RESEARCH PAPER

Translation 1:1, e24391; April/May/June 2013; © 2013 Landes Bioscience

# IRES-mediated translation of the pro-apoptotic Bcl2 family member PUMA

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**Keywords:** Cap-independent translation, PUMA, eIF2α, eIF4E-BP, IRES, skeletal myoblasts differentiation, apoptosis

The proapoptotic Bcl-2 family member PUMA is a critical regulator of apoptosis. We have previously shown that PUMA plays a pivotal role in the apoptosis associated with skeletal myoblast differentiation and that a MyoD-dependent mechanism is responsible for the increased expression of PUMA in these cells. Herein, we report that the increased expression of PUMA under these conditions involves regulation at the level of translation. Specifically, we have found that the increase in PUMA protein levels occurs under conditions of decreased total protein synthesis, eIF2-α phosphorylation and hypophosphorylation of eIF4E-BP, suggesting that PUMA translation is proceeding via an alternative initiation mechanism. Polyribosome analysis of PUMA mRNA further corroborated this suggestion. A combination of in vitro and ex vivo (cellular) approaches has provided evidence suggesting that PUMA mRNA 5'UTR harbors an Internal Ribosome Entry Site (IRES) element. Using mono- and bi-cistronic reporter constructs, we have delineated an mRNA fragment that allows for cap-independent translation in vitro and ex vivo (in skeletal myoblasts) in response to culture in differentiation media (DM), or in response to treatment with the DNA-damaging agent, etoposide. This mRNA fragment also supports translation in HeLa and 293T cells. Thus, our data has revealed a novel IRES-mediated regulation of PUMA expression in several cell types and in response to several stimuli. These findings contribute to our understanding and potential manipulation of any developmental or therapeutic scenario involving PUMA.

## Introduction

Differentiation and apoptosis are coordinately regulated in a variety of cell types.[1,2] In some cell types like skeletal myoblasts, apoptosis and differentiation are mutually exclusive biological endpoints.[3-5] During myogenesis and regeneration, apoptosis likely serves to eliminate myoblasts generated in excess.[6,7] However, such coordinated regulation of apoptosis and differentiation would likely decrease the efficacy of myoblast transfer to treat a variety of diseases. Identification of molecules involved in the apoptotic process, but not the differentiation process, could enable selective manipulation relevant to the regenerative potential of adult muscle stem cells and to the effectiveness of any treatment utilizing skeletal myoblasts.[8-10]

Studies in established myoblast cell lines have provided detailed information regarding the molecular regulation of differentiation. In vivo, myoblasts are maintained in a proliferative, undifferentiated state by certain mitogens. Cell cycle exit and differentiation are induced in response to decreasing gradients of these mitogens as a function of appropriate myoblast migration. Thus, ex vivo differentiation of myoblasts is induced by culture in differentiation media with reduced serum (DM).[4-7] We[3] and others[5,11,12] have reported that when induced to differentiate by culture in DM, roughly 30% of myoblasts undergo apoptosis rather than differentiation. Compared with the abundance of molecular information available with respect to skeletal myoblast specification and differentiation, the molecular mechanisms controlling the apoptosis associated with skeletal myoblast differentiation is sparce.[3-5,10-17] The necessity for bifurcated signaling pathways and distinct signaling molecules is suggested by our findings that, in skeletal myoblasts, differentiation and apoptosis in response to culture in DM are separable events.[3] In search of a molecule distinct to the process of apoptosis in skeletal myoblasts, we have identified the pro-apoptotic protein PUMA. Specifically, we have reported that PUMA plays a pivotal role in the apoptosis, but not in the differentiation induced by culture in DM.[18] During the course of these studies, we discovered that the expression of PUMA involves regulation at the level of translation.

Herein, we report the identification of an IRES element in PUMA mRNA that supports cap-independent translation in murine skeletal myoblasts and is responsive to conditions created by culture in DM. Our findings invite further investigation of this PUMA IRES that could identify additional molecules for therapeutic manipulation relevant to the regenerative potential of adult muscle stem cells and to the effectiveness of any treatment utilizing skeletal myoblast transfer. Further, as we report that this

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Submitted: 01/08/13; Revised: 03/06/13; Accepted: 03/20/13
Shaltouki A, Harford TJ, Komar AA, Weyman CM. IRES-mediated translation of the pro-apoptotic Bcl2 family member PUMA. Translation 2013; 1:e24391; http://dx.doi.org/10.4161/trla.24391

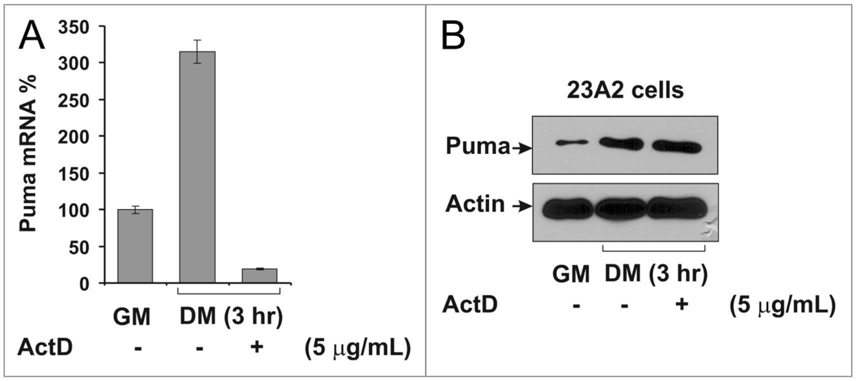


**Figure 1.** Actinomycin D treatment blocks the increase in PUMA mRNA but not protein in response to culture in differentiation media (DM). Equal cell numbers were plated and the next day incubated in growth media (GM), DM or DM supplemented with actinomycin D (5 μg/mL) for three hours. In (**A**) total RNA was prepared using 1 mL of Trizol reagent (Invitrogen) per 100 mm plate for lysis and following the manufacturer's instructions. Five hundred ng of RNA was then used for a 20 μL SuperScript III RT (Invitrogen) reverse transcription reaction. Quantitative PCR was then performed for PUMA as described in "Materials and Methods." Shown is an average of 3 experiments (mean +/- SEM). In (**B**), whole cell extracts were prepared and 100 μg of total protein was separated by SDS-PAGE. Western analysis was performed using anti-Puma or anti-actin (load control) antibodies as described in Materials and Methods. Shown are results from one experiment that are representative of three independent experiments.

PUMA IRES supports translation in several other cell types of human origin and in response to conditions created by a more traditional and widely applicable apoptotic agent, etoposide, these findings could have implications in other developmental and therapeutic scenarios involving PUMA.

## Results

**Enhanced translation plays a role in the increased expression of PUMA in skeletal myoblasts cultured in differentiation media.** We have previously reported that culture of 23A2 myoblasts in DM for three hours is sufficient to increase PUMA protein levels.[18] However, the molecular mechanisms contributing to such an increase remained unknown. We have found that both mRNA and PUMA protein levels increase under these conditions, suggesting that regulation of PUMA expression may occur at either the transcriptional and/or post-transcriptional levels, or both. During the course of our studies to document the importance of increased PUMA expression to the apoptosis that is initiated in a subset of myoblasts cultured in DM, we were prompted to explore both possibilities. To begin our exploration, we first assessed the ability of actinomycin D (a commonly used inhibitor of transcription) to alter the expression of PUMA in skeletal myoblasts following three hours of culture in DM. As expected, actinomycin D treatment prevented the increase in PUMA mRNA as a consequence of culture in DM (**Fig. 1A**). However, even under reduced levels of PUMA mRNA, PUMA protein levels were still increased (**Fig. 1A and B**), therefore suggesting post-transcriptional regulation. We also note that the increase in PUMA protein levels occurred during the global decrease (more than 2-fold) of total protein synthesis caused by culture in DM (**Fig. 2A**). This decrease in total protein synthesis was accompanied by increased phosphorylation of eIF2α (**Fig. 2B**) and hypophosphorylation of eIF4E-BP (**Fig. 2C**). Hypophosphorylated 4E-BP isoforms, such as 4E-BP phosphorylated on Thr37 and Thr46, interact strongly with eIF4E, thereby decreasing the efficiency of cap-dependent initiation.[19,20] Increased phosphorylation of eIF2α, likewise is known to repress global protein synthesis.[21,22] However, metabolic labeling of the cells followed by PUMA immunoprecipitation showed that, even in the presence of actinomycin D, de novo PUMA protein synthesis continues in DM (**Fig. 2D**). Morevover, we also show that rapamycin treatment inhibits mTOR and, in particular, also leads to dephosphorylation of 4E-BP, causing its increased association with eIF4E and general decrease in protein synthesis[23] does not affect the expression of PUMA in DM, while it decreases the expression of myogenin (**Fig. 2E**). Myogenin is a muscle-specific transcription factor that is induced during differentiation, a member of the MyoD family of transcription factors, which are involved in the coordination of skeletal muscle development and repair.[24] Taken together these data strongly suggest that translation of PUMA mRNA in DM is likely to proceed via an alternative initiation pathway.

We next performed comparative ribosomal profiling analysis to further assess the consequences of cell culture in GM relative to DM on PUMA translation. Attenuation of global translation after three hours of culture in DM compared with GM is indicated by the reduction in the polyribosomal fraction and the increase in 80S ribosomes (**Fig. 3A**). However, association of PUMA mRNA with polyribosomes increased in DM (**Fig. 3B**, upper panel). As a control, we also measured the association of GAPDH mRNA with polyribosomes in cells cultured in GM compared with DM. In contrast to the results obtained for PUMA mRNA, the association of GAPDH mRNA drastically decreased as a consequence of culture in DM and inhibition of general protein synthesis (**Fig. 3B**, bottom panel).

**IRES mediated translation of PUMA mRNA.** Results reported above suggest alternative initiation of translation of PUMA and the potential presence of an internal ribosome entry site (IRES). We also note that the 261 bp PUMA 5'UTR is extremely GC rich (66% compared with mean 46.1% in human genome[25]) and may form a highly stable structure with predicted (by mFold) ΔG value of -91.74 kcal/mol, which would be inhibitory to the conventional scanning mechanism employed by cap-dependent translation.[24] Structures with ΔG value of about -60–80 kcal/mol were shown to inhibit ribosome scanning in mammalian systems almost completely.[27] Moreover, we also note that PUMA 5'UTR contains 2 upstream out-of-frame AUGs. The first one starts 23 nucleotides downstream of the 5' mRNA end and may potentially encode a ~10 kDa protein product.

Therefore, to further assess potential cap-independent translation of PUMA mRNA, the effect of a competing cap analog on the in vitro translation of PUMA was measured. While increasing concentrations of a cap analog inhibited translation of the control CAT mRNA, almost no effect on the translation of capped and polydenylated PUMA mRNA can be detected (**Fig. 4A**).

To further explore the possibility that PUMA translation stems from internal initiation we performed a series of in vitro translation experiments utilizing a conventional CAT-LUC bicistronic construct that is widely used to assess the translation of both viral and cellular IRES elements.[26] We found that PUMA 5'UTR is able to support the translation of the second cistron and that the translation of the second cistron is elevated, when additional sequences downstream of the AUG PUMA start codon have been included (**Fig. 4B**). Several viral, such as, for example, Giardiavirus and human immunodeficiency virus type 2 IRESes,[29,30] as well as cellular (e.g., URE2 and p53) IRESes[31-33] were shown to require sequences downstream of the AUG start codon to modulate levels of internal initiation. Therefore, we chose to use the so-called 5'UTR+50 nt mRNA element in the subsequent ex vivo studies. We note, however, that further experiments would be required that will allow the dissection of the minimal PUMA IRES element sufficient and necessary to support PUMA translation.

To further probe for the existence of an IRES in the PUMA mRNA and measure for its efficiency *ex vivo,* we produced a number of reporter monocistronic and dicistronic DNA constructs depicted in **Figures 5A and B** and transfected them into various cell lines. We found that the PUMA 5'UTR+50 nt element supports enhanced expression of the reporter luciferase construct in 23A2 cells in DM, (**Fig. 5A**; compare expression from constructs 1 and 2) and that a stable hairpin-structure, known to abrogate cap-dependent initiation almost completely[34] (**Fig. 5A**, construct 3) is not able to inhibit the expression driven by the PUMA 5'UTR+50 nt element (**Fig. 5A**; compare expression from constructs 3 and 4). These data strongly indicate that the expression driven by PUMA 5'UTR+50 nt element proceeds in vivo in 23A2 cells in DM via an IRES. Please note that a β-galactosidase reporter construct has been used to normalize transfection efficiencies.

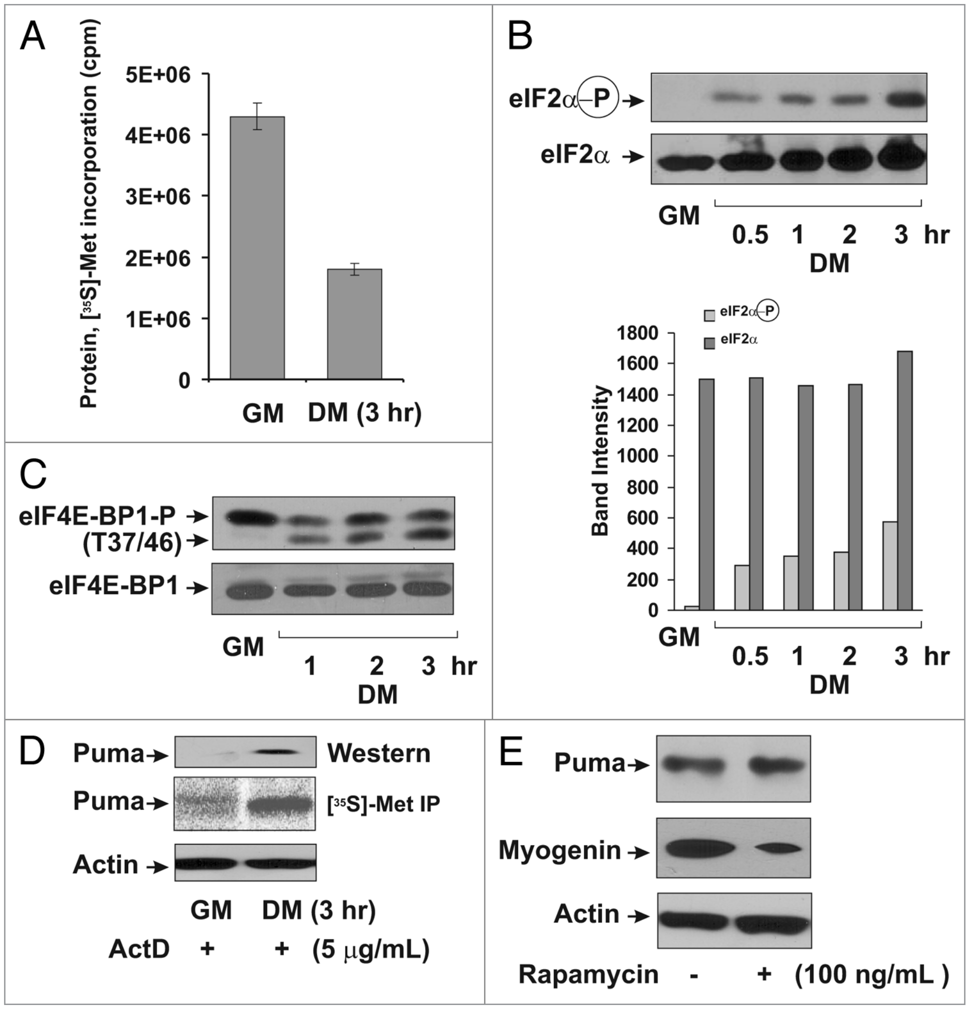


**Figure 2.** Culture in differentiation media leads to inhibition of total protein synthesis, eIF2-α phosphorylation and hypo-phosphorylation of 4E-BP, but does not prevent de novo PUMA protein synthesis. (**A**) Cells were plated at equal density and the next day incubated in cysteine- methionine-free media for 30 min, followed by incubation with 250 μCi/ml of [$^{35}$S]-methionine in GM or DM. Total protein synthesis was assessed as described in Materials and Methods. Shown are results from one experiment that are representative of two independent experiments. Error bars represent the mean +/- SEM of triplicate samples. In (**B** and **C**), cells were plated at equal density and the next day cultured in fresh GM or DM as indicated. Whole cell extracts were prepared and 100 μg of total protein was separated by SDS-PAGE. Western analysis was performed using anti-phospho eIF2α (**B**), anti-phospho 4EBP (**C**), or anti-actin (load control) antibodies as described in Materials and Methods. Shown are results from one experiment that are representative of three independent experiments. (**D**) De novo PUMA synthesis. As in (**A**) 23A2 cells were labeled with [$^{35}$S]-methionine in GM or DM additionally supplemented with Actinomycin D. PUMA protein was immunoprecipitated as described in "Materials and Methods" and resolved on 12% SDS-PAGE gel. Top panel- western blotting with anti-PUMA antibodies, Middle panel - [$^{35}$S]-labeled PUMA protein. Bottom panel–western blotting with anti-actin antibodies. (**E**) Cells were plated at equal density and the next day cultured in DM with or without rapamycin as indicated. Whole cell extracts were prepared and 100 μg of total protein was separated by SDS-PAGE. Western analysis was performed using anti-PUMA, anti-myogenin, or anti-actin (load control) antibodies as described in Materials and Methods. Shown are results from one experiment that are representative of two independent experiments.

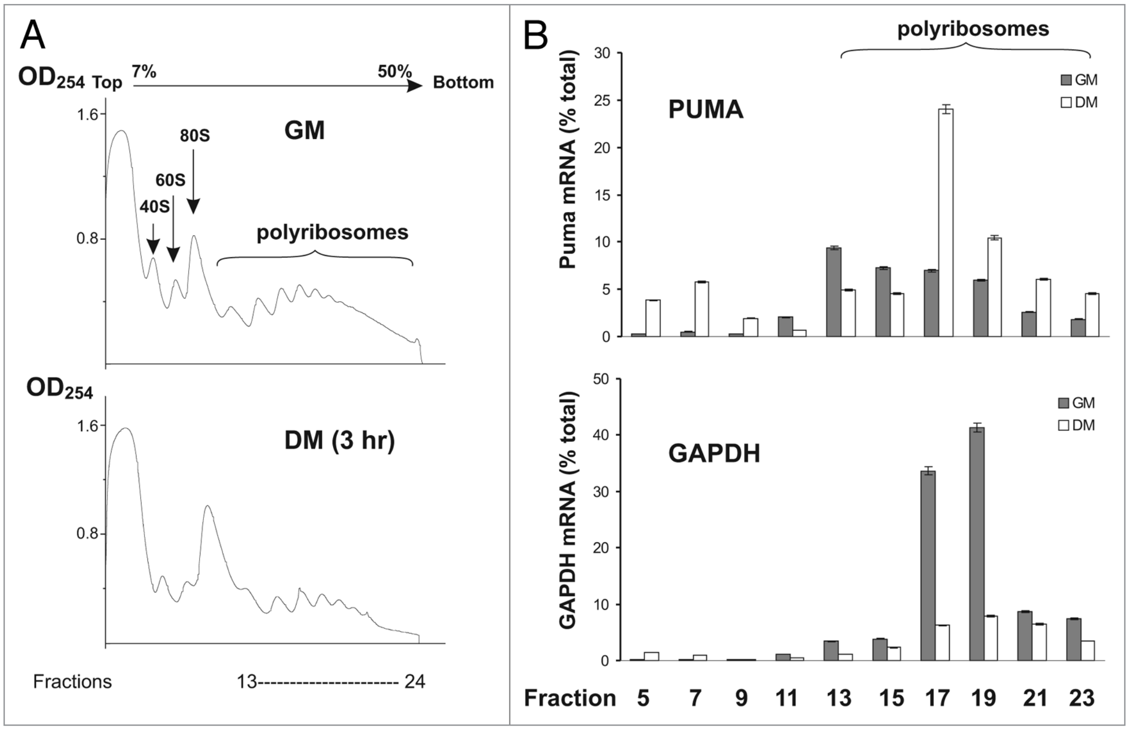


**Figure 3.** Ribosome profiles of 23A2 cells cultured in GM or DM and the expression of PUMA mRNA. (**A**) Extracts from 23A2 cells cultured in GM (top panel) or DM (bottom panel) respectively were resolved by velocity sedimentation on 7–50% sucrose gradients. Fractions were collected while scanning at A254. The positions of different ribosomal species are indicated. The data were recorded by the PeakTrak program (ISCO gradient density gradient fractionation system). (**B**) RT-PCR analysis of PUMA and GAPDH mRNA levels in polyribosomal fractions. (**C**) Cumulative relative abundance of PUMA and GAPDH mRNAs in polyribosomal fractions.

We also tested the PUMA 5'UTR+50 nt element in a bicistronic R-Luc-F-Luc pRF construct[35] (**Fig. 5B**, construct 1): a substantial increase in the expression of the second cistron was detected in 23A2 cell in DM (**Fig. 5B**; compare expression from constructs 1 and 3), when compared with cells transformed with the bicistronic construct devoid of PUMA 5'UTR+50 nt element. Similarly to the results obtained with the monocistronic construct, a stable hairpin-structure introduced in the 5'UTR of the bicistronic reporter[35] was not able to abrogate the expression of the second cistron driven by 5'UTR+50 nt element (**Fig. 5B**; compare expression from constructs 2 and 4). We note, however, that the putative PUMA IRES element is moderately active in GM and that a switch from GM to DM enhances its expression (**Fig. 5B** bottom panel, compare expression from constructs 1 and 3 in DM relative to GM). We have also found that the putative PUMA IRES element was active in HeLa and 293T cells (**Fig. 6**).

RT-PCR analysis of RNA from 23A2-transfected cells suggests that DM conditions do not induce alternative RNA transcription (**Fig. 7A**, right panel). The RT-PCR products had the expected sizes, and similar amounts were detected when three different forward primers were used, suggesting that alternative splicing or deletions do not occur in the intercistronic region. Also, DNase I pretreatment and PCR in the absence of RT proved that the RT-PCR products were derived from the mRNA and not from the plasmid DNA (**Fig. 7A**). When cells were transfected with the ancestor pRF plasmid, no products were obtained with F2 and F3 primers as expected (**Fig. 7A**, left panel). These data suggest that the integrity of Rluc-PUMA-Fluc transcripts is preserved ex vivo and, therefore, rule out the possibility of spurious splicing. Furthermore, the use of a promoterless construct (**Fig. 7B**) clearly indicates that the bicistronic construct utilized does not contain cryptic promoters. We conclude from the above data that PUMA mRNA possesses an IRES which allows for cap-independent translation initiation of PUMA protein. Having shown that this IRES is responsive to conditions created by culture in DM, when compared with GM, we next explored whether this IRES is responsive to conditions created by treatment with a traditional and widely applicable apoptotic agent, etoposide. We found that the apoptosis induction by etoposide (the topoisomerase II inhibitor) also leads to induction of the PUMA IRES (**Fig. 8**).

## Discussion

While the debate continues over whether expression of some cellular proteins/mRNAs can be explained by a true IRES-mediated translation[28,36,37] and/or alternative mechanisms, such as, for example, ribosome tethering and clustering[36] and/or cap-assisted internal initiation,[37] and/or even cap- and IRES-independent scanning mechanism[38] it is evident that a number of cellular mRNAs exist that do not follow the standard scheme of cap-dependent translation initiation[37] and that the regulation of their activity during nutritional stress, differentiation, and mitosis represent an important fine-tuning cellular mechanism controlling cell fate.[28,36] We and others suggested a number of criteria that may

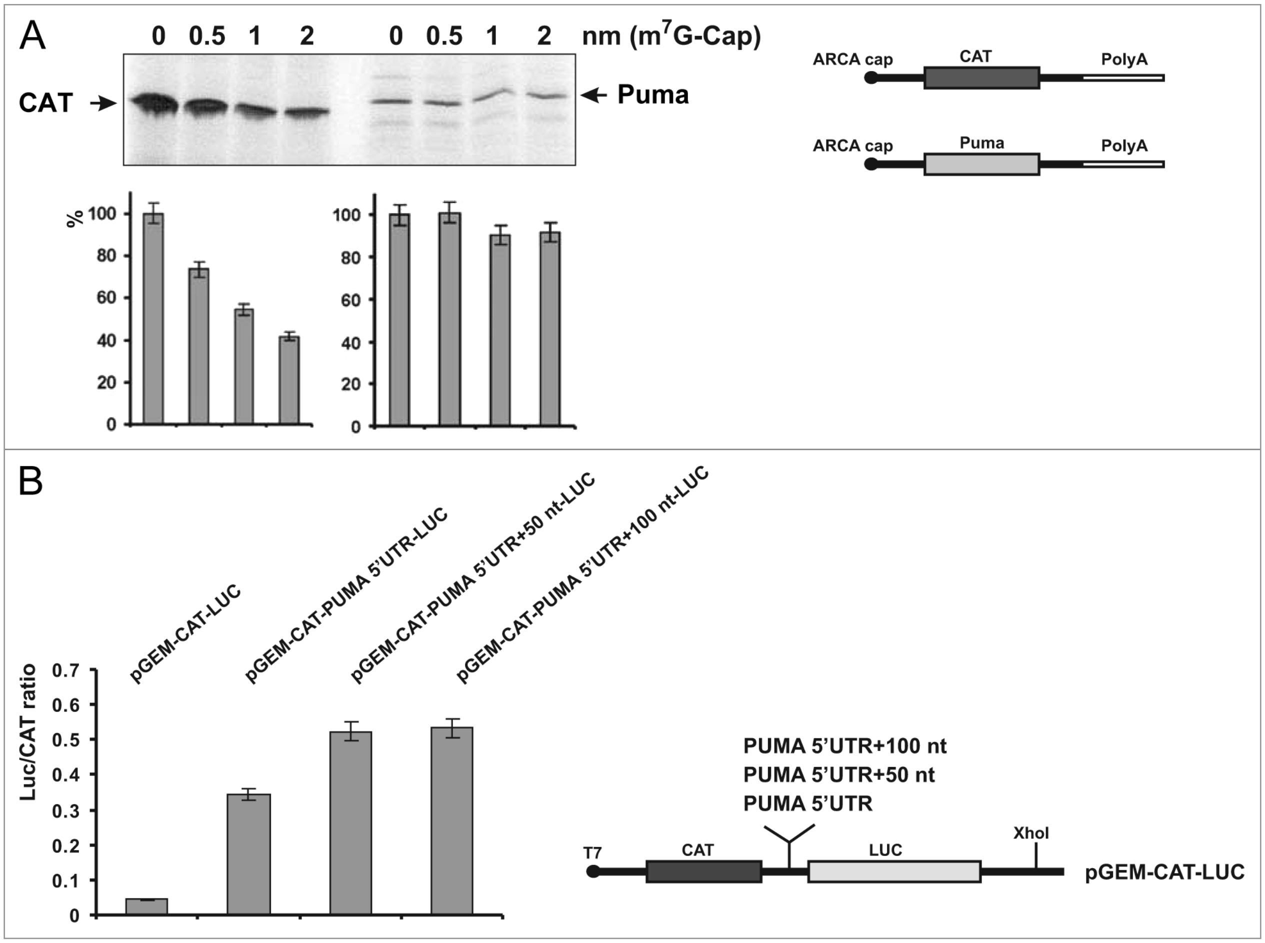


**Figure 4.** Cap-independent translation of PUMA mRNA in a cell-free system. To generate mRNA for use in cell-free translation assays, the plasmids were linearized with XhoI and transcribed in vitro with T7 polymerase. (**A**) Capped and polyadenylated chloramphenicol acetyl transferase (CAT) and PUMA mRNAs were subjected to translation in a Rabbit Reticulocyte Lysate cell-free system in the absence/presence of the increasing concentrations of the cap-analog ($m^7$GpppG, Ambion). Shown are results from one representative experiment. On the right - schematic diagram of the monocistronic pGEM-CAT and/or pGEM-PUMA plasmid constructs. Bottom - Quantitation of the translation products using a Typhoon 9410 imaging scanner. The mean value obtained from three independent sets of measurements is shown. Error bars indicate standard deviation. In each experiment values in the absence of $m^7$GpppG were set to 100%. (**B**) Relative translation efficiencies of CAT (cap-dependent initiation) and firefly luciferase (Luc) (PUMA IRES-dependent internal initiation) produced from CAT-PUMA-LUC mRNAs. On the right - Schematic diagrams of the bicistronic pGEM-CAT-PUMA-LUC plasmid constructs. Three different constructs containing different PUMA mRNA insertions were generated (see Material and Methods). The mean value obtained from four independent sets of measurements is shown. Error bars indicate standard deviation.

allow the determination and validation of a presence of an IRES element in a given mRNA.[37] These criteria include 1) the use of bicistronic test (in vivo and in vitro), 2) the use of monocistronic reporter mRNA containing hairpin structure in the 5' UTR to prevent scanning, 3) analysis of polyribosomal abundance of the endogenous IRES-containing mRNA, tested under normal conditions and conditions favoring IRES activity (i.e., under conditions of inhibition of cap-dependent translation) iv) verification of RNA integrity and the absence of cryptic promoters.

Our data strongly indicate that PUMA mRNA (its 5'UTR) passes all these tests and thus most likely contains a true IRES-element allowing for internal initiation in skeletal myoblasts in response to culture in differentiation media or in response to the DNA damaging agent, etoposde. Our data also show PUMA IRES is moderately active in GM and that a switch from GM to DM enhances PUMA IRES activity. Future experiments should, however, answer the question of whether activation of a PUMA IRES in DM requires any ITAFs. It remains to be also explored whether the mechanism supporting IRES-mediated translation of PUMA in skeletal myoblasts in response to culture in DM is similar to the mechanism initiated by culture with etoposide.

Culture of skeletal myoblasts in DM primarily elicits differentiation since apoptosis is induced in only roughly 30% of the cells.[3] IRES mediated translation has previously been reported to contribute to the increased expression of AMAP1 during the TPA-induced differentiation of monocytes,[39] to the increased expression of PDGF2 during the TPA-induced differentiation of megakaryocytes[40] and to the increased expression of FGF1 during the DM induced differentiation of C2C12 skeletal myoblasts.[41] Further, IRES-mediated translation for both FGF1 and utrophin A has been detected during muscle regeneration in response to cardiotoxin-induced damage and in differentiated myotubes in

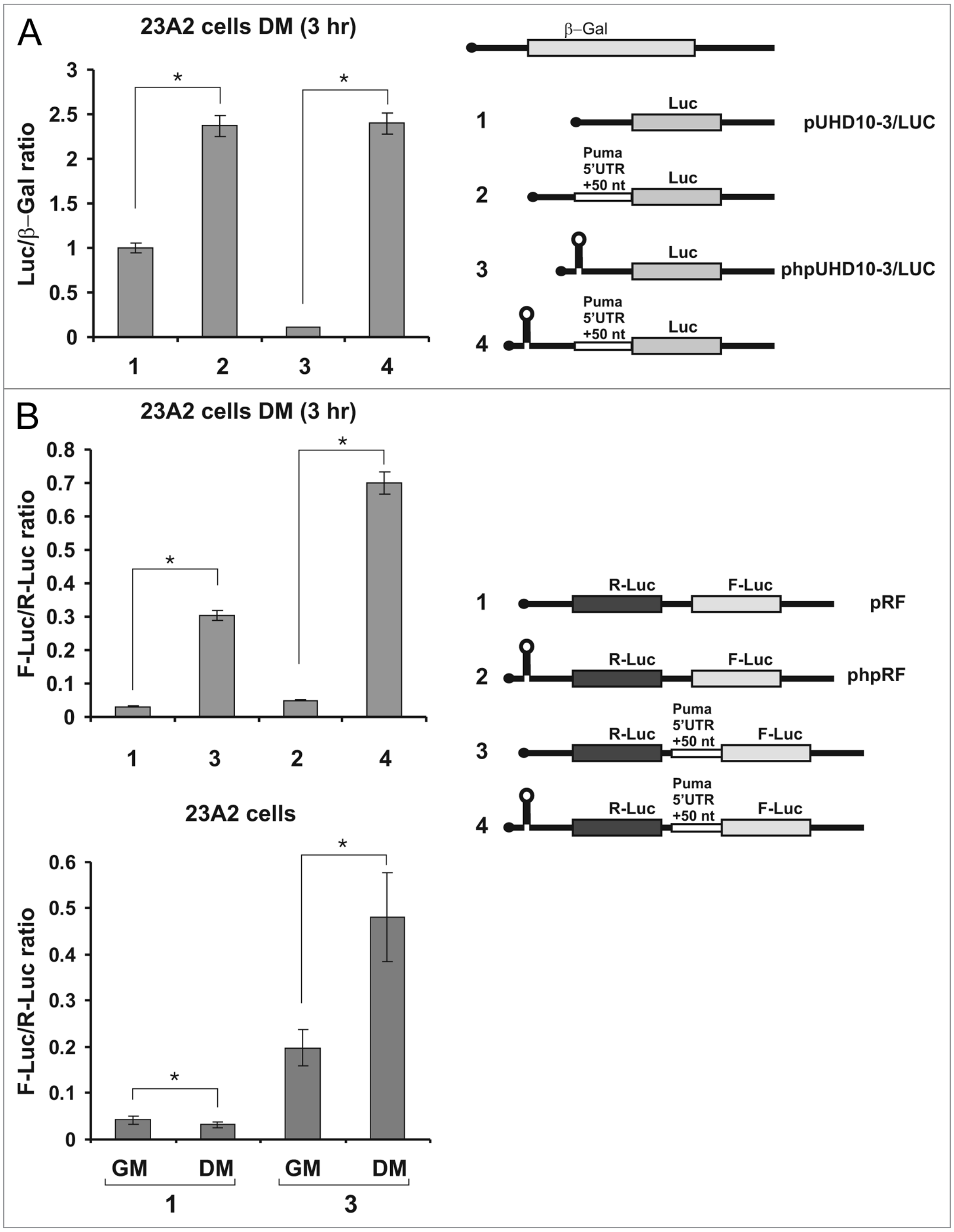


**Figure 5.** Cap-independent translation of PUMA mRNA ex vivo in 23A2 cells. (**A**) Expression of monocistronic reporter constructs in 23A2 cells. Equal cell numbers were plated and the next day co-transfected with the indicated monocistronic luciferase construct and a construct containing β-gal, to control for transfection efficiency, as described in "Materials and Methods." The next day, after culture in DM for 3 h, cultures were processed for analysis of luciferase and β-gal activity. On the right - schematic diagram of the monocistronic plasmid constructs. (**B**) Expression of bicistronic reporter constructs in 23A2 cells. Equal cell numbers were plated and the next day transfected with the indicated bicistronic luciferase constructs. The next day, after culture in fresh GM or DM for 3 h as indicated, cultures were processed for analysis of luciferase. Relative translation efficiencies of Renilla luciferase (RLuc) (cap-dependent initiation) and firefly luciferase (FLuc) are shown. On the right - Schematic diagrams of the bicistronic pRLuc-FLuc plasmid constructs. Error bars represent the mean +/- SEM of triplicate samples and * indicated $p < 0.05$.

response to glucocorticoid treatrment.[41-43] Whether the PUMA IRES is responsive to cardiotoxin-induced damage or glucocorticoid treatment awaits further investigation.

While previous reports have described IRES elements in several key anti-apoptotic molecules such as cIAP1, XIAP, Bcl2, Bcl-XL (for a review see refs. 45, 46), our results document an IRES in the pro-apoptotic molecule PUMA that supports translation initiation under conditions when cap-dependent translation is severely compromised. It is well documented that cap-dependent protein synthesis is greatly reduced under a variety of conditions such as starvation for growth factors/nutrients, hypoxia, endoplasmic reticulum stress and many others (for a review see refs. 28, 36). Rapid inhibition of protein synthesis under these conditions is believed to function as a protective homeostatic mechanism.[28,36,45,46] It should be noted however that only transient cellular stress favors expression from IRES elements that help cells to cope with these conditions (this include key anti-apoptotic molecules cIAP1, XIAP, Bcl2, Bcl-XL), while severe stress conditions are believed to result in activation of "pro-apoptotic" IRES elements [such as e.g., found in Apaf-1 and DAP5 mRNAs (for a review see refs. 45, 46)]. Obviously, the IRES element in the PUMA mRNA belongs to the latter cohort. The finality of the apoptotic process necessitates that the expression and/or activation of pro-apoptotic molecules be tightly controlled.[45,46] Our discovery that PUMA is regulated at the level of translation by an IRES, in addition to its well known regulation at the level of transcription, is further documentation of the layers of control surrounding key pro-apoptotic molecules.

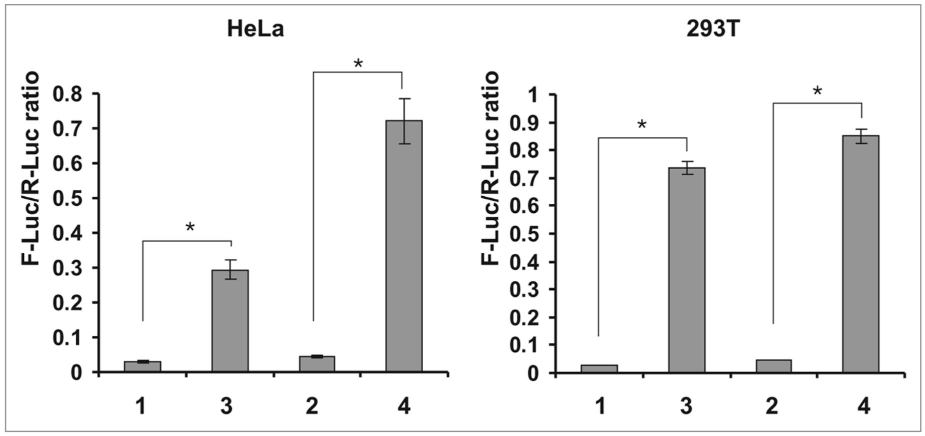


**Figure 6.** Cap-independent translation of PUMA mRNA ex vivo in HeLa and 293T cells. Equal cell numbers were plated and the next day transfected with the indicated bicistronic luciferase constructs (Schematic diagrams shown in **Figure 5**). The next day, cultures were processed for analysis of luciferase as described in "Materials and Methods." Relative translation efficiencies of Renilla luciferase (RLuc) (cap-dependent initiation) and firefly luciferase (FLuc) are shown. Error bars represent the mean +/- SEM of triplicate samples and * indicated $p < 0.05$.

The importance of PUMA as a mediator of apoptosis cannot be understated. While we have previously documented the critical role of PUMA in the apoptotic process that occurs in a subset of myoblasts induced to differentiate and in myoblasts in response to the DNA damaging agent etoposide or the ER-stress inducing agent thapsigargin,[18,48] others have demonstrated the importance of PUMA in many other cell types and in response to these and other stimuli.[49,50] Whether this PUMA IRES plays a role in any of these other cell types in response to any of the aforementioned stimuli awaits future investigation. Likewise, identification of the molecules required by this PUMA IRES to support translation in different cell types in response to distinct stimuli awaits future investigation, but could reveal additional therapeutic targets for manipulation in any of the myriad of physiologically important processes controlled by PUMA.

## Materials and Methods

**Cells and cell culture.** The growth and differentiation properties of 23A2 myoblasts have been reported previously.[3] 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 by switching cells from growth medium to differentiation medium (DM), which consists of BME, 1% P/S and 0% FBS. HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 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). Cells were incubated at 37°C in 5% $CO_2$. Cells were treated with actinomycin D (5 μg/mL), etoposide (200 μM), or rapamycin (100 ng/mL) dissolved in DMSO as indicated. Appropriate volumes of solvent alone were added to control cultures and did not exceed 0.15% v/v.

**Western blotting.** Myoblasts were plated at equal density and the next day switched to fresh GM or DM for three hours with or without additional treatment as indicated. Lysates were prepared as previously described.[3] Following protein determination, lysates (50 μg of total cellular lysate) were denatured in 5x sample buffer (10% SDS, 50% glycerol, 10% 2-mercaptoethanol, pH 6.8) and subjected to denaturing electrophoresis in 12% polyacrylamide gels. Following SDS PAGE (SDS-PAGE), samples were transferred to Hybond-P polyvinylidene difluoride membranes in transfer buffer containing 20% methanol and 1 g/L SDS. Membranes were blocked for one hour in 1x TBS/0.1% Tween 20 with 10% newborn calf serum and 5% dry milk. The following primary antibodies were incubated with the appropriate membranes: anti-PUMA antibody (Abcam, diluted 1:1000), anti-phospho 4E-BP (Cell Signaling Technology, Inc., diluted 1:1000), anti-phospho eIF2α (Cell Signaling, diluted 1:1000), anti-myogenin antibody (BD PharMingen, diluted 1:1000) and anti-actin for loading and transfer control of each Western analysis (Sigma-Aldrich Co. LLC, 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% Tween 20. Membranes were then incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Pierce Protein Biology Products) for 60 sec and bands were visualized using Kodak Scientific Imaging Film.

**Quantitative RT-PCR.** Myoblasts were plated at equal density and the next day switched to fresh GM or DM for three

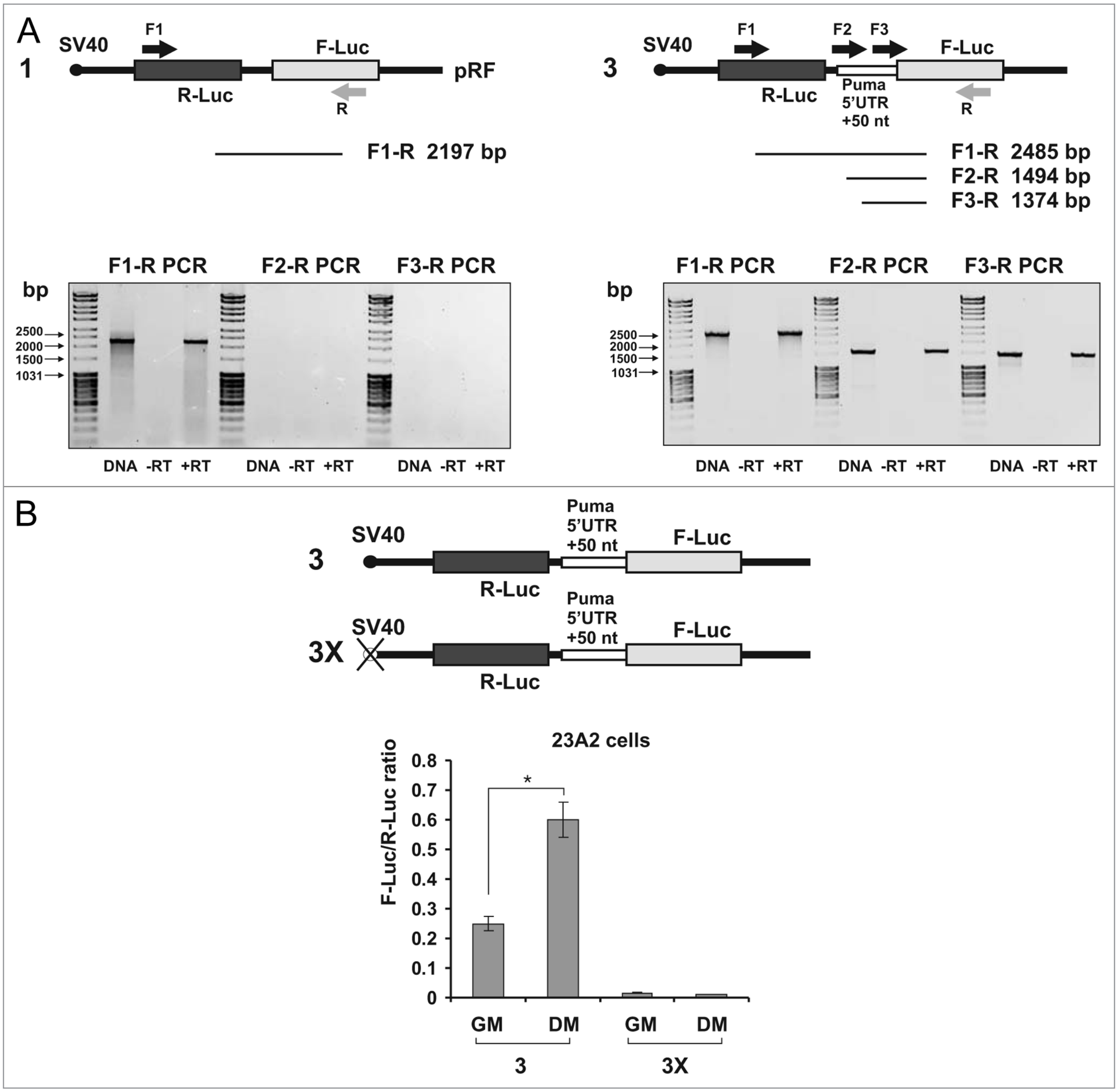


**Figure 7.** RLuc-Fluc bicistronic reporter construct containing PUMA IRES does not induce alternative RNA transcripts and does not contain cryptic promoters. (**A**) 23A2 cells were transfected with 2 μg of constructs 1 or 3 (**Fig. 5**). The next day RT-PCR analysis was performed on RNA from transfected 23A2 cells cultured in DM for 3 h. Relative primer positions used are indicated on the scheme top (for exact sequences see "Materials and Methods"). Reverse Transcription reactions were performed according to the manufacturer's protocol (Invitrogen, SuperScript III Reverse Transcriptase was used). DNase I pretreatment and PCR in the absence of RT served as a control. PUMA cDNA was used to control for the size of the amplified fragments (DNA control). (**B**) Equal cell numbers were plated and the next day transfected with 2 μg of bicistronic reporter constructs with or without SV40 promoter. The next day, cells were switched to either fresh GM or DM for three hours followed by analysis of luciferase activity. Relative translation efficiencies of Renilla luciferase (RLuc) (cap-dependent initiation) and firefly luciferase (FLuc) are shown. Error bars represent the mean +/- SEM of triplicate samples and * indicated $p < 0.05$.

hours with or without additional treatment as indicated. Total RNA was prepared using 1 mL of Trizol (Life Technologies) reagent per 100 mm plate for lysis and following the manufacturer's instructions. Five hundred ng of RNA was then used for a 20 μL SuperScript III RT (Life Technologies) reverse transcription reaction. Quantitative PCR was then performed for PUMA as described.[18]

**[$^{35}$S]-metabolic labeling and immunoprecipitation of PUMA.** 23A2 cells were plated at equal density 1X10$^5$ in 100 mm plates the night before. The next day, cells were washed with cold 1X PBS (Phosphate Buffer Saline) and preincubated in cysteine/methionine-free PRMI media (Thermo Scientific HyClone) for 30 min at 37°C. 23A2 cells were then incubated with cysteine/methionine-free medium containing [$^{35}$S]-methionine (500 μCi/

mL) (MP Biomedicals Inc.) with actinomycin D (5 μg/mL) in presence (GM) or absence (DM) of 10% dialyzed FBS (Thermo Scientific 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 1 mM, EDTA 1 mM, Triton X100 0.1% and 1X protease inhibitor). Further disruption of cells was achieved by repeated free-thaw cycles. [$^{35}$S]-labeled lysates were pre-cleared with Protein A/G agarose beads (Santa Cruz Biotechnology, Inc.) for one hour at 4°C. Pre-cleared [$^{35}$S]-labeled supernatant was removed after centrifugation at 2500 rpm for 30 sec at 4°C. Equal aliquots of protein from each pre-cleared supernatant were incubated with 10 μL anti-PUMA antibodies (Abcam) and 70 μL of Immunoprecipitation Matrix (ExactaCruz from Santa Cruz Biotechnology, Inc.) complex overnight at 4°C. Immunocomplexes were pelleted at 4°C the next day and washed four times with RIPA buffer and 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), boiled for 3 min at 95°C and sedimented at 13000 rpm for one minute. The resultant supernatant was subjected to SDS-PAGE. The gel was fixed in 50% methanol, 10% acetic acid fixation solution, soaked for 15 min in Amplify (GE Healthcare Biosciences), dried for 90 min at 65°C, and visualized using Typhoon imaging system (GE Healthcare).

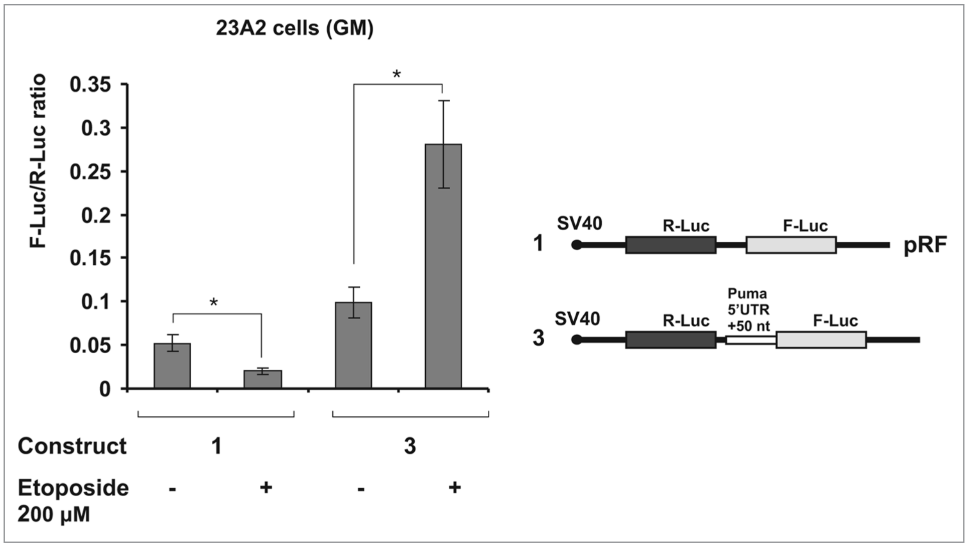


**Figure 8.** Etoposide stimulates the expression of PUMA. Expression of bicistronic reporter constructs 1 and 3 (from **Figure 5**) in 23A2 cells in GM either treated or not treated with etoposide (200μM). Relative translation efficiencies of Renilla luciferase (RLuc) and firefly luciferase (FLuc) are shown. Error bars represent the mean +/- SEM of triplicate samples and * indicated $p < 0.05$.

**Polyribosome analysis.** Equal cell numbers were plated in 15 cm tissue culture dishes. The next day, cells were switched to fresh GM or DM for 3 h. Prior to harvesting, cells were treated with 100 μg/mL of cycloheximide for 15 min at 37°C. Cells were washed twice with cold PBS containing cycloheximide and scraped in cold PBS containing cyclohexamide. Harvested cells were collected by centrifugation at 1.500 g for 10 min 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 (DTT), 0.1% Nonidet P-40, RNasin (100 units/mL) and 100 μg/mL cycloheximide for 15 min on ice). Further disruption of cells was achieved by repeated free-thaw cycles. Lysed cells were subjected to centrifugation at 10,000 g. Post-mitochondrial cytoplasmic extract (supernatant) was collected and equal optical density units (OD254) of each cytoplasmic extracts from GM or DM samples was determined, layered over 7–50% sucrose gradient (10 mM Hepes-KOH (pH 7.4), 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT) and centrifuged at 17,000 rpm for 18 h at 4°C in Beckman SW 28.1 Ti swinging-bucket rotor. Fractionation of gradients was performed using ISCO density gradient fractionator with absorbance monitor at 254 nm. RNA was collected from each of 24 fractions using Trizol reagent (Life Technologies) per manufacturer's instructions. mRNA levels in each fraction were quantified by reverse transcription-quantitative PCR (RT-qPCR) as previously described[18] and presented as % of the total specific mRNA.

**Analysis of global protein synthesis.** 23A2 cells were plated at equal density the night before. The next day, cells were washed with cold 1X PBS and pre-incubated in cysteine/methionine-free RPMI media (Thermo Scientific HyClone) for 30 min at 37°C. Cultures were then incubated with [$^{35}$S]-methionie (250 μCi/mL) in the presence or absence of 10% dialyzed FBS (Thermo Scientific HyClone). Cell lysates were prepared after three hours and an equal amount of protein from each sample was subjected to 10% trichloroacetic acid (TCA) precipitation. An equal volume of each sample was applied to GFC filter paper. Filters were washed twice with 5% TCA and once with ethanol. Radioactivity was quantified by scintillation counting.

**Plasmids.** Molecular cloning was performed following the general procedures described in Sambrook, et al.[51] DNA sequencing was performed by the Molecular Biology Core Facility at Cleveland Clinic, Cleveland, OH. Mono and bicistronic constructs were used to assess PUMA translation in vitro and ex vivo in various cellular systems. pBKS+PUMA monocsictronic vector carrying the entire PUMA cDNA was produced as follows: The cDNA was amplified by PCR using PUMA cDNA from Life Technologies, (Grand Island, NY: MG 67917) and the following primers: SacI forward: 5'-AAA AAG AGC TCC CAG GAG GCG GCG GCG ACA CCA GC-3' and EcoRI reverse: 5'-AAA AAG AAT TCC AAA GGA AAA GTT ATT TTA GTC TAA C-3' The resulting PCR product was digested with SacI and EcoRI and cloned in pBlueScript vector digested with SacI/EcoRI. pBIIKS-CAT vector carrying CAT cDNA has been previously described.[52] Bicistronic pGEM-CAT-PUMA-LUC constructs were produced as follows: The sequences spanning 5-UTR of PUMA, 5-UTR of PUMA + 50 nt and 5-UTR of PUMA + 100 nt passed initiation codon were amplified by PCR using PUMA cDNA (Life Technologies: MG 67917) as a template and the following primers: BamHI forward: 5'-AAA AGG ATC CCA GGA GGC GGC GGC GAC ACC AGC-3', BamHI +1 reverse: 5'-AAA AAG GAT CCA CAT GGC GCT CCC TGG AGC CCC-3', BamHI +50 reverse: 5'-AAA AAG GTC CAT ACA GCG GAG GGC ATC AGG CGG-3' and BamHI + 100 reverse 5'-AAA AGG ATC CAG CGG GCT AGA CCC TCT ACG GGC TCC-3', respectively. The resulting PCR fragments were digested with BamHI and cloned into pGEM-CAT-LUC vector[53] at BamHI site. pUHD10–3/LUC vector (kindly provided by Dr. Maria Hatzoglou) for cellular expression has been described previously.[34] This vector contains a minimal cytomegalovirus promoter and the SV40 polyadenylation signal.[34] The generation of the monocistronic expression vector, phpUHD10–3/LUC, containing a hairpin 70 nt downstream of transcription start site, has also been described previously.[34] To generate monocistronic vectors containing the 5'UTR of PUMA with an additional 50 nt passed the initiation codon, PUMA cDNA was amplified by PCR using the following primers: forward: 5'-AAA AAG AAT TCC CAG GAG GCG GCG GCG ACA CCA GC-3' and reverse: 5'-AAA AAT CAT GAT TAC AGC GGA GGG CAT CAG GCG G-3' digested with EcoRI and BspHI and inserted into the EcoRI/NcoI sites. pRF and phpRF plasmids (kindly provided by Dr. Anne Willis) have been described previously.[35] pRF-PUMA and phpRF-PUMA plasmids have been produced by inserting a PUMA cDNA fragment at the EcoRI/NcoI sites of the above mentioned vectors. To this end the PUMA cDNA was amplified using the following primers 5'-AAA AAG AAT TCC CAG GAG GCG GCG GCG ACA CCA GC-3' (forward) 5'-AAA AAT CAT GAA GCG GGC TAG ACC CTC TAC GGG CTC C-3' (reverse) digested with EcoRI and BspHI and inserted into the EcoRI/NcoI site of the bicistronic pRF/phpRF vectors respectively. Promoterless pRF-PUMA bicistronic vector was made by removing SmaI-EcoRV fragment harboring SV40 promoter from pRF-PUMA plasmid. All constructs were verified by sequencing.

**In vitro transcription/translation.** The mMESSAGE mMACHINE T7 Ultra Kit incorporating Anti-Reverse Cap Analog (ARCA) (Ambion®, Life Technologies) was used to produce capped and polyadenylated mRNAs. Mono and bicistronic mRNAs were further subjected to in vitro translation in Nuclease-Treated Rabbit Reticulocyte Lysate (Promega) cell-free system in the presence or absence of increasing concentrations of $m^7G(5')ppp(5')G$ cap analog (Ambion®, Life Technologies) and 10 μCi of Trans [$^{35}$S]-Label Met/Cys (MP Biomedicals Inc.). Reactions were resolved on a 12% SDS-PAGE. The gel was fixed, dried and visualized/quantified using Typhoon Imaging System (GE Healthcare).

**Transient transfection and reporter assays.** Equal cell numbers were plated in 6 well plates and the next day transfected with 2 μg of either monocistronic of dicistronic reporter constructs using Lipofectamine Plus per manufacturer's instructions (Gibco®, Life Technologies, Grand Island, NY). Measurements of chloramphenicol acetyltransferase, luciferase and β-galactosidase activities were done as previously described.[15,31,50]

**RT-PCR analysis of RNA integrity.** To verify the integrity of bicistronic pRF-PUMA mRNA, we examined the size of the reverse transcribed PCR products. The following primers were used: F1 5'-GGT CCG CAG TGG TGG GC-3' (forward), F2 5'-AGC AGC AAG GTG CCT CAA TAG-3' (forward), F3 5'-ATG GCC CGC GCA CGC CAG G-3' (forward) and R 5'- GCG GTC AAC GAT GAA GAA GTG-3' reverse. Reverse Transcription reactions were performed according to the manufacturer's protocol (Invitogen, Life Technologies SuperScript III Reverse Transcriptase).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


### Acknowledgments

We thank Drs. Graham Belsham, Maria Hatzoglou and Anne Willis for their generous gifts of plasmids used in this study. We also thank Drs. Maria Hatzoglou and William Merrick for many helpful discussions.

### Financial Support

This work was supported by NIH grant AR053857 awarded to C.M.W. and in part by National American Heart Association Grant 0730120N (to A.A.K.).


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