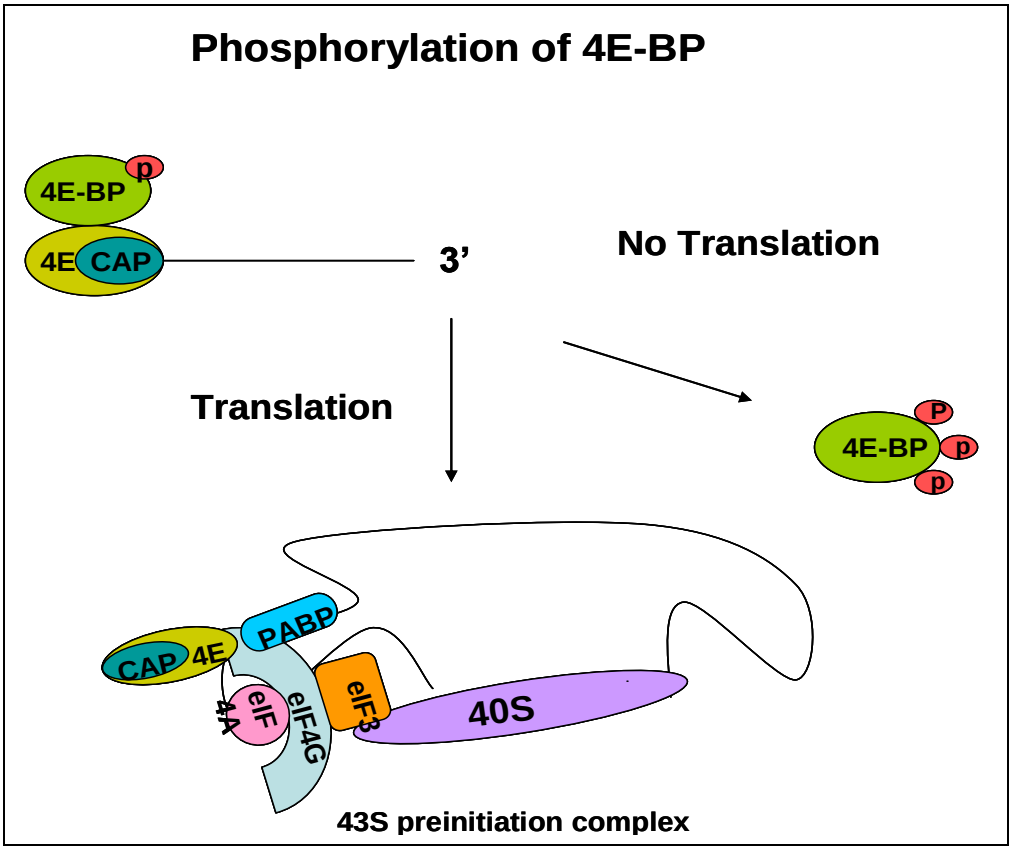


Figure 3.1 Phosphorylation of 4E-BP allows translation.
Hypophosphorylated 4E-BP binds to eIF4E with high affinity and sequesters it from entering into the preinitiation complex, while phosphorylation of 4E-BP in response to growth factors or insulin releases eIF4E and allows translation.

eIF2α

eIF2α is a eukaryotic translational initiation factor that is involved in formation of the 43S preinitiation complex (Fig. 3.2). Binding of eIF2α with GTP and Met-tRNA^{Met} forms a ternary complex which mediates the recruitment of Met-tRNA^{Met} to the small ribosomal subunit, forming the 43S preinitiation complex. An alternative mechanism for the regulation of global translation depends on the availability of this translational ternary complex which only forms when eIF2α is in its GTP- bound state. Reinitiation of translation requires recycling of eIF2α -GDP which is produced at the end of each initiation step to eIF2α -GTP is catalyzed by eIF2B, a guanine nucleotide exchange factor (Fig 3.2). Phosphorylation of the alpha subunit of eIF2α on Ser-51, in response to different physiological stimuli inhibits eIF2B activity. Thus, phosphorylation of the α subunit of eIF2-GDP prevents GDP-GTP exchange of nonphosphorylated eIF2-GDP therefore impairing ternary complex formation (43, 44). Currently, four eIF2α kinases are known : the double-stranded RNA-dependent eIF2 kinase (PKR) (45, 46), the eIF2 like kinase-like endoplasmic reticulum kinase (PERK) (47), heme regulated eIF2 kinase (HRI) (48) and the mammalian orthologue of the yeast GCN2 protein kinase (mGCN2), only GCN2 activation has been linked to culture in the absence of serum (49).

dsRNA-activated protein kinase (PKR) is an interferon inducible serine-threonine kinase which was originally linked to the antiviral response. Recently,

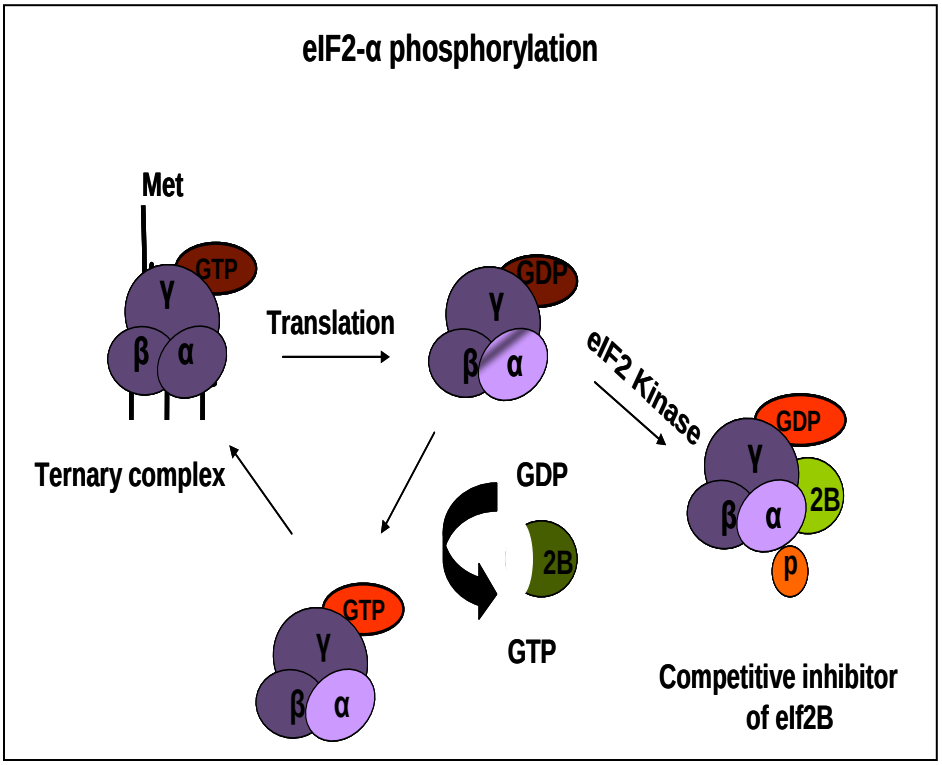


Figure 3.2 Phosphorylation of of serine 51 on the α subunit of eIF2 by eIF2 specific stress kinases in response to different stimuli makes it a competitive inhibitor of eIF2B preventing the exchange of GDP to GTP and inhibit translation initiation.

caspase-dependent activation of PKR has been reported. In this cleavage-induced mode of activation, cleavage of PKR at an Asp residue frees the catalytic domain from the control of the regulatory domain resulting in a free kinase domain which is constitutively active (50). PKR-mediated phosphorylation of eIF2α in a caspase-dependent manner lends support to previous published data reporting inhibition of total protein synthesis during apoptosis (51). PERK is an ER resident kinase that is activated by stimuli triggering unfolded protein response within ER (52-58). HRI is a heme-regulated translational inihibitor and plays a role in inhibition of total protein synthesis during heme deficiency (59).

Internal Ribosome Entry Site (IRES)

Initiation of translation of eukaryotic mRNAs results in the formation of the 80s ribosome-initiation complex at a start codon within the mRNA. For the majority of cellular mRNAs, initiation is achieved by a scanning mechanism, which involves ribosomal loading on the mRNA through recognition of the 5′ cap structure, a 7-methyl-guanylic acid residue, by a cap-binding complex known as eIF4F(7, 60). Recently, an alternative mechanism has been identified for translation initiation which is independent of cap structure. Some viral and eukaryotic cellular mRNAs contain IRES (internal ribosomal entry sites) in their 5′ untranslated region (5′ UTR) and translation of these mRNAs can be initiated by direct recruitment of the 43S ribosome-preinitiation complex to this cis-element which is generally in close proximity to the initiation codon (61-63).

IRES elements were originally identified in viral mRNAs and it was shown that a variety of viral mRNAs are capable of internal initiation. Recent findings suggest however, that many cellular mRNAs with diverse cellular functions use IRES-mediated internal initiation to maintain their protein synthesis under stress conditions where the cap function is compromised (64-67). For instance, it was shown that during apoptosis, cleavage or alteration of many initiation factors results in a decrease in cap-dependent translation. However, synthesis of proteins such as Apaf1, Xiaps, and c-myc are maintained (68, 69). Analysis of IRES elements within the 5′UTR of several viral mRNAs has revealed an RNA fold profile displaying several structural domains and short motifs important for internal ribosomal entry. In viruses, these structural features are reported to be conserved among closely related species. In contrast to viral IRESs, conservation of secondary structures is not observed among IRESs of cellular mRNAs (70-73).

The requirement for canonical initiation factors to mediate internal initiation varies among different IRES-containing mRNAs. The requirement of eIF4G and eIF4A for picornaviral IRES-mediated translation has been established (74). Recent discoveries suggest that the efficiency of some IRES-mediated translation for some mRNA requires transacting factors which are RNA binding proteins. The first example of these cellular proteins was a 57 kDa polypyrimidine –tract-binding protein (PTB) known previously as a splicesome

component. PTB was found to be essential for the IRES-dependent translation of EMCV and poliovirus (62, 75, 76). Several other IRES Transacting Factors (ITAFs) have been identified by *in vitro* reconstituted translation assays, including LA autoantigen (77), Poly(rC) binding protein 2 (PCBP2) (78, 79)and upstream of N ras (unr) (80).

As it was mentioned earlier, the inhibitory effect of serum deprivation or apoptosis on total protein synthesis has been described. We have shown that the expression of PUMA is induced concurrently with apoptosis as we switch the culture of 23A2 cells to DM. Although transcriptional regulation of PUMA is well established, we noticed that enhanced PUMA expression could occur in the absence of newly synthesized PUMA transcript; therefore, we began to investigate the translational regulation of PUMA expression in response to culture in DM.

MATERIALS AND METHODS

Cell lines and cell culture

23A2 murine skeletal myoblasts are derived from 5-azacytidine treatment of 10T1/2 fibroblast (81). They were cultured on gelatin coated plates and maintained in growth medium consisting of basal modified Eagle's medium (BME), 10% fetal bovine serum (FBS) and 1% penicillin/streptopmycin (P/S). (P/S =10000 I.U./ml of penicillin and 10000 μg/ml of streptomycin). HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium

supplemented with 10% FBS and 1% of P/S. Differentiation was induced by switching 23A2 cells to DM which lacks serum. Cells were maintained at 37°C in 5% CO_2. Actinimycin D was purchased from Sigma and was used at final concentration of 5 μg/ml or 10 μg/ml in DMSO. Rapamycin was purchased from Sigma and was used at final concentration of 100ng/ml in DMSO.

Statistical analysis

Error bars in our data represent the mean ± standard deviations. Statistical analysis was performed using a student's t test (paired t test) and p values less then 0.05 were considered significant.

Silencing of PUMA using siRNA

Custom SMARTpool plus small interfering RNA (siRNA) to target mouse PUMA (GenBankTM accession number NM_133234) was designed and synthesized by Dharmacon (Lafayette,CO). siRNA (1.5 μg) was transfected into 23A2 cells in GM using RNAiFect Transfection Reagent (Qiagen) as described by the manufacturer's protocol. siCONTROL Non-Targeting siRNAs were included as a control. After 48 hours of transfection, cells were treated for 3 hours in DM. Total RNA was isolated from cells using TriZol (Invitrogen) and 0.5 μg of total RNA was reverse-transcribed using SuperScript III RNase H reverse transcriptase (Invitrogen) to produce cDNA according to the manufacturer's protocol. Silencing was monitored at the mRNA level by Real-time PCR. Quantitative Real-time PCR was performed using Opticon & QuantiTect™ SYBR®

GREEN™ PCR Master Mix (Qiagen). Analysis of each sample was performed in duplicate or triplicate and normalized to actin mRNA levels.

Real-time analysis

Total RNA was prepared using 1 mL of Trizol (Invitrogen) reagent per plate for lysis following the manufacturer's instructions. 500 ng of total RNA was used to synthesize the first cDNA strand using reverse transcriptase SuperScript III (Invitrogen) in a 20 μl reaction. 2 μl of the synthesized cDNA was used in subsequent quantitative PCR analysis using SyberGreen (Invitrogen). Primer pairs used for amplification of PUMA mRNA were a forward PUMA primer: CCTCAGCCCTCCCTGTCACCAG and a reverse PUMA primer: CCGCCGCTCGTACTGCGCGTTG. Briefly, 2 μL of synthesized cDNA was mixed with 0.3 μM of each PUMA primer and 10 μl SYBR green reagent (Invitrogen) in a total of 20 μL reaction. cDNA was amplified for 39 cycles at an annealing temperature of 55 °C. Fluorescence was detected using an Opticon Monitor (MJ Research). The cycle number at which the amplification of product began to increase linearly (cycle threshold (C_t) value) was set manually and taken from the Opticon software. The fold difference between the C_t values of PUMA and GAPDH was determined.

Western blot analysis

The cells were washed with 1X PBS and lysed with 1X p21 lysis buffer (20 mM MOPS pH 7.4, 4 mM magnesium chloride, 200 mM sucrose, 0.1 mM EDTA,

0.001% DNase,1 M phenyl methyl sulfonyl floride, 50 μg/ml each of aprotinin, pepstatin, and leupeptin) with 1% CHAPS. Protein concentration was determined using Coomassie Protein Assay Reagent (Pierce) as per manufacturer's instruction and equal amounts of lysate were denatured in 5X reducing buffer (50% glycerol, 10% 2-β-mercaptoethanol, 10% SDS, pH 6.8). Samples were heated in sample buffer to 95°C for 5 minutes and loaded on a 12% SDS-PAGE. Samples from the gel were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane in transfer buffer (containing 5.8 g/L of Tris, 29 g/L glycine, 20% methanol, 1 g/L of SDS) for 40 minutes at 1500 mAmp. The membrane was blocked with 5% milk in wash buffer (1X TBS with 1% NP-40) for one hour and incubated with primary antibody in wash buffer overnight at 4°C. The following primary antibodies were used for incubation with the appropriate membranes at the specified dilution: anti-PUMA antibody (Abcam) at 1:1000 dilution, anti-phospho 4E-BP antibody and anti-4E-BP anti-body (Cell Signaling) at 1:1000 dilution, anti-phospho mTOR antibody (Cell Signaling) at 1:1000 , anti- anti-phospho eIF2α (Ser51) antibody (Cell Signaling) at 1:1000, anti-eIF2α (Cell Signaling) antibody at 1:1000, and anti-PKR antibody 1:1000 and Anti-ß-actin (Sigma) was used at a dilution of 1:30, 000 for one hour as the control for equal loading. The membrane was washed three times with wash buffer after incubation with the primary antibody. The membrane was then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz) diluted at 1:1000 for one hour. Prior to visualization, the membrane was washed again 3 times with wash buffer. SuperSignal West Pico Chemiluminescent

Substrate (Pierce) was incubated with the membrane for 2 minutes and visualization was performed using a Kodak Scientific imaging film.

Immunoprecipitation of ^{35}S-metabolically labeled PUMA

23A2 cells were plated at equal density at 1X10^5 cells per 100 mm plate the night before. The next day, the cells were washed with cold 1X PBS (Phosphate Buffer Saline) and preincubated in cysteine/methionine-free PRMI media (HyClone) for 30 minutes at 37°C. 23A2 cells were then incubated with cysteine/methionine-free medium containing ^{35}S-methionine (0.5 mCi.ml^{-1} in total of 4 ml of medium) (MP Biomedicals Inc) with actinomycin (5 μg/ml) in the presence or absence of 10% dialyzed fetal bovine serum (HyClone). After three hours, 23A2 cells were washed with cold 1X PBS and then lysed by scraping in 70 μl of 1X RIPA lysis buffer (Tris 50 mM, NaCl 150 mM, SDS 0.1 %, phenyl methyl sulfonyl floride 1mM, EDTA 1 mM, Triton X100 0.1% and 1X protease inhibitor).

Further disruption of cells was achieved by repeated freeze-thaw procedures. For immunoprecipitation, ^{35}S-labeled lysates were depleted of endogenous Igs by preclearing them with Protein A/G agarose beads (Santa Cruz) for one hour at 4°C. Precleared ^{35}S-labeled GM or DM supernatant was removed after centrifugation at 2500 rpm for 30 sec at 4°C. Equal aliquots of protein from each precleared supernatant were incubated with 10 μl immunoprecipitation antibody (rabbit polyclonal antibody against PUMA (Abcam):70 μl of Immunoprecipitation matrix (ExactaCruz from Santa Cruz)

complex overnight at 4°C. Immunocomplexes were pelleted at maximum speed at 4°C the next day and were washed four times with RIPA buffer containing protease inhibitors. The final pelleted complex from each lysate was resuspended in 70 μl of 2X reducing electrophoresis buffer (50% glycerol, 10% 2-β-mercaptoethanol, 10% SDS) and boiled for 3 minutes at 95°C. To elute ^{35}S-labeled PUMA proteins, the sample loading buffer was centrifuged at 13000 rpm for 1 minute. The ^{35}S-labeled precipitated PUMA proteins was subjected to SDS-polyacrylamide gel electrophoresis (12%). The Gel was fixed in 50% methanol, 10% acetic acid fixation solution, soaked for 15 minutes in Amplify (Amersham Pharmacia Biotech), dried for 90 minutes at 65 °C, and visualized by a Typhoon scanner.

Total protein synthesis (Metabolic labeling with ^{35}S-methionine)

23A2 cells were plated at equal density in 6-well plates the night before. The next day, cells were washed with cold 1X PBS (Phosphate Buffered Saline) and preincubated in cysteine/methionine-free PRMI media (MP Biomedicals) for 30 minutes at 37°C. To measure newly synthesized protein under different conditions, after brief amino acid starvation, 23A2 cells were incubated with cysteine/methionine depleted medium containing ^{35}S-methionine (40 mCi.ml-1) (MP Bipmedicals) in the presence or absence of 10% dialyzed fetal bovine serum (HyClone). At the various time points indicated on the graph, cells were washed with cold 1X PBS and then lysed in 50 μl of 1X p21 lysis buffer (20 mM MOPS pH 7.4, 4 mM magnesium chloride, 200 mM sucrose, 0.1 mM EDTA, 0.001%

DNase, 1M phenyl methyl sulfonyl floride, 50μg/ml each of aprotinin, pepstatin, and leupeptin) with 1 % CHAPS. The lysates were subject to further lysing by two consecutive freeze-thaw procedures. Protein determination was performed using Coomassie Protein Assay Reagent (Pierce) as per the manufacturer's instructions and equal amounts of protein from each lysates was subject to trichloroacetic acid (TCA) precipitation (10%). 5 μl of ^{35}S-labeled precipitated protein from each lysate was placed onto a 25-mm-diameter glass fiber filter (Fisherscientific Inc). The filters were washed three times in 5% TCA and once in 100% ethanol, air dried and subjected to scintillation counting.

Polyribosome Analysis.

A. Sucrose gradient prepration

Polysome buffer was made by mixing 100 mM KCL, 2.5 mM $MgCl_2$, 10 mM Hepes-KOH (pH 7.4), 1 mM DTT in a 250 ml volume of DEPC-treated water.

To make a sucrose gradient of 5-50% using a gradient maker, 6 g of sucrose was dissolved in 120 ml of polysome buffer (5% sucrose solution) and 60 g of sucrose was dissolved in 120 ml of polysome buffer (50% sucrose solution). 60 ml of each sucrose solution was then loaded into the gradient maker (in different channels of the gradient maker) to make 2 gradients. Gradient maker's pump was set at 1 revolution /20sec. The gradient was collected in collection tubes with 5% solution moving upward as the concentration of sucrose solution was increased. The gradient was poured at least 4 hours before use and allowed to equilibrate at 4°C.

B. Equal number of cells were plated in 15cm tissue culture dishes. The next day, cells were switched to fresh GM (growth media containing 10% FBS) or DM (differentiation media) for 3 hours. Prior to harvesting, cells were treated with 100 μg/ml of cycloheximide for 15 minutes at 37°C. Cells were washed twice with cold 1X PBS containing cycloheximide. Cells were then scraped in cold 1X PBS containing cycloheximide. Harvested cells were collected by centrifugation at 1500 xg for 10 minutes at 4°C.

Pelleted cells were lysed by incubation with 500 μl of lysis buffer (10 mM HEPES-KOH (pH 7.4), 2.5 mM $MgCl_2$,100 mM KCL, 1 mM dithiothreitol, 0.1% Nonidet P-40, RNasin (100 units/ml) and 100 μg/ml cycloheximide for 15 minutes on ice. Further lysing of cells was insured by freeze-thaw procedure. Lysed cells were then subject to centrifugation at 10000 xg to remove cellular debris and mitochondria. Post-mitochondrial cytoplasmic extract (supernatant) was collected and an equal optical density unit (260A) of each cytoplasmic extracts from GM or DM samples was determined and layered over 5-50% sucrose gradient (10 mM Hepes-KOH (pH 7.4), 100 mMKcl, 2.5 mM $MgCl_2$, 1mMdithiothreitol) and centrifuged at 17,000 rpm for 18 hours at 4°C using a Beckman rotor. Fractionation of gradients was performed using ISCO density gradient fractionator with absorbance monitor set at 260 nm. Twenty five fractions were collected. RNA was collected from each fraction using TriZol reagent (Invitrogen) per the manufacturer's instruction. 500 ng of total RNA from each fraction was used in a reverse transcription reaction using reverse

transcriptase SuperScript III (Invitrogen). 2 μl of each RT reaction was used for quantitative Real-time PCR analysis as described above using PUMA or GAPDH specific primers.

Cap analog experiment

pBluescript vector (Invitrogen) was a generous gift from Dr. Anton Komar (Cleveland State University). The entire PUMA cDNA was amplified by PCR using primers: EcoRI forward: AAAAAGAATTCCCAGGAGGCGGCGGCGACACCAGC

Sac I reverse: AAAAAGAGCTCTTACAGCGGAGGGCATCAGGCGG. The resulting PCR product was digested with SacI and EcoRI and was cloned

.in pBluescript backbone vector digested with SacI/EcoRI. The final construct was verified by sequencing and was linearized with HindIII. The pCAT vector was used as a positive control since its translation is strictly cap-dependent. 100 ng of linearized DNA was used in an *in vitro* transcription reaction to produce capped and polyadenylated RNA using Archacap Ultra Machine Message (Promega). 500 ng of RNA was subjected to an *in vitro* translation reaction in the presence or absence of increasing concentrations of $m^7G(5')ppp(5')G$ cap analog (Ambion) and 10 μCi of Trans ^{35}S-Label Met/Cys (MP Biomedicals) . Reactions were resolved on a 12% SDS-PAGE . Gel was fixed in 50% methanol and 10% acetic acid fixation solution, soaked for 15 minutes in Amplify (Amersham Pharmacia Biotech), dried for 90 minutes at 65°C and visualized on a Typhoon scanner and the amount of radioactivity was quantified for each band.

In vitro transcription and translation

pGEM-CAT-fLuc was a gift from Dr Komar (Cleveland State University). Its construction has been described previously (82). The sequences spanning 5′UTR of PUMA , 5′UTR of PUMA + 50 nt and 5′UTR of PUMA + 100 nt past the initiation codon were each amplified by PCR using PUMA cDNA (Invitrogen MG 67917) as a template and with the following primers: BamHI bbc3 forward: AAAAAGGATCCCCAGGAGGCGGCGGCGACACCAGC, BamHI bbc3 +1 reverse : AAAAAGGATCCACATGGCGCTCCCTGGAGCCCC, BamHI bbc3 +54 reverse: AAAAAGGATCCATACAGCGGAGGGCATCAGGCGG and BamHI bbc3 +102+1 reverse:AAAAAGGATCCAGCGGGCTAGACCCTCTACGGGCTCC

The resulting PCR fragments were digested with BamHI and were each cloned into a pGEM-CAT-fLuc vector at BamHI site. Constructs were verified by sequencing. The resulting vectors were linearized with XhoI (Invitrogen). A total of 1 μg of each DNA construct (pGEM bicistronic vector control, pGEM- PUMA 5′UTR , PGEM- PUMA-5UTR-(+50) and pGEM-PUMA-5UTR-(+100)) was linearized and transcribed *in vitro* using mMESSAGE mMACHINE T7 Ultra kit (Ambion) according to the manufacturer's instructions. The integrity of RNA transcripts was verified by running them on an urea-PAGE. 2 μg of each capped RNA was *in vitro* translated using nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-methionine (10 μl). The resulting radiolabeled protein products were resolved on a 12 % SDS-PAGE. The gel was fixed in 50% methanol and10% acetic acid fixation solution, soaked for 15 min in Amplify

(Amersham Pharmacia Biotech), dried for 90 minutes at 65°C, and visualized on a Typhoon scanner. The amount of radioactivity was quantified for each band.

Monocistronic contructs

We used the monocistronic expression vector pUHD10-3/LUC. This vector was a generous gift from Dr. Hatzoglou (Case Western University) and its generation has been described in detail (83). It was generated by cloning the chimeric LUC cDNA into the Xba/EcoRI site of pUDH10-3. This vector contains a minimal cytomegalovirus promoter and the SV40 polyadenylation signal. To generate the monocistronic vector harboring the 5′ UTR of PUMA with an additional 50 nt or 100 nt past the initiation codon, we amplified PUMA cDNA by polymerase chain reaction using the following primer sequences: EcoRI forward: AAAAAGAATTCCCAGGAGGCGGCGGCGACACCAGC

and

AAAAATCATGAATACAGCGGAGGGCATCAGGCGG; Pag I reverse:

AAAAATCATGAAGCGGGCTAGACCCTCTACGGGCTCC. A 300 or a 350-bp PCR product was amplified from PUMA cDNA (Invitrogen) using this approach. The PCR products were digested with EcoRI and BspHI and inserted into the EcoRI/NcoI site of the pUHD10-3/LUC. We also obtained phpUDH10-3/luc from Dr. Hatzoglou. The hairpin was generated as described previously (83) using phosphorylated primers: 5′- GGAAGCTTATCGATTTCGAACCCGGGTACCG′

AND 5′- AATTCGGTACCCCGGGTTCGAAATCGATAAGCTTCCGC ′

which were annealed and inserted at a unique SacII site 70 nt downstream of the transcription start site. The identity of each vector was verified by sequencing.

Dicistronic constructs

To make dicistronic constructs harboring the PUMA 5′ UTR and different regions past the initiation codon, we obtained the pRF vector from Dr. Jian-Ting Zang, (Indiana University). This vector is a generous gift from Dr. Stonely. It contains both *Renilla* and Firefly luciferase genes and its generation is described previously (68). We also obtained a dicistronic control php-RF vector from Dr. Jian-Ting-Zang which was made by inserting a stable hairpin upstream of the *Renilla* gene at the *EcoRV* site in the pRF vector using a double-stranded oligonucleotide with sense strand of 5′-ATCAAAAGCGCAGGTCGCGACCGCGCATGCGCGGTCGCGACCTGCGCTAAAGAT-3′ (84). We amplified the 5′UTR of PUMA and 100 nucleotides past the initiation codon from PUMA cDNA using primers:

EcoRI forward: AAAAAGAATTCCCAGGAGGCGGCGGCGACACCAGC

Pag I reverse: AAAAATCATGAAGCGGGCTAGACCCTCTACGGGCTCC

The PCR product was digested with EcoRI and BspHI and inserted at EcoRI/NcoI sites in pRF and phpRF vectors. The resulting vectors were verified by sequencing.

Cell Transfections and repoter analysis

23A2 Cells were plated at a density of $1X10^5$ cells per plate (6 Well) in GM. The next day, cells were transfected with 2 μg/well of either monocistronic or dicistronic reporter plasmids using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's instructions. In monocistronic reporter assay, cells were cotransfected with 800 ng/well of pSV- β-galagtosidase expression vector (Promega). Hela cells were transfected with 500ng of bicistronic vector while 293T cells were transfected with 2ug of bicistronic plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. After incubation at 37° for 24 h, the cells were switched to fresh GM or DM for 3 hours (in case of 23A2 cells) and treated or untreated cells were collected for reporter assays. For monocistronic repoter assays, Firefly luciferase activity was measured using Luciferase Assay System (Promega) and was normalized to β-galagtosidase activities as measured by the β-galagtosidase Enzyme Assay System (Promega). For dicistronic reporter assays, Firefly and *Renilla* luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions

Promotorless bicistronic vector. pRF vector was digested with SmaI and EcoRV to remove the simian virus 40 (SV40) promoter sequence and the chimeric intron from pRF dicistronic vector. The resulting digestion product was religated using T4 ligase to generate a promoterless dicistronic pRF(-p) vector. PUMA 5′UTR and 100 nt past the initiation codon was amplified as described

above and the resulting PCR product was inserted into an EcoRI/NcoI site within the promoterless pRF to generate pR-PUMA- F(-P).

RT-PCR analysis of the transfected bicistronic pRF vector harboring PUMA 5′UTR. 23A2 cells were transfected with 2 μg of pRF vector containing PUMA 5′UTR in the intercistronic region using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's protocol. 24 hours after transfection, total RNA was isolated using TriZol (Invitrogen) reagent. 5 μg of RNA was subjected to DNase treatment (Promega) followed by phenol/chloroform extraction. 0.5 μg of DNase-treated RNA was used for a 20 μL reverse transcription reaction in the presence or absence of SuperScript III reverse transcriptase (Invitrogen). 2 μl of each RT and –RT reaction was used for PCR using the following primer set:

Fwd AGCAGCAAGGTGCCTCAATAG and Rev TGATGTCCACCTCGATATGTG

RESULTS

Increased levels of PUMA protein in the absence of increased PUMA message

We have previously reported that an increase in PUMA mRNA (Fig 3.3A) and protein (Fig 3.3B) is detected in lysates from 23A2 myoblasts cultured in DM as compared to GM. This increased level of PUMA mRNA can be abrogated by prior incubation with siRNA targeting PUMA but not by prior incubation with non-targeting control siRNA (Fig. 3.3A). However, this suppression of increased levels of PUMA message did not correspond to a suppression of PUMA protein (Fig. 3.3B). Further, treatment with actinomycin D abrogated the increase in PUMA mRNA but had no effect on the increased level of PUMA protein (Fig. 3.4 A & B).

These results suggested that post-transcriptional regulation contributes to the increased expression of PUMA, either through increased protein stability or increased translation. We therefore assessed the half-life of PUMA protein in GM compared to DM. Myoblasts were cultured in either GM or DM, each supplemented with the protein synthesis inhibitor cycloheximide (CHX). PUMA protein levels were then measured by Western analysis after various times. The half-life of PUMA (Fig. 3.5) was greater than 24 hours. Thus, we have focused on the translational regulation of PUMA.

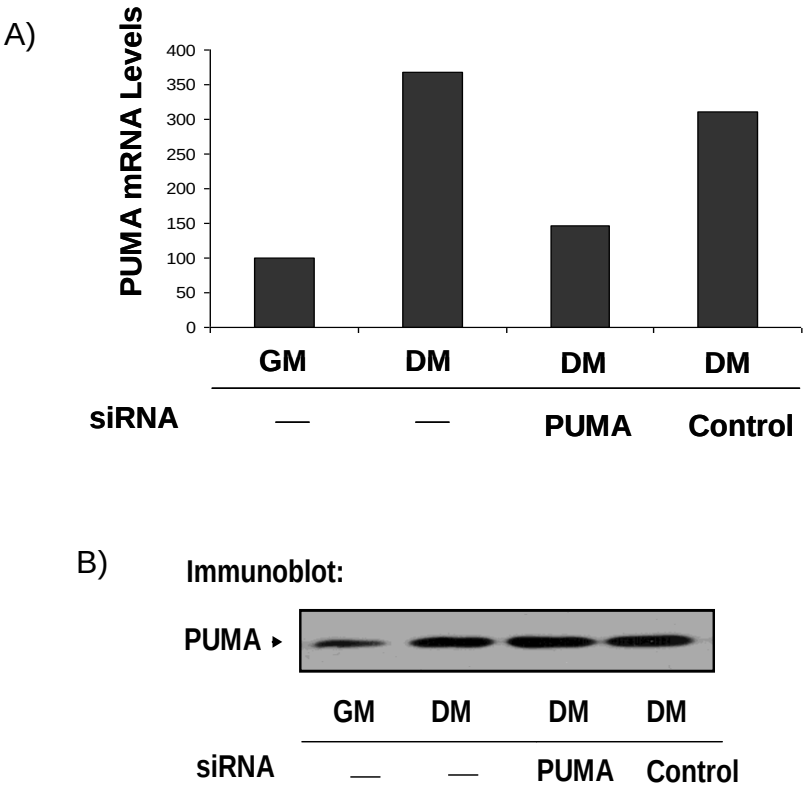


Figure 3.3 siRNA treatment of 23A2 cells suppresses the increase in PUMA transcripts but does not prevent the increase of PUMA protein in DM. (A) Custom SMARTpool plus small interfering RNA (siRNA) to target mouse PUMA was designed and synthesized by Dharmacon (Lafayette,CO). siRNA (1.5 ug) was transfected into 23A2 cells in GM using RNAiFect Transfection Reagent (Qiagen) as described by the manufacturer's protocol. siCONTROL Non-Targeting siRNAs were included as a control. After 48 hours of transfection, cells were treated for 3 hours in DM. Total RNA was isolated from cells using TriZol (Invitrogen) and 0.5 μg of total RNA was reverse-transcribed using SuperScript III RNase H reverse transcriptase (Invitrogen) to produce cDNA according to the manufacturer's protocol. Silencing was monitored at the mRNA level by Real-time PCR. (B) Western blot analysis using anti-PUMA and anti-actin antibodies. This is a representative of 4 independent experiments. Error bars represent mean $\pm$ SEM from triplicate samples.

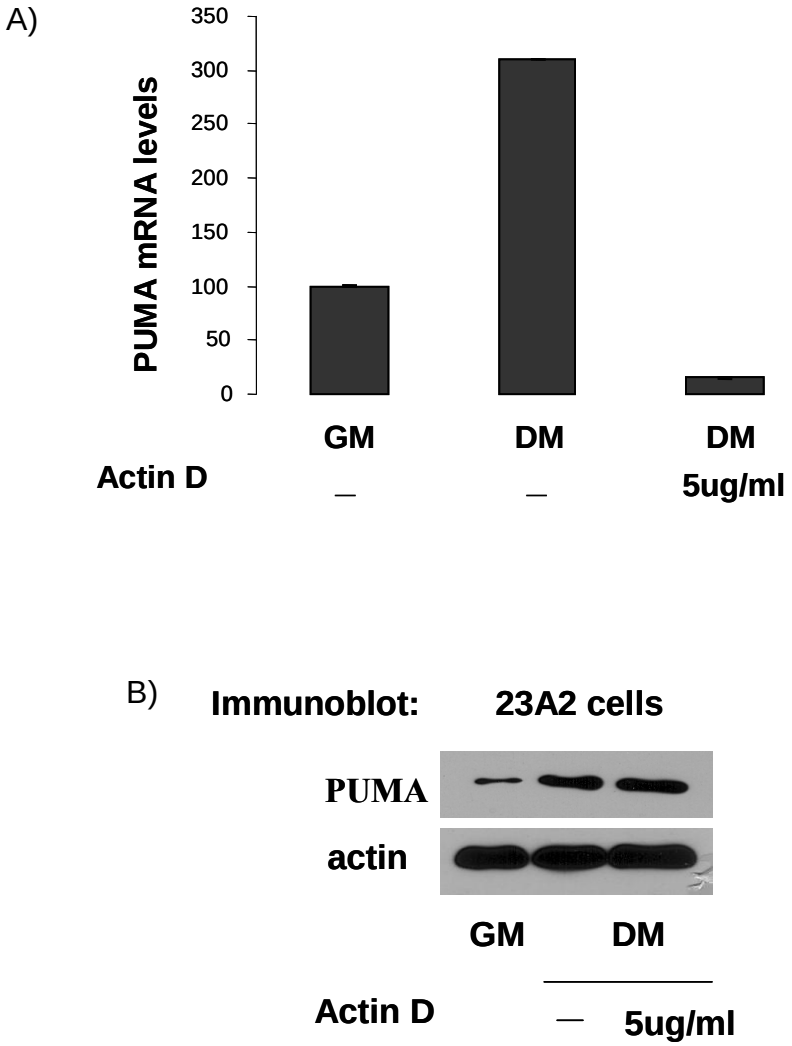


Figure 3.4 Actinomyocin treatment of 23A2 cells does not prevent the increase of PUMA protein in DM. Cells were plated at equal density and the next day switched to fresh GM or DM in the presence or absence of actinomycin D. (A) Total RNA was isolated from cells using TriZol (Invitrogen) and 0.5 μg of total RNA was reverse-transcribed using SuperScript III RNase H reverse transcriptase (Invitrogen) to produce cDNA according to manufacturer's protocol. mRNA levels quantified by Real-time PCR. (B) Western blot analysis using anti-PUMA and anti-actin antibodies. This is a representative of 3 independent experiments. Error bars represent mean ± SEM from triplicate samples.

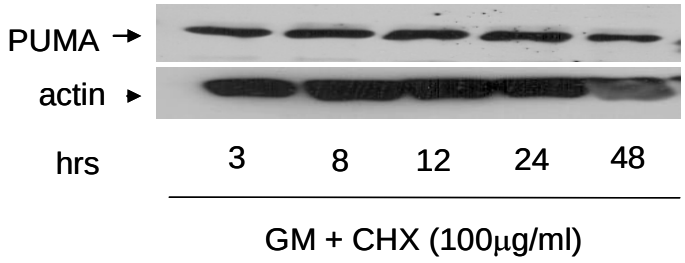


Figure 3.5 PUMA Protein has a half-life greater than 24 hours. 23A2 Cells were plated at equal density the next day switched to fresh GM in the presence of 100 μg/ml of cyclohexamide. Lysates were collected at various time points as indicated. Western blot analysis was performed using anti-PUMA and anti-actin antibodies. This is a representative of two independent experiments.

PUMA protein is translationally regulated

To show that increase translation contributes to the observed increased PUMA expression in DM, 23A2 cells were starved in cysteine/methionine-free PRMI media for a brief time and pulsed with ^{35}S-methionine in the presence of actinomycin D, either in the presence or absence of 10% dialyzed serum for 3 hours. Immunoprecipitation of PUMA was performed on precleared lysates using a rabbit polyclonal antibody against PUMA coupled to an immunoprecipition matrix. The ^{35}S-labeled precipitated PUMA protein was eluted from the resulting immunocomplex and analyzed by electrophoresis on a 12% SDS-polyacrylamide gel. Fig. 3.6 shows enhanced incorporation of the label, indicative of new PUMA protein synthesis in the absence of serum. Further, this enhancement was not affected by the presence of actinomycin D. This is consistent with the observed increase in steady-state levels of PUMA protein in DM even when the increase in PUMA mRNA is blocked and suggests that translational regulation of PUMA is the mechanism responsible for increased expression of PUMA protein.

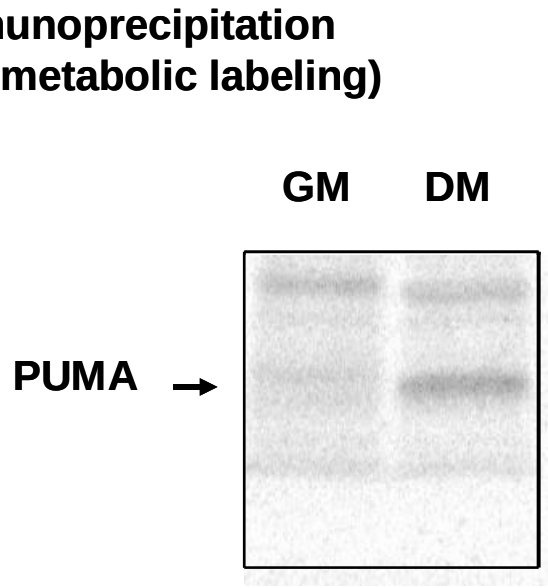


Figure 3.6 PUMA expression is translationally regulated 23A2 cells were plated at equal density and preincubated with media free of cysteine and methionine. Actinomycin D treated 23A2 cells were then labeled with ^{35}S-meth in the presence or absence of 10% dialyzed serum. PUMA protein was immunoprecipited as described under "Experimental Procedure" from lysates treated with GM (growth factor) or DM (differentiation) medium. Immunoprecipited ^{35}S-labeled PUMA was loaded on a 12% SDS-PAGE gel and the band corresponding to ^{35}S-labeled PUMA was visualized by a Typhoon scanner. Data shown is representative of two independent experiments.

PUMA translation is regulated at initiation step

In order to determine if the translation of PUMA is enhanced at the level of translation initiation, we performed ribosomal profiling. Cytoplasmic extracts of 23A2 cells incubated in GM or DM were layered, after treatment with 0.1 mM cycloheximide, on a 5-50% sucrose gradient followed by centrifugation. Gradients were then fractionated with continuous monitoring at 260 nm using an ISCO fractionator. Analysis of ribosomal profiles reveals an inhibition of total protein synthesis in DM as indicated by smaller polysome peaks and a higher 80S peak (Fig. 3.7). Furthermore, it suggests that initiation attenuation is responsible for this global decrease in translation. Analysis of PUMA mRNA across the gradient by Real-time PCR using PUMA specific primers reveals differential distribution of PUMA mRNA levels along polysomes in GM versus DM. As previously reported, an increase in translation initiation of a specific mRNA is indicated by an increase in its association with polyribosomes (85). In agreement with this, our data indicates that more PUMA mRNAs are associated with polysomes in the absence of serum and hence more PUMA mRNAs are being actively translated. As a control, we also determined the distribution of mRNAs are actively translated and therefore more GAPDH mRNAs are associated with polysome fractions, giving a similar distribution to that of PUMA mRNAs in DM. Removal of serum reduces the association of GAPDH mRNAs with polysomes consistent with its cap-dependent mode of translation initiation.

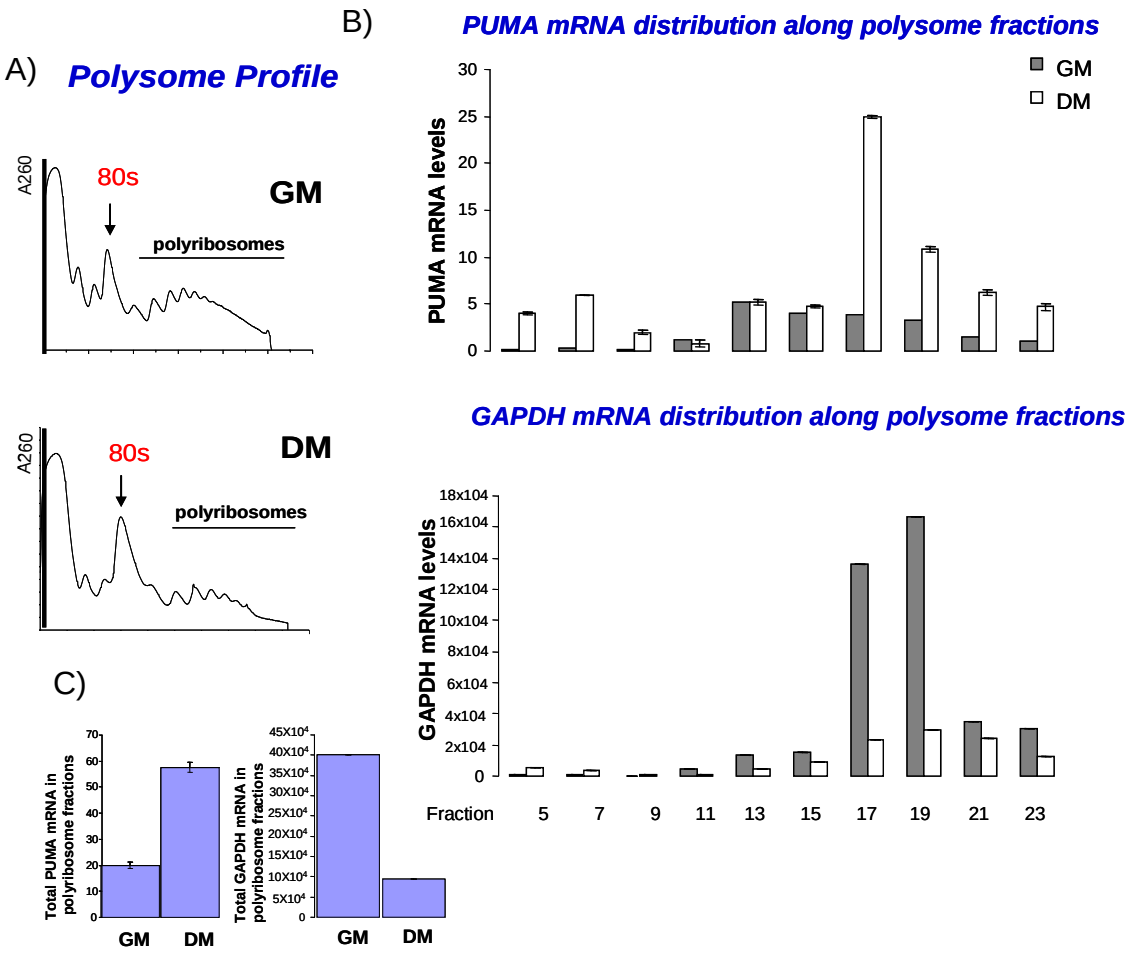


Figure 3.7 Translational increase in PUMA expression occurs at initiation step. A) Polysome profiles of mRNAs in 23A2 cells in presence and absence of serum Lysates from 23A2 cells in presence and absence of serum were separated by centrifugation over a 5-50 % linear sucrose gradient. Fractionation of gradients was performed with the absorbance monitor set at 260 nm. 25 fractions were collected. RNA was collected from each fraction using TriZol reagent (Invitrogen) as per the manufacturer's instructions. **B) PUMA mRNAs and GAPDH mRNAs are translocated to polyribosomal fractions in the absence or presence of serum respectively** RNA from each of the two adjacent fractions were combined and distributions of PUMA and GAPDH mRNAs were analyzed by real-time PCR using PUMA or GAPDH specific primers. Results are representative of triplicate samples from two independent experiments. Error bars represent mean ± SEM from triplicate samples. C) Graph bars representing total polyribosomes for PUMA and GAPDH in the presence or absence of serum.

Cap-dependent translation is initially decreased in DM but then recovers to levels comparable to that observed in GM

We next investigated global translational regulation as a consequence of culture in DM. Culture in DM is equivalent to serum deprivation. Many studies have shown that total protein synthesis is subject to regulation by environmental cues and that serum deprivation results in decreased cap-dependent translation (86, 87). We have discovered, however, that total protein synthesis as assessed through ^{35}S-methionine incorporation is initially decreased after 3 h of culture in DM but recovers to levels comparable to that observed in GM after 18 hours (Fig. 3.8).

The presence of hypophosphorylated 4E-BP correlates with initial decrease but not recovery of cap-dependent translation

To elucidate the molecular mechanism responsible for the global effect on translation in 23A2 cells, we began to investigate the phosphorylation state of 4E-BP as a consequence of culture in DM. Hypophosphorylated 4E-BP binds to eIF4E and prevents formation of the 43S pre-initiation complex. The appearance of hypophosphorylated 4E-BP correlated with the decrease in global translation after 3 hours of culture in DM (Fig. 3.9). However, hypophosphorylated 4E-BP persists even after 18 h of culture in DM when global translation has returned to levels detected in GM. Thus, the phosphorylation status of 4E-BP correlates with the initial decrease, but not the recovery, in global translation.

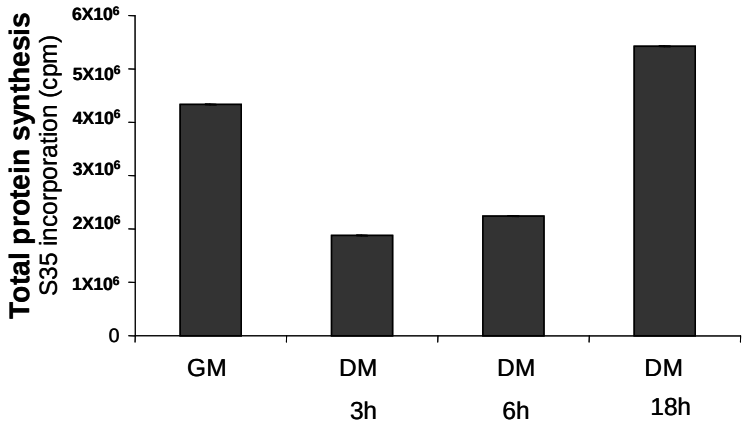


Figure 3.8 Global translation of 23A2 cells decreases after 3 hours in DM but then recovers to that level observed in GM after 18 hours 23A2 cells were cultured in GM. The next day 23A2 cells were starved in cysteine- methionine-free RPMI for 60 minutes. 23A2 cells were then incubated with ^{35}S-methionie (250 μCi/ml) in the presence or absence of 10% dialyzed serum (Hyclone). Cell lysates were prepared after the indicated time and equal amounts of protein were incubated with 500 μl of NaOH at 37°C for 15 minutes followed by 10% TCA precipitatation. Equal volume of each sample was applied on GFC filter paper. Each filter was washed with 5% TCA twice and 10 ml of ethanol then the amount of radioactivity was quantified by scintillation counter. Shown are results from one experiment which are representative of two independent experiments. Error bars represent mean ± SEM from triplicate samples.

Others in our lab have also shown that PI3K activity in myoblasts cultured in DM initially decreased but then recovered to that found in GM (33). It has been suggested that PI3K lies upstream of mTOR in other systems and signaling by mTOR phosphorylates 4E-BP and allows protein synthesis (41). To assess mTOR activation as a consequence of culture in DM, lysates prepared from 23A2 myoblasts cultured in DM for various times were assessed for phosphorylated active mTOR by Western analysis. The phosphorylation status of mTOR follows that of AKT, suggesting that mTOR might lie downstream of the AKT signaling pathway. However, the recovery of AKT signaling to mTOR does not correspond with a recovery in hyperphosphorylated 4E-BP and corresponding loss of hypophosphorylated 4E-BP. Hence, we continued searching for a key translational regulator whose activation status corresponded to observed fluctuation in global protein synthesis.

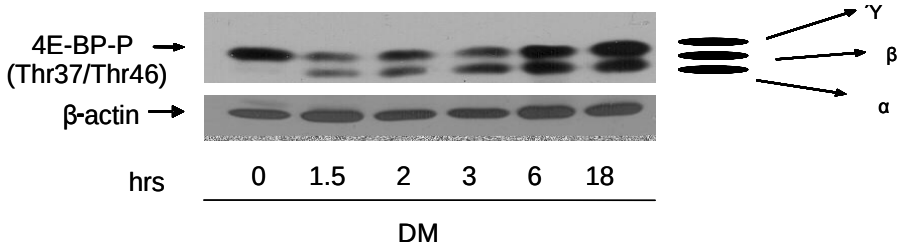


Figure 3.9 4E-BP phosphorylation fails to correlate with the recovery of the global protein synthesis in 23A2 cells. 23A2 cells were plated at equal density and the next day switched to fresh GM or DM for the indicated time. Western blot analysis was performed on equal amounts of lysates with a phospho-specific antibody against 4E-BP (Cell Signaling) and an anti-β-actin antibody (SantaCruz). This is a representative of three independent experiments.

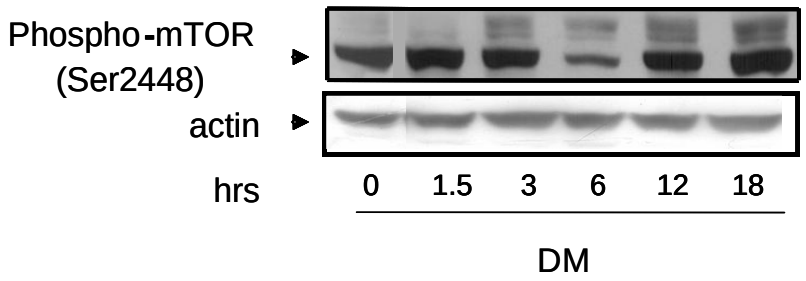


Figure 3.10 mTOR phosphorylation levels correlate with total protein translation and PI3 kinase activity in 23A2 cells upon culture in differentiation media. 23A2 cells were plated at equal density and the next day switched to fresh GM or DM for the indicated time. Whole cell protein lysate was prepared and subjected to SDS-PAGE. Western blot analysis was performed using an anti-phospho mTOR (Cell Signaling) antibody and visualized by autoradiography. Anti ß-actin (Sigma) was used as the control for equal loading. The results shown here are representative of three independent experiments.

De-phosphorylation of eIF2α correlates with the recovery of Cap-dependent translation

Since eIF2α phosphorylation regulates the rate of protein synthesis (7, 8), we next assessed the phosphorylation status of eIF2α in 23A2 cells as a consequence of culture in DM by performing Western blot analysis using a phospho-specific antibody to eIF2α (Ser-51). Phosphorylated eIF2α prevents the exchange of GDP for GTP by eIF2B and blocks translation initiation. phosphorylation of eIF2α is below the level of detection in presence of serum. Phosphorylation can be detected as early as 30 minutes upon removal of serum and is maximal from 3 to 6 hours in DM (Fig. 3.11). This is in agreement with our previous data showing a decrease in total protein synthesis after 3 hours in DM. Furthermore, phosphorylation of eIF2α decreases after 18 hours in DM and this correlates to the recovery of global translation.

To identify the kinase responsible for eIF2α a phosphorylation, we focused our attention on PKR. Recently, caspase-dependent activation of PKR has been reported. In this cleavage-induced mode of activation, cleavage of PKR at an Asp residue frees the catalytic domain from the control of the regulatory domain resulting in a free kinase domain which is constitutively active (50). Since our lab has previously shown activation of caspases in 23A2 cells as a consequence of culture in DM, we monitored PKR cleavage by performing Western blot analysis using an antibody that recognizes its N-terminal domain (Fig. 3.12). We confirmed the cleavage of PKR in DM after 3 hours by detecting a 38 KD

fragment which is similar in size to the estimated fragment of the regulatory domain of PKR. This 38 KD fragment is absent in GM. Since PKR activation coincides in time with eIF2α phosphorylation, these results allow us to speculate that eIF2α phosphorylation could be mediated by PKR activation in DM. Although these data suggest that caspase-mediated activation of PKR and subsequent phosphorylation of eIF2α in 23A2 cells might be responsible for the inhibition of total protein synthesis during apoptosis, future studies using caspase inhibitors are necessary to confirm that PKR activation and eIF2α phosphorylation are indeed capspase-mediated.

Identification of secondary structures in 5 ′ UTR of PUMA using MFOLD algorithm

We have previously shown an increase in eIF2α phosphorylation levels which correlated with a decrease in total protein synthesis after switching the culture of 23A2 cells to DM for 3 hours. We have also demonstrated that PUMA protein expression levels increase under the condition of decreased global translation. These data led us to hypothesize that the increase in PUMA expression under the condition of decreased total protein synthesis could be IRES-mediated.

Analysis of IRES elements within the 5′UTR of several viral mRNA has revealed several general structural features important for internal ribosomal entry (70, 71). We performed computational analysis to predict the possible secondary

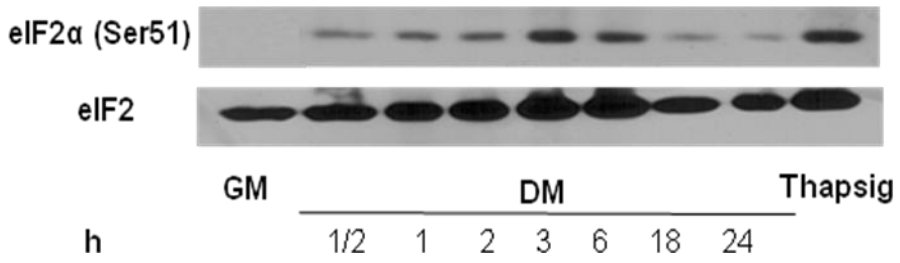


Figure 3.11 The phosphorylation status of eIF2α correlates with the level of total translation in 23A2 cells upon removal of serum. 23A2 cells were plated at equal density and the next day switched to fresh GM or DM for the indicated time. Whole cell protein lysate was prepared and subjected to SDS-PAGE. Western blot analysis was performed using an anti-phospho eIF2α (Ser51) (Cell Signaling) antibody or anti-eIF2α antibody and visualized by autoradiography. Anti-ß-actin (Sigma) was used as the control for equal loading. The results shown here are representative of four experiments.

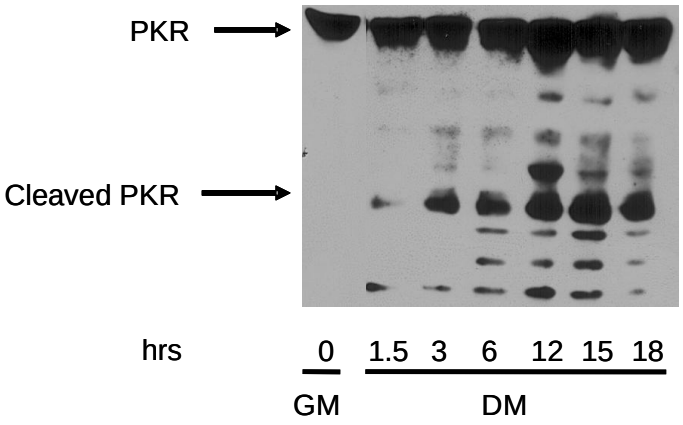


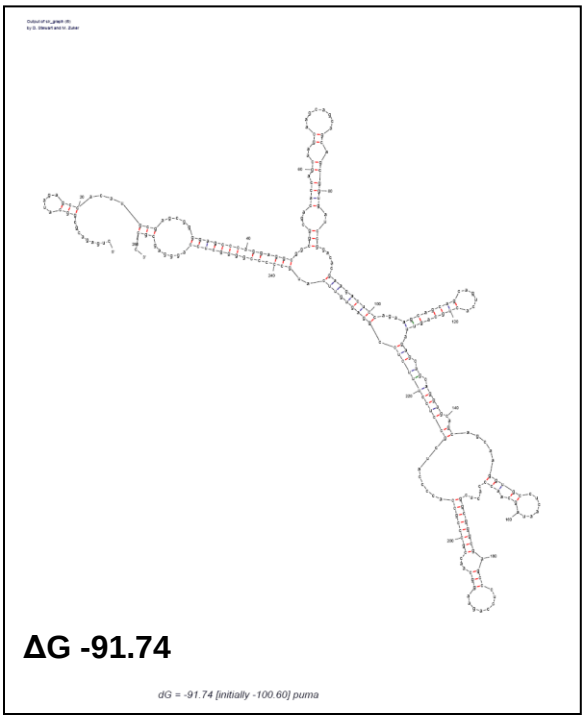
Figure 3.12 The appearance of active PKR fragments upon culture of 23A2 cells in DM correlates with the phosphorylation status of eIF2α and total protein synthesis. 23A2 cells were plated at equal density and the next day switched to fresh GM or DM for the indicated time. Whole cell protein lysate was prepared and subjected to SDS-PAGE. Western blot analysis was performed using an anti-PKR (N-terminal 1-515 amino acid) antibody (Santa Cruz) and visualized by autoradiography. The results shown are representative of two separate experiments.

structures within the 5′UTR of PUMA RNA. Secondary structural elements were generated using MFOLD algorithm (88). This method predicts RNA folds that are thermodynamically stable based on their minimal free energy. Using the MFOLD algorithm, we identified the presence of predicted secondary structures in the 5′ UTR of PUMA (Fig. 3.13). It is suggested that the presence of such highly stable stem loops with a predicted ΔG value of -91 Kcal/mol in the 5′UTR of PUMA could be inhibitory to the conventional translation initiation by scanning mechanism (89, 90).

Competition by cap analog failed to inhibit the translation of capped PUMA RNA

To test the hypothesis that PUMA protein is translated via a cap-independent mechanism, the whole sequence of PUMA cDNA, including poly(A) sequence, was cloned into the pBluescript (Stratagene) vector as described in Material and Methods. The resulting plasmid was linearized and subjected to an *in vitro* transcription program using the mMESSAGE mMACHINE T7 ULTRA kit (Ambion). The resulting capped PUMA RNA transcript was used to program *in vitro* translation reactions with rabbit reticulocyte lysate (RRL) in the absence or presence of increasing concentrations (0.200 μm and 1 mM) of cap analog m7GpppG (Ambion). To monitor the inhibition of cap-dependent translation, a

A)



B)

CUGAGACGCGGCAUAGAGCCACAU
GCGAGCGGGGAGCCCAGGAGGCG
GCGGCGACACCAGCAAGCAAGCAG
CAGCAGCGGUGAUCCGGACACGAA
GACUCCAGAAGCAGCAGCAGUCAC
UGCAGUUAGAGCAGCAGGAGCAGC
AGCAAGGUGCCUCAAUAGCAACCC
ACUCGGCGGGCGAGCCCUCCAGA
AGGCAACCGCCCGCCACCCCAUCG
CCUCCUUUCUCCGGAGUGUUCAU
GCCCCCGGGGCUCCAGGGAGCGC
C

Figure 3.13 A) Predicted secondary structures of PUMA 5′ UTR were generated by MFOLD. A strong secondary structure with ΔG of -91 kcal/mol was identified. B) Nucleotide sequence of structural elements within PUMA 5′UTR.

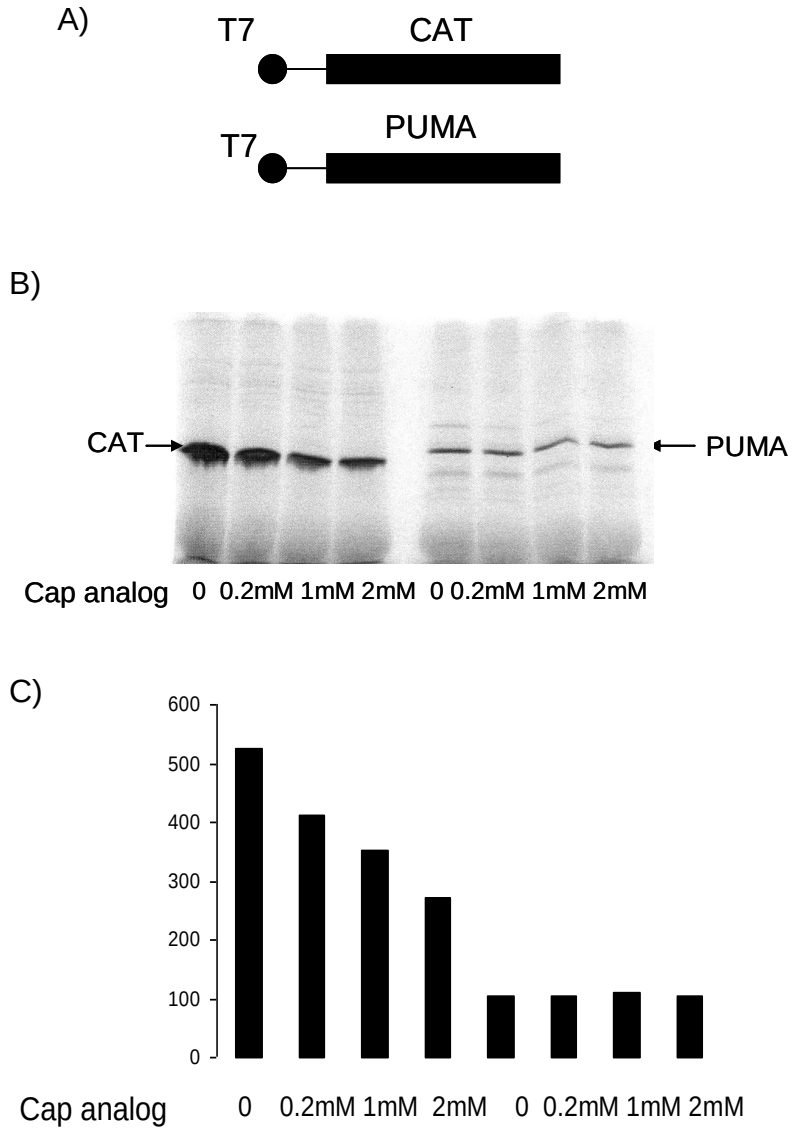


Figure 3.14 Competition by cap analog failed to inhibit the translation of PUMA RNA A) schematic diagram of constructs used in this experiment. B)*In vitro* translation of capped PUMA RNA in the presence or absence of exogenously added cap analog. pBluescript containing the entire PUMA cDNA and pCAT were linearized. Capped RNAs were transcribed *in vitro* (mMachine mMassage T7 transcription kit (Ambion). Capped RNA transcripts were used to program *in vitro* translation reactions with RRL in the presence of increasing concentrations of cap analog m7GpppG (Ambion) as indicated. Data shown here is representative of two independent experiments. C) quantification of band shown in B by a typhon scanner.

pBluescript vector containing the CAT enzyme was used as a positive control. The addition of a cap analog decreases the cap-dependent translation of CAT enzyme in a dose-dependent manner; however, it fails to inhibit the translation of capped PUMA RNA, suggesting that PUMA mRNA can be translated in a cap-independent manner (Fig 3.14).

PUMA 5′-UTR functions as an IRES in a bicistronic vector *in vitro*

We also determined the sequence within the 5′UTR of the PUMA mRNA that would be sufficient to mediate IRES-dependent translation of PUMA mRNA. The complementary DNA sequence corresponding to the 5′UTR of PUMA mRNA was cloned between CAT and LUC genes in a pGEM bicistronic vector developed by Drew & Belsham (82) (Fig 3.15A). The constructs containing either the full length 5′UTR or full length 5′UTR and additional nucleotides past the initiation codon were subject to an *in vitro* translation reaction and the translation efficiency of each construct was determined as the ratio of fLUC/CAT. Insertion of the 5′UTR of PUMA resulted in an increase in the ratio of fLUC/CAT; however, addition of 50 or 100 nucleotides past the coding sequence resulted in an increase of this ratio, three to four times that observed with vector alone (Fig 3.18C). This data is in agreement with previous published reports showing that sequences within the protein coding region were sometimes required for IRES function (91-93).

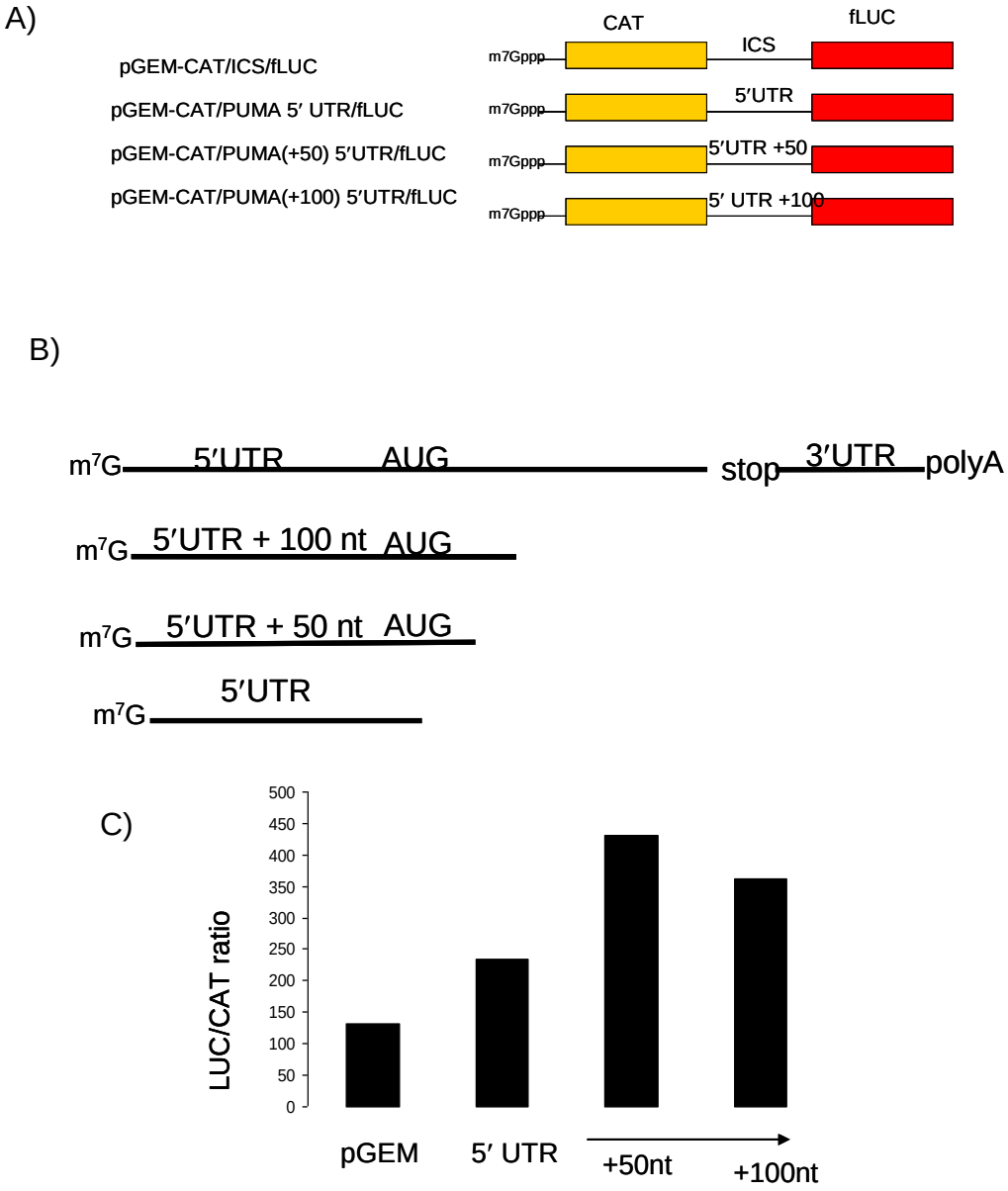


Figure 3.15 PUMA 5′UTR shows IRES activity *in vitro*. A) A diagrammatic representation of the bicistronic control (pGEM-CAT-ICS-LUC) and PUMA 5′UTR containing vectors. B) A diagrammatic representation of region of PUMA cDNA tested in these bicistronic constructs. C) pGEM bicistronic vector control, harboring PUMA 5′UTR and PUMA(+50) 5′UTR, and PUMA(+100) 5′UTR were each linearized and transcribed *in vitro* using the mMESSAGE mMACHINE T7 ULTRA kit (Ambion). Capped RNA was *in vitro* translated using RRL. Radiolabeled protein was resolved on a 12% SDS-PAGE and the amount of radioactivity was quantified. Data shown here is representative of two independent experiments.

PUMA protein expression in DM is cap-independent

To investigate if the increase in PUMA protein levels observed after 3 hours of culture in DM is cap-dependent, 23A2 cells were treated with rapamycin. Rapamycin inhibits the function of the mammalian target of rapamycin, mTOR, a serine/thereoine kinase responsible for phosphorylating 4E-BP (94-96). Inhibition of mTOR results in hypophosphorylation of 4E-BP, which becomes active and is then able to inhibit the formation of the eIF4F initiation complex by sequestering eIF4E, thereby blocking cap-dependent translation of mRNA (19, 40, 97). To determine the concentration of rapamycin necessary to inhibit cap-dependent translation, we assessed the level of MyoD, a protein with half-life of 45 minutes, following treatment with rapamycin. Blocking cap-dependent translation allowed MyoD protein levels to be reduced to nearly below detectable levels after 3 hours (Fig. 3.16A). We showed that the inclusion of rapamycin at a concentration sufficient to prevent cap-dependent translation of MyoD did not affect the expression of PUMA protein in DM (Fig. 3.16A). We also showed that myogenin induction in DM could be inhibited by inclusion of rapamycin (Fig 3.16B).

PUMA 5′-untranslated region functions as an IRES *in vivo*

To investigate whether PUMA is translated in an IRES-mediated manner *in vivo*, we used the monocitronic construct pUHD10-3/LUC in which PUMA 5′UTR and different regions past the initiation codon were each cloned in frame upstream of the Firefly luciferase gene. The resulting monocistronic vector, if

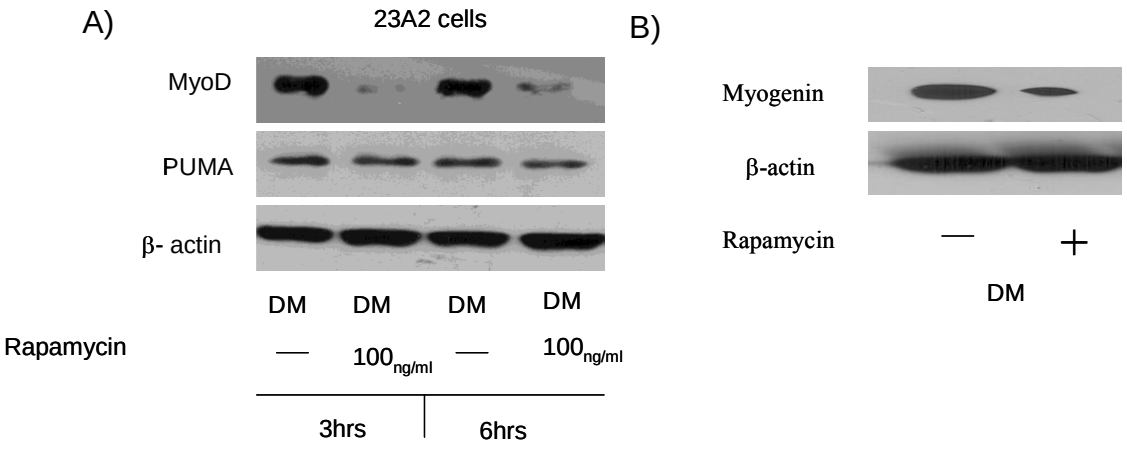


Figure 3.16 PUMA expression in DM is cap-independent. A) 23A2 cells were treated in the presence or absence of rapamycin (100 nM) in DM for the indicated time, A) total protein lysates were prepared and resolved on 10% for MyoD which has a short protein half life) or 12% (for PUMA) SDS-PAGE. Steady state level of PUMA , MyoD and β-actin were determined by Western blot analysis using antibodies against PUMA, MyoD and β-actin, respectively B) Western blot analysis of 23A2 cells treated in presence or absence of rapamycin in DM for 12 hours. Lysates were resolved on 10% SDS-PAGE and immunoblot analysis was performed using anti-myogenin antibodies. Data shown here is representative of two independent experiments.

expressed, produces a monocistronic chimeric mRNA (83). To distinguish between scanning- dependent and independent translation initiation, we utilized a pUHD10-3/LUC vector that harbors a stable RNA hairpin upstream of fLUC. Using this vector, PUMA 5′UTR was cloned downstream of the hairpin in-frame with the fLUC gene. 23A2 cells were transfected in GM with these monocistronic constructs along with a β-galctosidase expression vector. The cells were switched to DM for 3 hours. Luciferase activity was determined for each construct and normalized to β-galctosidase to account for differences in transfection efficiency. Upon switching 23A2 cells to DM, the ratio of fLuc/β-gal was enhanced significantly if PUMA 5′UTR +100 nt was inserted into this monocitronic vector (Fig. 3.17B); however, this ratio did not change if only 50 nt past the initiation codon was added to PUMA 5′UTR (data not shown). In addition, the hairpin was effective in inhibiting cap-dependent translation as determined by a sharp decrease in fLUC/β-gal ratio for the control vector harboring the RNA hairpin (phpRF). The presence of the hairpin, however, did not affect the expression of the vector harboring PUMA 5′UTR indicating that PUMA 5′UTR can support translation independent of the cap (Fig. 3.17C). The fact that an additional sequence from the coding region is required for IRES activity of PUMA *in vivo* also suggests the involvement of trans-acting factors *in vivo* which are absent *in vitro.* We performed RT-PCR analysis on RNA extracted from these transfected cells using Firefly luciferase specific primers (Fig. 3.17D) to exclude the possibility of a transcriptional increase as a mechanism responsible for enhanced expression of the construct harboring the putative

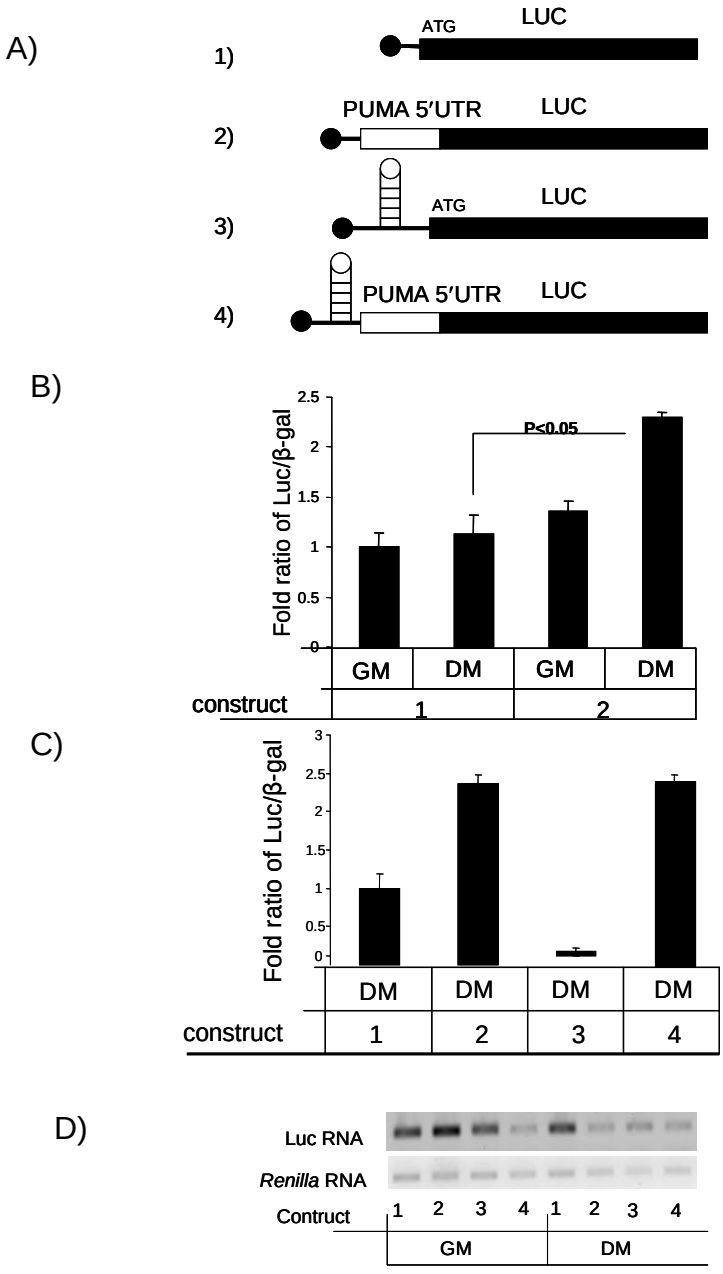


Figure 3.17 IRES-mediated translation of PUMA A) A diagrammatic representation of constructs used in this experiment.A&B) 23A2 cells were transfected independently with monocistronic constructs along with a β-gal control vector as indicated. 24 hours after transfection, cells were switched to fresh GM or DM for 3 hours. Cell extracts were prepared and luciferase activities were measured according to the manufacturer's instructions (Promega) and normalized to β-gal using a β-gal assay (Promega). D) RT-PCR analysis using RNA from 23A2 cells transfected in GM or DM with the indicated constructs and a *Renilla* expression vector. Error bars represent mean ± SEM from triplicate samples.

PUMA IRES element.

PUMA 5′-untranslated region mediates internal ribosome entry site-dependent translation *in vivo*

Since the standard test for detecting IRES activity utilizes a bicistronic vector (61), we cloned PUMA 5′UTR +100 nt of coding region from AUG into a pRF Vector. In this bicistronic vector, the *Renilla* LUC is translated from the first cistron in a cap-dependent manner whereas the Firefly LUC enzyme is translated from the second cistron. Enhanced expression of the second cistron is only achieved if the intercistronic region harbors elements with IRES activity. 23A2 cells were transfected with these bicistronic constructs separately in growth medium. Twenty four hours after transfection, 23A2 cells were switched to fresh GM or DM for 3 hours and the luciferase activity of each cistron was determined. Similar to results obtained using monocistronc constructs, the pRF harboring PUMA 5′UTR in the intercistronic region displays enhanced IRES activity as determined by fLUC/RLUC ratio which is significant compared to pRF control vector (Fig. 3.18 B&C). To distinguish reinitiation from IRES activity, we cloned the sequence representing PUMA IRES into a phpRF vector. This vector contains a RNA hairpin upstream of the *Renilla* coding region. Addition of a hairpin upstream of the *Renilla* cistron suppressed the cap dependent translation as determined by a sharp decrease in *Renilla* luciferase expression (Fig. 3.18D).

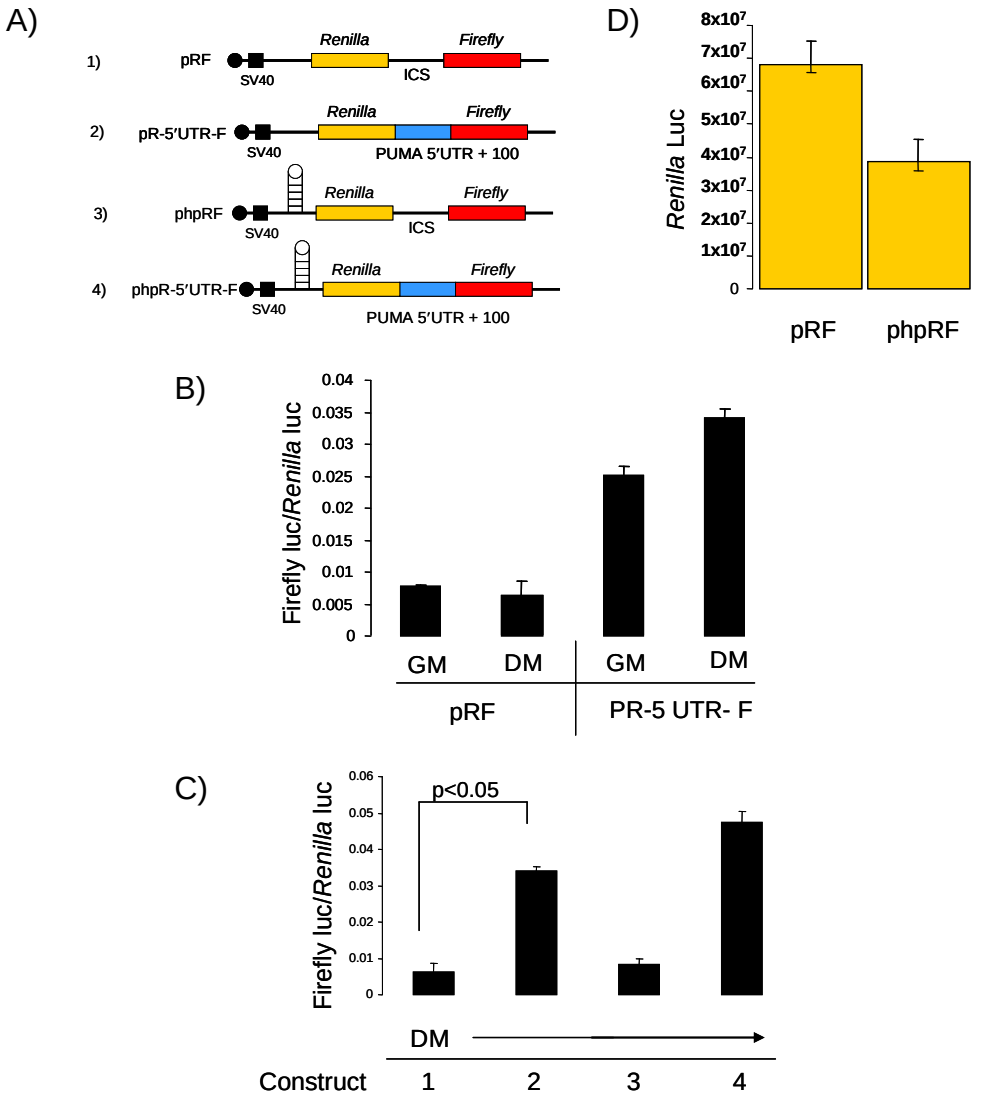


Figure 3.18 5′UTR of PUMA shows IRES activity in the absence of serum. A) Schematic representation of the constructs used in this experiment. B) 23A2 cells were transfected with the indicated vectors. 24 hours after transfection, cells were switched to fresh GM or DM for 3 hours. IRES activities were determined as a ratio of Firefly to *Renilla* activities for each transfection. C) The presence of the hairpin upstream of the Renilla cistron inhibits its cap-dependent translation in DM (phpRF) but has no effect on the translation of PUMA 5′UTR. D) *Renilla* activity is decreased in phpRF transfected cell as compared to the pRF control vector transfected cells. Error bars represent mean $\pm$ SEM from triplicate samples.

However, the hairpin did not affect Firefly luciferase expression of the bicistronic vector containing the PUMA IRES (Fig. 3.18C).

5′ UTR of PUMA does not contain elements with promoter activity

Recently, it has been demonstrated that in some cases the activity that defined the presence of an IRES in a few bicistronic mRNAs using bicistronic DNA constructs was actually due to the presence of a cryptic promoter in intercistronic elements (98). Indeed, the 5′UTR of several genes, including mouse Bad and human Sno, have been shown to possess promoter sequences (84, 98). It has been argued that the increase in expression of the second cistron could be due to production of a monocitronic mRNA transcribed from the second cistron (99, 100). To rule out the possibility of the presence of a cryptic promoter in the 5′UTR of PUMA, we generated a promoterless dicistronic vector as described in Material and Methods by removing the simian virus 40 promoter as well as the chimeric intron (Fig. 3.19A). Removal of the promoter should prevent the false positive detection of IRES activity from the intercistronic region of this bicistronic construct (101). 23A2 cells were transfected with these promoterless bicistronic constructs with or without PUMA 5′UTR cloned into their intercistronic region. No enhancement of the second cistron was observed even when the PUMA IRES element was present in the construct (Fig. 3.19). Furthermore, switching the cells to DM failed to activate the IRES element. These observations ruled out the possibility of the existence of a promoter sequence in 5′UTR of PUMA.

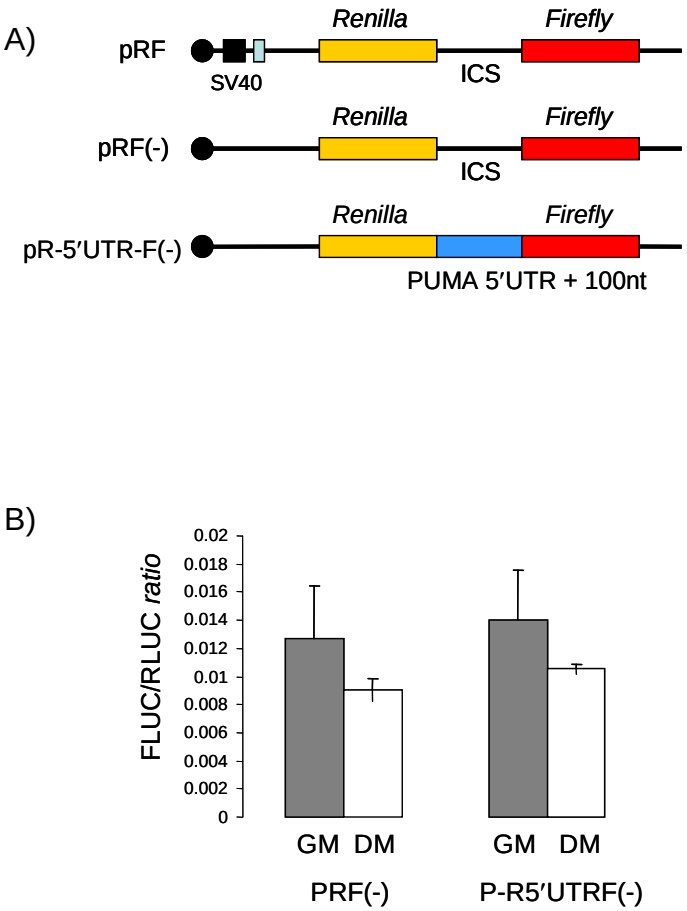


Figure 3.19 5′UTR of PUMA does not contain promoter elements. A) A diagrammatic representation of the bicitronic construct with the SV40 promoter and chimeric intron. B) 23 A2 cells were transfected with 2 μg of pRF(-) control vector in which the promoter was removed in addition to pR 5UTR F(-) vector independently. 24 hours after transfection, 23A2 cells were switched to fresh GM or DM and lucifearse activities were determined using a Dual Luciferase Assay System (Promega). Fluc activity was normalized to Rluc activity for each transfection. This is representative of two independent experiments. Error bars represent mean ± SEM from triplicate samples.

5′UTR of PUMA does not contain splicing acceptor sites

To rule out the possibility that the observed fLuc activity is due to the presence of cryptic splice sites within the bicistronic RNA which would generate monocistronic fluc RNAs *in vivo*, we performed RT-PCR analysis on bicistronic RNAs extracted from 23A2 cells in GM using three sets of primers as defined in Fig 3.20. This method was originally suggested by Van Eden (102) in assessing aberrant RNA species by splicing produced from bicistronic DNA constructs. PCR products were subjected to electrophoresis on a 1% agarose gel. Amplification of the RNAs using these three set of primers should result in single bands of 524 bp, 383 bp and 161 bp. No additional PCR products of smaller size were detected. We also performed PCR amplification on a –RT sample to exclude the possibility of genomic DNA contamination. These results provide further support that the 5′UTR of PUMA does not contain cryptic splice sites.

PUMA IRES activity is enhanced in response to etoposide treatment

Increased PUMA expression is well documented in response to DNA damaging agents. Etoposide is a DNA damaging agent that activates the signaling pathways associated with DNA repair and apoptosis (103). Etoposide-induced apoptosis is shown to be mediated through the intrinsic pathway and involves activation of p53 (104, 105). Recently, it has been reported that global reduction of translation in response to etoposide treatment coincides with increased levels of hypophosphorylated 4E-BP. This increased

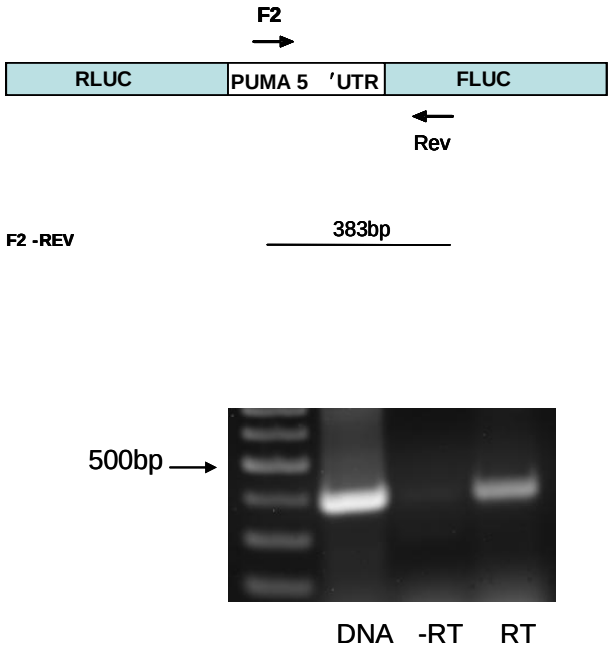


Figure 3.20 RT-PCR analysis ruled out production of any aberrant RNA species from the dicistronic construct A) Schematic representation of the location of the primers and expected size of the corresponding product. B) RT-PCR analysis of total RNA extracted from 23A2 cells transfected with a bicistronic construct harboring the PUMA IRES using the shown set of primers. DNA (bicistronic plasmid harboring PUMA 5′UTR), –RT (no reverse transcriptase) and RT (reverse transcriptase).

hypophosphorylation of 4E-BP is p53-mediated (106, 107). To assess the effect of etoposide on PUMA IRES activity, 23A2 cells were transfected with the bicistronic vector into which the PUMA IRES was cloned in the intercistronic region and the control vector independently. Twenty four hours after transfection, the cells were treated with etoposide for 8 hours in GM. Etoposide treatment of 23A2 cells significantly enhanced the IRES-mediated translation of PUMA RNA as compared to the control vector (Fig 3.21).

PUMA IRES functions in HeLa cells and 293T cells.

Sequence analysis of the 5′UTR of PUMA showed a 93% homology between mice and humans. This data prompted us to test the IRES in cell lines of human origin. HeLa cells and 293T cells were transfected with the indicated vectors separately. Twenty four hours after transfection, the expression of both Firefly and *Renilla* luciferase enzymes was determined. PUMA IRES activity is functional in Hela cells and 293T cells even in absence of treatment, unlike 23A2 cells which require culture in DM or treatment with etoposide. Furthermore, the efficiency of the IRES as determined by the ratio of the Firefly to *Renilla* was even further enhanced in the presence of a RNA hairpin (Fig. 3.22).

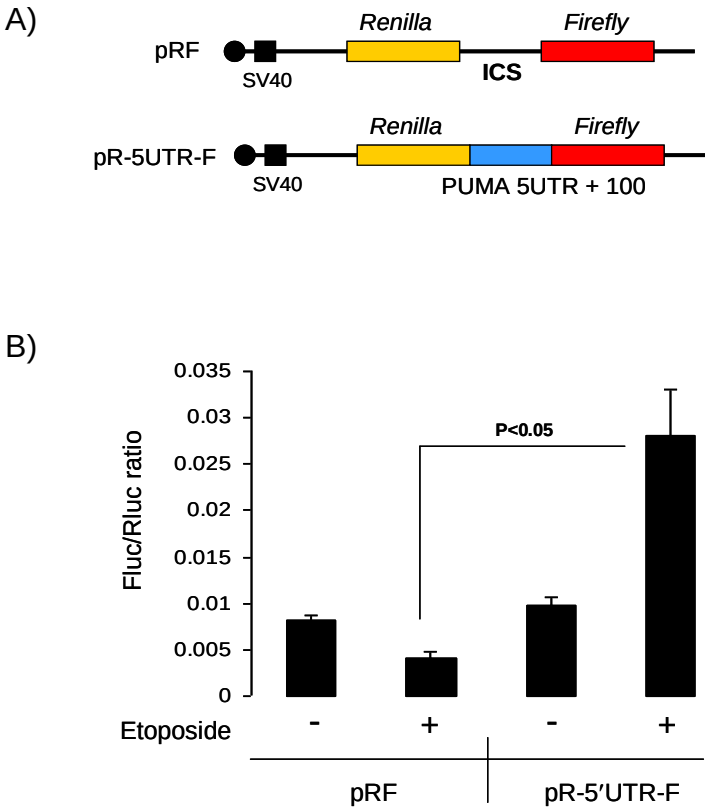


Figure 3.21 PUMA IRES activity is increased in response to etoposide. A) A diagrammatic representation of bicistronic constructs. B) 23A2 cells were transfected with the indicated vectors independently. 24 hours after transfection, cells were incubated with fresh GM in the presence or absence of 3 μM/ml of etoposide or DMSO for 8 hours. Activity was determined as a ratio of FLuc/RLuc for each transfection. This experiment was done in triplicate. Error bars represent mean ± SEM from triplicate samples.

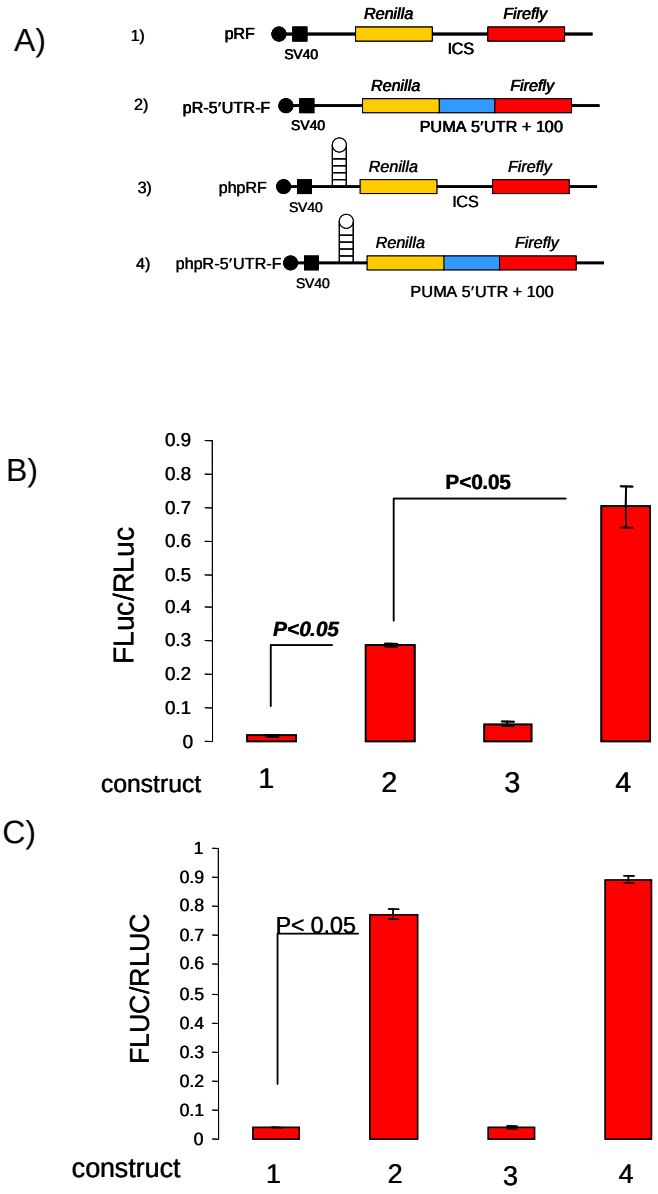


Figure 3.22 The 5′UTR of PUMA shows IRES activity in human HeLa and 293T cells. A) A diagrammatic representation of constructs used in this experiment. 5′UTR + the first 100 nt of the coding region of PUMA was cloned into the intercistronic regions of the bicistronic pRF or phpRF vectors. phpRF vector contains a hairpin upstream of the *Renilla* coding sequence. B) HeLa cells & C) 293T cells were plated at equal density in 24 well plates. The next day, cells were transfected with 2 μg of the bicistronic pRF, phpRF , pRF(5′UTR of PUMA) and phpRF(5′UTR of PUMA) vectors separately. 24 hours after transfection, both Firefly and *Renilla* luciferase activies were determined using a Dual Luciferase Assay System (Promega). Error Bar represent mean ± SEM from triplicate samples. Data shown here is representative of two independent experiments.

DISCUSSION

We have previously documented a critical role for the pro-apoptotic Bc2 family PUMA in the apoptosis that occurs in a subset of myoblasts induced to differentiate as a consequence of culture in DM (media lacking serum) (108). Others have documented a critical role for PUMA in the apoptosis that occurs in response to DNA damaging agents such as etoposide (109-111). To date, the known regulation of PUMA expression has been at the level of increased transcription in response to the activation of the transcription factor p53 in response to a number of stimuli (111-113). We report herein the novel cap-independent translation of PUMA and we have identified the IRES element in the PUMA RNA. Further, we have shown that this IRES element facilitates translation in myoblasts cultured in DM (media lacking serum) or treated with etpopside.

It is well documented from studies in other systems that culturing cells without serum leads to a decrease in total protein synthesis and inhibition of cap-dependent translation in serum starved cells can in part be responsible for this decrease (114-116). We too observe a decrease in total protein synthesis in skeletal myoblasts cultured in DM (media lacking serum). However, in skeletal myoblasts, culture in DM leads to apoptosis in a subset of cells. The onset of apoptosis is likewise associated with inhibition of global translation (86, 117). This regulation has been explained in part by caspase-dependent cleavage of a number of initiation factors involved in cap-dependent translation (86, 114, 118,

119). Since the decrease in total protein synthesis that we observe coincides with the onset of apoptosis, at this time we cannot distinguish between a decrease in protein synthesis in all cells or a decrease in protein synthesis in only those cells destined to undergo apoptosis.

Interestingly, while we initially detect a decrease in total protein synthesis with maximal inhibition after 3 hours of culture in DM, this inhibition recovers after 18 hours of culture in DM to levels of synthesis comparable to that detected in growth media (GM). At this time, there are no remaining apoptotic cells. Thus, there are at least three possible explanations for the recovery of protein synthesis. Firstly, it is possible that only the apoptotic cells underwent a decrease in total protein synthesis. Alternatively, it is possible that the entire population initially displayed a decrease in protein synthesis and that this recovered with time due to the auto secretion of insulin like growth factor I (IGF-I). An increased secretion of IGF-I during differentiation is well documented (120, 121). Finally, it is possible that a combination of these possibilities is responsible for the observed levels of total protein synthesis.

Several key molecules are known to regulate translation. Serum-induced phosphorylation of 4E-BP allows translation initiation by preventing its association with eIF4E (20, 28, 40, 60, 122-128). Using a phosphospecific antibody against 4E-BP, we demonstrated that the appearance of hypophosphorylated 4E-BP correlated with the decrease in global translation

after 3 hours of culture in DM; however, hypophosphorylated 4E-BP persisted even when global translation was recovered after 18 hours of culture in DM.

Serum-induced phosphorylation of 4E-BP is mediated by PI3K/Akt/mTOR signaling in other system (122-128)**.** Signaling by PI3 kinase is also required for skeletal myoblast differentiation. Specifically, treatment with LY294002 (the PI3K inhibitor) blocks the increase in myogenin mRNA (129). In correlation with results obtained from our studies on global translation, we have determined that PI3K signaling to Akt decreases after 3 hours in DM and recovers by 18 hours (130). Further, herein we report that mTOR activity similarly decreases after 3 hours in DM and recovers by 18 hours (unpuplished). Although the suppression of PI3K/Akt/mTOR signaling concomitant with culture in DM and the onset of apoptosis could explain the decrease in 4E-BP phosphorylation and resulting decrease in total protein synthesis, the persistent hypophosphorylation status of 4E-BP in DM could not be explained by the recovery of PI3K/Akt/mTOR signaling.

eIF2 is another initiation factor whose phosphorylation is known to impair translation initiation (44, 131). Our kinetic analysis of eIF2α phosphorylation demonstrated that the phosphorylation status of eIF2α correlates with the decrease and recovery of global translation. In our attempt to identify the kinase responsible for eIF2α phosphorylation, we showed that PKR cleavage, indicative of its activation (50, 118), is detected in 23A2 cells as a consequence of culture

in DM. Although these data suggest that caspase-mediated activation of PKR and subsequent phosphorylation of eIF2α in 23A2 cells might be responsible for the inhibition of total protein synthesis during apoptosis, future studies using caspase inhibitors are necessary to confirm that PKR activation and eIF2α phosphorylation are indeed capspase-mediated. Nonetheless, even if caspase-mediated cleavage and activation of PKR is confirmed to mediate the phosphorylation of eIF2α, the mechanism responsible for the decrease in phosphorylation of eIF2α and the recovery of global translation remains to be determined since cleaved PKR persists. Interestingly, recent reports suggest the involvement of mTOR in eIF2α phosphorylation. It has also been shown that GCN2 phosphorylates eIF2α in yeast in a rapamycin-depdent manner (132). Therefore, future work is necessary to determine the extent of GCN2 activation as a consequence of culture in DM and to explore the role of GCN2 in the phosphorylation of eIF2α.

To our knowledge, we are the first to report the IRES mediated translation of a BH3-only proapoptotic Bcl2 family member. Nonetheless, IRES activity has been discovered in several other molecules involved in apoptosis such as such as XIAP (133), Apaf-1 (134) and cMyc (135). However, the activation of these IRESs during apoptosis is specific to the apoptotic stimuli and the cell type (64). For instance, it was shown that Apaf-1 translation, which is constitutively IRES-mediated, was only enhanced in cell lines of neural origin or in response to genotoxic stress (136). This differential activity of IRES elements with respect to

cell type and stimuli are attributed to the presence of cell specific *trans-acting* factors (ITAFs), which in turn are subject to regulation by internal or external stimuli (73).

Thus, the requirement of an additional 50 nucleotides downstream of the AUG for PUMA IRES activity *in vivo* implies that there are cell-specific trans-acting factors that can regulate the activity of this IRES (136). We believe that tissue specific ITAF expression can also explain our result of enhanced PUMA IRES activity in HeLa and 293 T cells even in the presence of serum. PTB is an ITAF that is known to be associated with many cellular IRES-containing mRNAs during apoptosis. PTB is highly expressed in HeLa cells as compared to other cell type (137). Future studies are necessary to identify the minimal IRES element in PUMA mRNA to identify the ITAFs associated with this PUMA IRES and to investigate their possible regulation in response to culture in DM or treatment with etoposide.

Although our analysis indicates that PUMA translation can occur under compromised conditions where cap-dependent translation is inhibited, we cannot at this point speculate whether PUMA translation utilizes both cap-dependent and IRES-mediated translation during apoptosis or if PUMA translation is solely IRES mediated under these conditions.

In other cell types, etoposide treatment results in the activation of the transcription factor p53 (138). Translational regulation in response to etoposide in other cell types has been reported and involves an increased level of hypophosphorylated 4E-BP. It has been suggested that p53-mediated pathway might be responsible for modulation of 4E-BP phosphorylation in response to etoposide (107, 139); however, the exact mechanism is not understood. We have previously shown, however, that p53 does not play a role in skeletal myoblast apoptosis in response to serum deprivation (108). Furthermore, we have determined a role for MyoD in the increase of PUMA expression in response to etoposide (T. Harford, unpublished). These data allow us to speculate that MyoD might be responsible for the increase in the PUMA IRES activity in response to culture in DM or treatment with etoposide. It is unlikely that a role for MyoD in the regulation of translation involves its ability to function as a transcription factor since we have shown that increased translation of PUMA is detected in the presence of actinomycin D. Several p53 mediated events are independent of its transcriptional activity (140). Future studies are required to determine the role of MyoD in this process.

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VI. SUMMARY

Programmed cell death (apoptosis) is induced concomitant with the induction of differentiation in skeletal myoblasts (1, 2). Skeletal myoblast transplantation is a potential treatment for muscular dystrophies (3), acute myocardial infarction (4) and chronic end-stage heart dystrophies (5). Transplanted myoblast survival rates remain low despite great advances in stem cell therapy (3). Thus, identifying therapeutic targets for manipulation to block apoptosis associated with differentiation might improve efficacy of myoblast transfer. Herein we report a functional role for increased PUMA expression in the release of cytochrome C and apoptosis in skeletal myoblasts. By modulation of PUMA expression we showed, for the first time, that we can successfully abrogate apoptosis without affecting differentiation. These findings could be significant in the utilization of PUMA as a therapeutic target in the effort to suppress myoblast apoptosis and increase the efficacy of myoblast transfer. Therefore, the assessment of survivability of PUMA -/- satellite cells in myoblast transfer should be considered. We speculate that myoblasts derived from satellite cells deficient for PUMA may improve the regenerative potential of myoblast stem cells for the purpose of the cell therapy.

PUMA is involved in mitochondrial mediated apoptosis in response to a vast number of stimuli in a p53-dependent and independent manner (6). While exogenous PUMA expression induces apoptosis in a number of human cancer cells (7, 8), its deletion has been shown to abrogate apoptosis in response to hypoxia, p53 signaling and DNA damaging agents (9). Impairment of apoptosis renders cancer cells resistant to chemotherapy and radiation therapy. In many cases this resistance is attributed to the p53 status of the tumors (10). Modulation of PUMA expression has been shown to restore the therapeutic response of cancer cells to anticancer agents, particularly when combined with DNA-damaging agents (11). Our findings suggest that PUMA is translated in a cap-independent manner using an IRES element in its 5′UTR. We showed that PUMA is synthesized under compromised conditions where total protein synthesis is inhibited. Exploring the molecular mechanism(s) responsible for this IRES-mediated translation will identify additional therapeutic targets. We believe that this selective translation as well as tissue specific expression of translational factors will allow more precise manipulation of PUMA in anticancer therapy regardless of p53 status.

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