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Investigation of Ires-mediated Translation of Puma Mrna: Initiation Factor Requirements And Search For Itafs

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INVESTIGATION OF IRES-MEDIATED TRANSLATION OF PUMA mRNA: INITIATION FACTOR REQUIREMENTS AND SEARCH FOR ITAFs

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Dedicated to my parents

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INVESTIGATION OF IRES-MEDIATED TRANSLATION OF PUMA mRNA: INITIATION FACTOR REQUIREMENTS AND SEARCH FOR ITAFs

AMRA ISMAIL

ABSTRACT

Translation initiation is the rate-limiting and tightly regulated step of protein synthesis. Cap-dependent translation initiation accounts for about 95% of cellular mRNAs. Around 3-5% of cellular mRNAs have been found to contain a cis-regulatory element (IRES) which can recruit ribosomes in a cap-independent manner. IRESs support protein synthesis under cellular stress conditions when cap-dependent translation is inhibited. Differentiation in 23A2 myoblast cells can be induced by culturing cells in serum-free differentiating media (DM). During 23A2 cellular myoblast differentiation, approximately 30% of cells undergo apoptosis as a result of stress caused by serum withdrawal in order to induce differentiation. The expression of the pro-apoptotic Bcl2 family member PUMA was found to be elevated when myoblasts cells were cultured in differentiating media. PUMA was identified as a critical regulator for apoptosis associated with skeletal myoblast differentiation. It was reported earlier that translation of PUMA was found to be regulated during skeletal myoblast differentiation. PUMA was found to be translated by an IRES-mediated mechanism when skeletal myoblast cells were switched from growth media to differentiating media conditions. PUMA was found to be actively translated despite reduced global protein synthesis. PUMA translation was found to proceed by a cap-independent mechanism precluding the need for canonical translation initiation factors like eIF4E and eIF2α. Experimental analyses confirmed the presence of an IRES element located within PUMA 5'UTR. In this study, we attempted

to understand the mechanism of PUMA internal initiation and identify canonical and non-canonical factors required for PUMA translation. Unlike Hepatitis C viral IRES, the 40S ribosomal subunit is not recruited directly to PUMA IRES during initiation. We also investigated the canonical initiation factors required by PUMA IRES. Using specific inhibitors for eIF4A and eIF4G, we found that PUMA translation requires eIF4A and intact eIF4G for its activity. We further attempted to identify a specific RNA-binding protein which may act as a co-factor and assists PUMA translation under conditions that inhibit cap-dependent translation and activate IRES mediated translation. RNA affinity pull-down assay and mass spectrometric analysis helped us to identify Hsp70 binding to PUMA IRES with high affinity in differentiating media. We confirmed PUMA IRES and Hsp70 protein interaction by gel retardation assay and competition assay. Further, antibody depletion assay confirmed that Hsp70 is an essential factor for PUMA translation.

TABLE OF CONTENTS

Page

ASBTRACT……vi

LIST OF TABLES……xiii

LIST OF FIGURES……xiv

LIST OF ABBREVIATIONS……xvi

CHAPTER

I. INTRODUCTION……1

1.1 Overview of translation……1

1.2 Translation initiation……4

1.3 Initiation factors and their role in translation……9

1.4 Alternative modes of translation initiation……14

1.4.1 Leaky scanning……14

1.4.2 Ribosome shunting……16

1.4.3 Re-initiation……17

1.4.4 Internal initiation……19

1.5 IRES elements in viruses……22

1.6 IRES trans-activating factors (ITAFs)……23

1.7 Classification of viral IRES……25

1.8 Cellular IRES……34

1.8.1 Significance of IRES-mediated translation……35

1.8.2 Structure of cellular IRES……39

1.8.3 Initiation factors required for cellular IRESs……42

1.8.4 Role of ITAFs in cellular IRES-mediated translation.....49

1.9 PUMA (p53 Upregulated Modulator of Apoptosis).....52

1.9.1 PUMA is required for apoptosis during cellular myoblast Differentiation.....55

1.9.2 Translational regulation of PUMA.....56

II. Mechanism of 40S recruitment to PUMA IRES and initiation factor requirements for translation.....58

2.1 Abstract.....58

2.2 Materials and methods.....60

Antibodies and reagents.....60

Plasmids.....60

In vitro transcription.....61

In vitro translation.....62

HRV2 2A protease protein expression and purification.....62

2.3 eIF4A is essential for PUMA translation.....64

2.3.1 Introduction.....64

2.3.2 Result.....67

2.4 Intact eIF4G is required for PUMA translation.....69

2.4.1 Introduction.....69

2.4.2 Result.....71

2.5 43S ribosomal complex recruitment to PUMA IRES.....74

2.5.1 Introduction.....74

2.5.2 Materials and methods.....76

In vitro translation assay……76

Sucrose density gradient analysis……76

2.5.3 Result……78

2.6 Discussion……81

III. Identification of specific ITAFs involved in upregulated PUMA expression in differentiating media……85

3.1 Introduction……85

3.2 Materials and Methods……87

In vitro transcription……87

Cell culture and preparation of 23A2 cell lysates……87

RNA affinity pull down assay……88

RNA gel shift assay and competition assay……89

3.3 Results……91

3.3.1 Identification of RNA-binding proteins bound to PUMA IRES in DM conditions……91

3.3.2 PUMA IRES (5'UTR+50 nt) binds to Hsp70 protein in vitro……95

3.4 Discussion……101

IV. Hsp70 is essential for PUMA translation……104

4.1 Introduction……104

4.2 Materials and Methods……106

Immuno depletion assay……106

In vitro translation assay……106

Translation rescue experiment……107

In vitro translation assay with GM/DM……107

4.3 Results……108

4.3.1 In vitro translation of PUMA mRNA requires Hsp70……108

4.3.2 PUMA translation is stimulated by DM cellular extract……113

4.4 Discussion……115

BIBLIOGRAPHY……119

LIST OF TABLES

Table Page

I. Viral IRES Classification and initiation factor requirements……..…32

II. Cellular IRES and identified ITAFs……....…51

III. Sequence analysis of proteins from a silver stained SDS PAGE gel……....93

LIST OF FIGURES

Figures		Page
1.1	Overview of mRNA translation in eukaryotes	3
1.2	Model of canonical translation initiation	8
1.3	Modes of translation initiation	20
1.4	Model of internal initiation	21
1.5	IRES trans-acting factors (ITAFs)	24
1.6	Major classes of IRES in RNA Viruses	33
1.7	ITAF binding to Bag-1 IRES changes its structural conformation	41
1.8	Cellular stress conditions induce eIF4E inactivation	44
1.9	Cellular stress conditions induce eIF2 inactivation	46
1.10	Cleavage of eIF4G	48
1.11	Apoptotic pathway	54
2.1	Hippuristanol inhibits in vitro translation of PUMA in a cell-free system	68
2.2	Cleavage of eIF4G by 2A protease	72
2.3	eIF4G cleavage affects PUMA translation in vitro	73
2.4	Assembly of translation initiation complexes on PUMA IRES in rabbit reticulocyte lysate cell-free extract and analysis by sucrose density gradient centrifugation	80
3.1	Identification of RNA-binding proteins in DM that binds to PUMA IRES	94
3.2	Binding of Hsp70 to PUMA RNA sequence	97
3.3	Cold PUMA IRES (5'UTR + 50 nt) competes to bind with Hsp70	98
3.4	Nucleotide sequence of PUMA ORF in mammals	99

3.5 Predicted secondary structure of PUMA ORF (1-50 nt) generated by MFOLD.....100

4.1 Hsp70 Immuno-depletion.....110

4.2 In vitro translation of PUMA mRNA and control Luciferase mRNA in Hsp70 depleted RRL extract.....111

4.3 In vitro translation of PUMA mRNA in Hsp70 depleted RRL, supplemented with Hsp70.....112

4.4 In vitro translation of PUMA mRNA in a cell-free RRL system, supplemented with GM or DM extracts.....114

LIST OF ABBREVIATIONS

aa	amino acid
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine triphosphate
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- X_L	B-cell Lymphoma extra large
BH	Bcl-2 homology domain
Bim	Bcl-2 interacting mediator of cell death
BMEM	Basal modified Eagle's medium
Caspases	Cysteine-aspartic acid specific proteases
cDNA	complementary deoxyribonucleic acid
CTD	C-terminal domain
DM	Differentiation medium

DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleo triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation facor
FBS	Fetal bovine serum
GCN4	General control nonderepressible 4
GDP	Guanosine diphosphate
GM	Growth medium
GMP-PNP	Guanosine-5' [β,γ-imido]triphosphate
GTP	Guanosine triphosphate
HRI	Heme regulated eIF2 kinase inhibitor
HRV 2A	Human Rhinovirus 2A protease
Hsp70	Heat shock protein 70

IGF	Insulin-like growth factor
IRES	Internal ribosomal entry sites
ITAF	IRES-trans acting factor
kDa	Kilo Dalton
Luc	Luciferase
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
nt	nucleotide
NTD	N- terminal domain
ORF	Open Reading Frame
PABP	Poly-A-binding protein
PBS	Phosphate buffered saline
PCBP2	Poly (rC) binding protein 2
PERK	Protein kinase-like endoplasmic reticulum kinase
Pi	Inorganic phosphate
PIC	Pre-initiation complex

PI3K	Phosphatidyl inositol 3-kinase
P/S	Penicillin/Streptomycin
PTB	Polypyrimidine tract binding protein
PUMA	p53 upregulated modulator of apoptosis
RF	Release factor
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulfate
TC	Ternary complex
tRNA	Transfer RNA
uORF	Upstream open reading frame
UTR	Untranslated region
WT	Wild type
XIAP	X-linked Inhibitor of Apoptosis

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW OF TRANSLATION

Translation is the process of protein synthesis in a cell which involves conversion of the information embedded in the genetic message/ nucleotide sequence of mRNA into amino acid sequence of a protein. In eukaryotes, the messenger RNA, which is made in the nucleus, is transported into the cytoplasm to be further engaged with the ribosome and specific translation factors for peptide synthesis. The process of protein synthesis is complex and consists of four major phases: Initiation, Elongation and Termination and Ribosome Recycling (Fig. 1.1).

Initiation stage involves the recruitment of ribosome-initiator tRNA complex to the initiation codon of the messenger RNA. With the help of specific initiation factors, the methionyl-initiator tRNA is carried to the P (peptidyl) site of the ribosome to base-pair with the initiator AUG of the mRNA. During the initiation stage, the Met-$tRNA_i$ brings methionine as the first amino acid of the peptide sequence (Poulin and Sonenberg, 2013).

Elongation stage involves systematic addition of amino acids as dictated by the codon sequence in the mRNA. The new amino acids enter the decoding site which is the

A (acceptor) site of the ribosome. The amino acyl tRNA that is recruited to the 40S subunit moves from the A site to the P site on the ribosome. This makes the ribosome A site available for the arrival of the next aminoacyl tRNA and ultimately a peptide bond formation between the previous amino acid and the current amino acid at the P site. (Kasinath et. al, 2006).

Termination phase involves the arrival of the 80S ribosome at the stop codon on the messenger RNA and the release of the synthesized protein.

Ribosome recycling involves splitting of the post-termination 80S ribosome and stripping off the mRNA, deacylated tRNAs and factors from the ribosome. The ribosomes are then prepared for the next round of translation (Kasinath et. al, 2006).

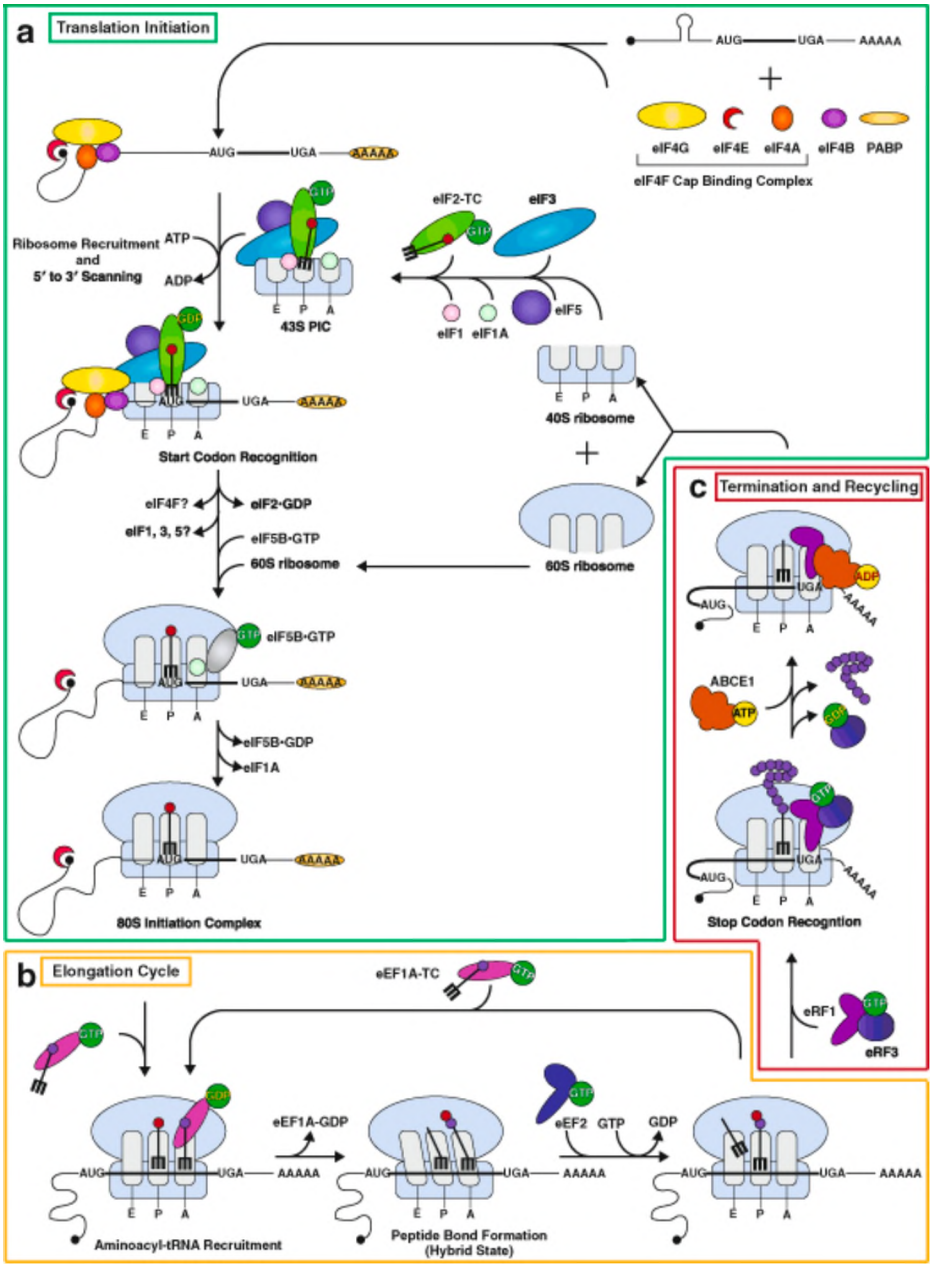


Fig 1.1. Overview of mRNA translation in eukaryotes. a) Initiation: The 40S ribosome bound to eukaryotic translation initiation factor eIF1, eIF1A, the eIF3 complex and eIF5 is loaded with initiator methionine tRNA (Met-tRNA$_i$) in the P site by eIF2·GTP, to form a 43S pre-initiation complex. The 43S complex is then positioned onto the 5′ end of a capped mRNA by eIF4F. The assembled 48S complex then scans the mRNA to locate the AUG start codon. After AUG recognition, facilitated by eIF3, eIF1 and 1A, 60S subunit joins and triggers initiation factor release. b) Elongation: Each charged tRNA is delivered to the 80S ribosome A site by eEF1A·GTP. After peptide bond formation, eukaryotic elongation factor 2 (eEF2) catalyzes 80S translocation, transferring the deacetylated tRNA to the E site, positioning the peptidyl-tRNA in the P site and re-exposing the A site. c)Termination and Recycling: Eukaryotic release factor 1 (eRF1) recognizes the stop codon in the A site, triggering 80S arrest and polypeptide release. Several initiation factors are released, dismantling the complex and recycling ribosome subunits. (Villa N. and Fraser C, 2014)

1.2 TRANSLATION INITIATION

The translation initiation process is divided into 8 steps:

(a) Binding of eIF3 to 40S subunits. This step blocks joining of the 40S subunit with the 60S subunit.

(b) Binding of the ternary complex (eIF2•GTP•Met-tRNA$_i$) to the 40S subunit to form the 43S pre-initiation complex.

(c) Binding of the eIF4F (and eIF4A and eIF4B) to the mRNA. This activates mRNA and removes any secondary structure.

(d) 43S pre-initiation complex recruitment to the 5' m^{7}G cap of the activated mRNA.

(e) Scanning of the mRNA-bound 43S pre-initiation complex along the 5' non-translated region and recognition of the AUG codon, resulting in the formation of 48 initiation complex.

(f) GTP hydrolysis of the ternary complex, release of initiation factors and joining of the 60S ribosome subunit.

(g) Recycling of eIF2•GDP to eIF2•GTP.

(h) Reassembly of the eIF4F from released eIF4A, eIF4E, and eIF4G (Komar et. al., 2012) (Fig. 1.2).

43S Pre-Initiation Complex formation

Translation initiation in eukaryotes begins with the identification of the initiation codon by the translation machinery (Ternary complex, the 40S ribosomal subunit and eukaryotic initiation factors). Ternary complex (TC) consists of- initiator methionyl tRNA (Met-tRNA$_i$), the eukaryotic initiation factor 2 (eIF2) and GTP. The ternary

complex binds to the small 40S ribosomal subunit to form the 43S pre-initiation complex. A number of initiation factors like eIF1, eIF1A, eIF5 and eIF3 are involved for the binding of the TC to the 40S subunit. The 40S ribosome along with initiator Met-tRNA bound to eIF2-GTP, eIF1, eIF1A, eIF5 and eIF3 forms the 43S pre-initiation complex. This 43S pre-initiation complex binds to the messenger RNA at the 5'-7-methylguanosine cap. This process is facilitated by the cap-binding complex eIF4F, eIF3 and the poly (A)- binding protein (PABP). eIF4F is a 3-subunit protein complex composed of eIF4E, eIF4A, and eIF4G, which binds at the 5'terminus of the mRNA. The cap-binding protein eIF4E associates with eIF4G and brings the eIF4F complex in proximity of the cap structure. eIF4G is the scaffolding protein which binds to eIF4E, eIF3 and PABP. It is believed that eIF3 present in the pre-initiation complex associates with the eIF4G present in the eIF4F complex, to facilitate the binding between 43S pre-initiation complex and mRNA. The initiation factor eIF4A acts as a RNA helicase and facilitates scanning of the 5'UTR region by the 43S complex to locate the initiation AUG codon (Hinnebusch, 2012; Browning and Bailey-Serres, 2015).

Start site Recognition

After binding at the 5'cap structure, the 43S complex scans the 5' untranslated region of the messenger RNA to locate the initiation AUG codon in a suitable sequence context, leading to the formation of the 48S initiation complex. The eIF1A binds to the open or scanning 43S pre-initiation complex and facilitates the process of scanning. A crucial event in the recognition of the start codon is the base-pairing between the anticodon of Met-tRNA$_i$ and the AUG in the peptidyl-tRNA (P) site of the 40S ribosome.

eIF5 interacts with eIF1A and promotes GTP hydrolysis from the ternary complex. The release of P_i from the ternary complex results in scanning arrest at the initiation site and a closed pre-initiation complex (PIC). eIF5 also interacts with eIF2 and this interaction serves two purposes- to stimulate the GTPase activity of eIF2 and to prevent early release of P_i from the ternary complex during at the time of scanning (Hinnebusch, 2014).

The most suitable initiation codon sequence is a purine residue (A or G) at position -3 and a guanine (G) residue at position +4 relative to the start AUG codon. The nucleotides surrounding the initiation AUG codon are thought to play a role in maintaining a closed PIC conformation during start site recognition (Browning and Bailey-Serres, 2015).

Assembly of the 80S ribosome

The scanning initiation complex gets arrested at the initiation AUG codon and the eIF2 in the TC is converted to its GDP-bound state with the help of initiation factor eIF5. eIF5 is released along with eIF2•GDP, once a closed PIC is formed. eIF5 binds to eIF2•GDP and acts as a GDP dissociation inhibitor for eIF2•GDP. eIF2B then acts to replace GDP to GTP. eIF5 plays a role in GTP hydrolysis of eIF2 and dissociation of initiation factors from the ribosome-mRNA complex. After eIF2•GDP and other initiation factors are dissociated from the 48S complex, the large 60S combines with the small 40S subunit and results in 80S complex formation. The release of eIF5 and eIF2•GDP opens the 40S subunit surface for 60S subunit binding. In addition, eIF5B•GTP interacts with eIF1A and facilitates 60S subunit joining, and later eIF5B•GDP dissociates from the complex. The 80S complex contains the Met-$tRNA_i$

base paired to AUG in the ribosomal P-site. This step marks the end of initiation phase and the beginning of elongation phase of translation. The cell is now ready for peptide elongation (Browning and Bailey-Serres, 2015).

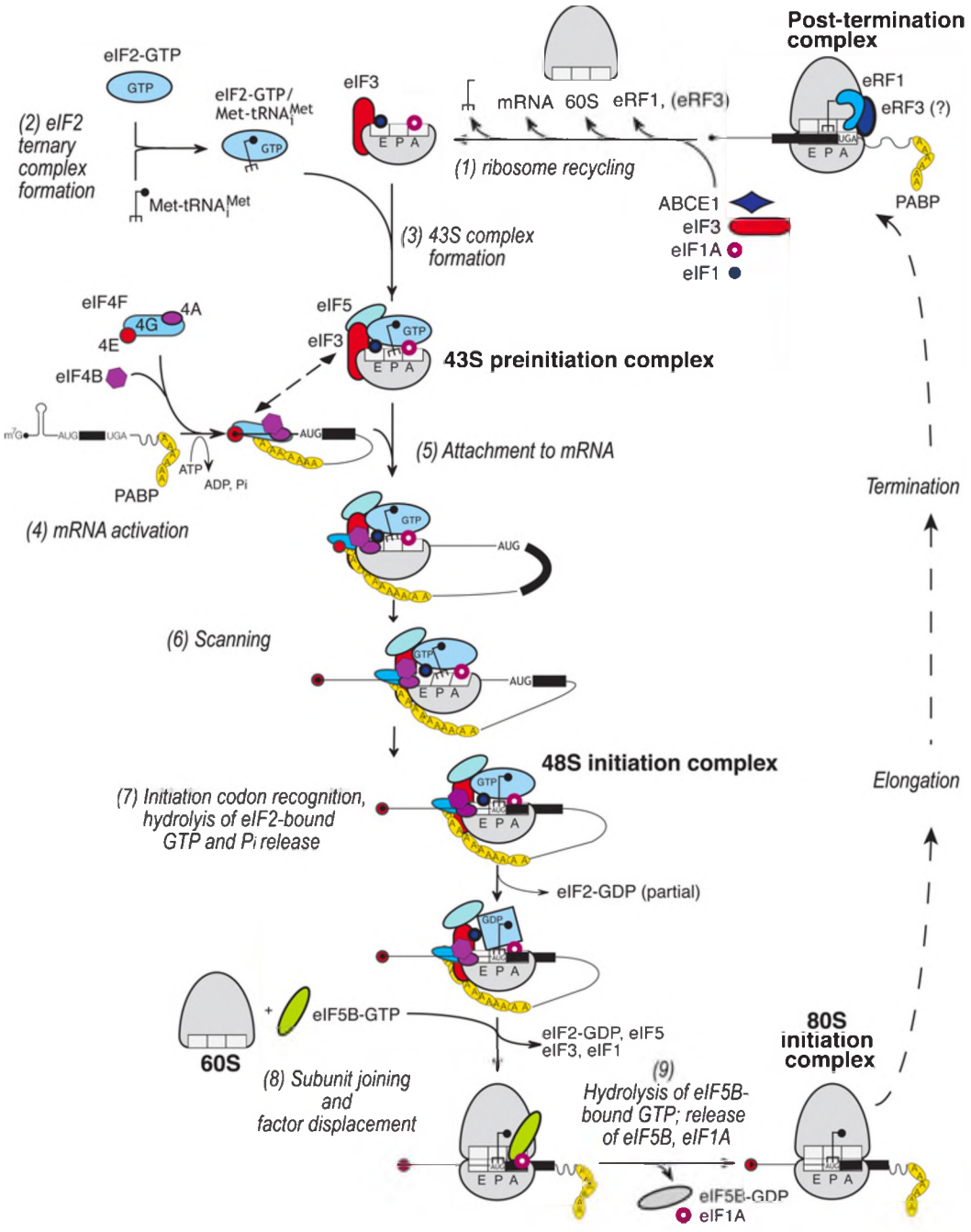


Fig 1.2. Model of canonical eukaryotic translation initiation- The stages include: (1) Ribosome recycling from post-termination complexes (2) Formation of the ternary complex (3) Formation of a 43S preinitiation complex (4) mRNA activation (5) Attachment of the 43S complex to mRNA (6) Scanning of the 5'UTR in a 5'→3'direction by 43S complexes (7) Recognition of the initiation codon and 48S initiation complex formation (8) Joining of 60S subunits to 48S complexes and concomitant displacement of initiation factors (9) GTP hydrolysis from elongation-competent 80S ribosomes (Jackson et al., Nat Rev Mol Cell Biol. 2010).

1.3 INITIATION FACTORS AND THEIR ROLE IN TRANSLATION

eIF1 and 1A- The eIF1 group includes 2 proteins: eIF1 and eIF1A. These are small, single polypeptide proteins of about 12-17 kDa and highly conserved among eukaryotes (Browning and Bailey-Serres, 2015).

eIF1 is believed to play a role in PIC scanning, destabilization of incorrectly positioned complexes and stabilization of the correctly positioned PICs. eIF1 is an essential requirement for the formation of 48S complex and is involved in promoting the correct positioning of the 43S complex at the initiation codon. eIF1 binds near the P-site of the 40S ribosomal subunit and prevents the ternary complex from engaging with the P-site of the 43S PIC, until the correct initiation codon is identified (Browning and Bailey-Serres, 2015; Poulin and Sonenberg, 2013; Pestova et. al, 1998).

eIF1A have different functions during translation initiation. eIF1A, together with eIF1 promotes the 48S complex assembly at the initiation site. The two proteins interact and stabilize the open PIC, also preventing full engagement of Met-tRNA$_i$ at the P-site. eIF1A promotes ternary complex binding to the 40S subunit. eIF1A binds at the A-site of the 40S subunit and directs the Met-tRNA$_i$ to the P-site and also prevents complete engagement of Met-tRNA$_i$ with the P-site until the correct initiation codon is located. After the identification of the initiation codon, eIF1A interacts with eIF5 and promotes eIF2•GTP hydrolysis. Finally, eIF1A also has a role to facilitate 80S ribosome assembly (Browning and Bailey-Serres, 2015; Poulin and Sonenberg, 2013; Pestova et. al, 1998).

eIF2- Eukaryotic initiation factor eIF2 is a heterotrimeric G protein made up of α, β and γ subunits. In eukaryotes, the ternary complex is assembled by interaction of the initiator tRNA with eIF2•GTP complex, which delivers Met-$tRNA_i$ directly at the 40S ribosomal P-site. The base pairing between the initiation AUG codon and the initiator tRNA anticodon leads to GTP hydrolysis by eIF2. This process involves the assistance of a GTPase activating protein eIF5, which facilitates the hydrolysis of GTP-bound eIF2. eIF2α is activated when GDP is exchanged to GTP. GTP exchange and recycling is facilitated by the guanine nucleotide exchange factor eIF2B and the activated eIF2α protein is available to take part in another round of initiation. eIF2α also plays a role in translation regulation (Kimball, 1999).

eIF3- Eukaryotic initiation factor eIF3 is the largest and most complex initiation factor (consisting on its own in higher eukaryotes of 13 different subunits) and has multiple functions. It is required both for the recruitment of the ternary complex to the 40S subunit and mRNA recruitment to the pre-initiation complex. eIF3 interacts with eIF4F and acts as a bridge to the mRNA through the interaction of eIF4E with the 5'cap structure. It is also believed that eIF3 plays a role in dissociation of 40S and 60S ribosomal subunits. Previous studies have suggested that eIF3 stabilizes the ternary complex binding to the 40S subunit by preventing the 60S subunit from disrupting the 43S preinitiation complex (Browning and Bailey-Serres, 2015).

The main role of eIF3 is to bind to the 40S subunit and participate in the formation of the 43S pre-initiation complex, along with other initiation factors like eIF1, eIF1A and eIF5, the 40S subunit and the ternary complex. In addition, eIF3 acts as a

bridge which functions to facilitate mRNA binding with initiation factors (eIF4A, eIF4G) and with the pre-initiation complex, to form a 48S scanning complex (Browning and Bailey-Serres, 2015).

eIF4F family- This family includes 3 major proteins- eIF4E, eIF4G and eIF4FA. The role of eIF4F family of proteins is to interact with the mRNA and prepare it for binding with the 43S PIC. The 5' m^7G cap binds to the eIF4F through the eIF4E subunit. The eIF4G acts as a scaffold, binding to both eIF4A and eIF3 and, preparing the mRNA for binding to the 43 PIC and its scanning along the 5' untranslated region for the initiation codon (Browning and Bailey-Serres, 2015).

eIF4E- eIF4E is the mRNA cap-binding protein that binds to the 5' m^7G cap structure and is one of the proteins that belongs to the eIF4F complex. eIF4E along with eIF4G and m^7G cap structure forms a complex that directs the 43S preinitiation complex to the mRNA 5'terminal (Proud, 2005).

eIF4G- eIF4G is a scaffolding protein that is a part of the eIF4F complex. It functions as an adaptor protein which functions in mediating a series of protein-protein interactions that eventually result in 43S complex recruitment to the mRNA 5'end (Sharma et. al., 2016). The amino terminal region of eIF4G contains an interaction domain that binds poly (A) binding protein. The middle fragment of the eIF4G possess two interaction domains; the first domain interacts with RNA helicase eIF4A and the other is the eIF3 interaction domain. The central portion of eIF4G also includes the

eIF4E-binding domain that is essential for canonical translation initiation. The 4E-binding site is necessary for targeting eIF4G to the mRNA. Finally, the carboxy terminus of eIF4G harbors a second interaction domain for eIF4A. This domain is not found to be present in yeast eIF4G and is not essential for eukaryotic translation. A key role of eIF4G is to bridge the 40S ribosomal subunit (via eIF3) and mRNA cap (via eIF4E) (Sharma et. al., 2016).

eIF4A- eIF4A is a RNA helicase protein that belongs to the DEAD-box family of RNA helicases (Rogers et. al., 2002). eIF4A is an RNA-dependent ATPase which functions to unwind the RNA duplexes. The helicase activity of eIF4A is strongly stimulated by eIF4B due to its intrinsic weak helicase activity. eIF4A is also a part of the eIF4F complex. It is believed that eIF4F targets eIF4A to the mRNA, and eIF4A unwinds the mRNA secondary structure, which allows efficient ribosome recruitment. eIF4A undergoes ATP hydrolysis and in turn utilizes the energy to rearrange complex mRNA structures. (Rogers et. al., 2002, Browning and Bailey-Serres, 2015).

eIF4A unwinds short RNA duplexes, without being specific to any RNA sequence. In the presence of RNA and ATP, eIF4A forms a closed, catalytically active conformation. It is believed that this closed conformation is favored due to eIF4G and eIF4B binding to eIF4A. Interaction of eIF4A with eIF4G and eIF4B also leads to mRNA complex secondary structure relaxation, for efficient scanning and binding of the 43S PIC (Browning and Bailey-Serres, 2015).

eIF5 family- This family includes three proteins, eIF5, eIF5A and eIF5B. eIF5 and eIF5B are involved in selection of the start site and codon-anticodon base-pairing. The third member of this group, eIF5A functions in elongation (Hinnebusch and Lorsch, 2012).

eIF5- It has GTPase activating function and promotes GTP hydrolysis of the ternary complex after initiation site recognition. eIF5 interacts with the GTP-binding region of eIF2 and promotes GTP hydrolysis. eIF5 is released after the formation of the 48S complex along with eIF2•GDP (Browning and Bailey-Serres, 2015).

eIF5B- This protein has function similar to prokaryotic IF2. eIF5B in eukaryotes does not bind Met-tRNA$_i$, but functions in 60S subunit binding. After the recognition of the initiation codon, eIF1 is released from the PIC and GTP hydrolysis takes place which leads to conformational changes in eIF2. eIF5B•GTP then binds to the PIC via its interaction with eIF1A. This interaction is believed to stabilize Met-tRNA$_i$ binding at the P-site and also dissociation of the remaining initiation factors- eIF2, eIF3, and eIF5 from the complex. Upon joining of the 60S subunit, eIF5B•GTP is hydrolyzed and release of eIF5B•GDP and eIF1A occurs, as 80S ribosome is formed (Browning and Bailey-Serres, 2015).

1.4 ALTERNATIVE MODES OF TRANSLATION INITIATION

Canonical translation initiation involves the 5' end of the mRNA and a linear scanning mechanism to locate the start codon. The scanning complex (40S ribosomal subunit bound to initiation factors and Met-tRNA$_i$) is recruited at the capped 5'-end and scans along the 5'-untranslated region to find the start AUG codon. Apart from the canonical/predominant mode of translation initiation, there are several other modes of translation initiation. These include- Leaky scanning, Re-initiation, Ribosome shunting and Internal initiation (which on its own also includes a variety of different mechanisms) (Ryabova et. al, 2002, Ryabova et. al, 2006, Firth and Brierley, 2012) (Fig. 1.3).

1.4.1 LEAKY SCANNING

This mechanism is mostly used by viruses to express polycistronic RNAs. The mechanism of leaky scanning involves the ribosomal complex bypassing the first start codon and initiating translation at a downstream start codon. An optimal translation initiation site has a purine (R) at position -3 and a guanine (G) at position +4. These residues are highly conserved in eukaryotes and have a great importance in translation. If the initiation codon lacks R residue at position -3 and G at +4, or if the initiation codon is non-AUG, the scanning ribosomes bypass the first start codon (Ryabova et. al, 2006). The scanning ribosomes fail to initiate at the first AUG site and continue scanning downstream until they reach an alternative initiation site. The process of leaky scanning therefore results in expression of two or more proteins with a common C-terminal region. These protein isoforms are translated from the same open reading frame and have a common in-frame initiation site. Leaky scanning can also result in different proteins if

start codons sites are located in different ORFs. Translation initiation may begin at a non-AUG codon, resulting in three or four different proteins being translated from a single transcript. The mechanism of leaky scanning is mostly used by RNA viruses to translate multicistronic messages (Ryabova et. al, 2002, Ryabova et. al, 2006).

The distance of the AUG codon from the 5' m^7G cap is another deciding factor for leaky scanning. The AUG codon is not recognized efficiently and correctly if it is too close to the 5'end of the transcript and may lead to leaky scanning of ribosomes. A distance of 30 nt or less drastically reduces the chances of recognizing the AUG codon. Transcripts having short upstream ORFs may also encounter leaky scanning. In case of short ORFs, the scanning ribosomes resume scanning and re-initiate on a downstream ORF. However, it may take time for the ribosomes to reacquire initiation factors and some intervening AUG codons may be bypassed, resulting in leaky scanning (Firth and Brierley, 2012).

1.4.2 RIBOSOME SHUNTING

Ribosomal Shunting is a discontinuous type of ribosome scanning, first described in Cauliflower mosaic virus. This form of translation initiation is mostly prevalent in RNA viruses and many plant viruses. Ribosome scanning is a non-linear scanning mechanism in which the scanning ribosomes are transferred from a 5'donor site to a 3' acceptor site, bypassing scanning of an intervening region (Ryabova et. al, 2002). Ribosome shunting is a result of discontinuous ribosome scanning due to strong secondary structure downstream of a small ORF (less than 30 codons). A short complex secondary structure makes this region inhibitory to scanning, and hence results in ribosome shunting (as such ribosome scanning resumes downstream of the secondary structure). Ribosome shunting is predominantly found in plant RNA viruses which have a long 5'UTR that folds into a stem-loop structure. The 40S ribosomes scans and translates the short ORF upstream of the stem-loop structure and terminates just before the stem-loop. The ribosome bypasses the stem-loop and lands at a site 3' of the stem-loop structure and resumes scanning. It is believed that the scanning is resumed because of the ability of the 40S ribosomal subunit to retain some initiation factors while translating the short ORF. There also seems to be a temporary loss of some initiation factors which promotes discontinuous scanning across the stem-loop structure. Another factor which determines efficient shunting is the length and position of the short ORF, but not its sequence (Firth and Brierley, 2012).

Ribosome shunting provides an opportunity for scanning ribosomes to bypass a leader region that may contain inhibitory elements such as multiple short ORFs and stem-loop structure and resume translation downstream of it (Ryabova et. al, 2006).

1.4.3 RE-INITIATION

After translation termination, dissociation of the 40S and the 60S ribosomal subunits from the message takes place. However, the 40S subunit may remain associated with the mRNA and resume scanning after translating a short ORF. Translation is re-initiated at a downstream AUG codon, if a short ORF (less than 30 codons) is present upstream (Ryabova et. al, 2002).

The process of Re-initiation involves a round of initiation at a downstream start codon after translation of several upstream short ORFs. After translating the short ORFs, the terminating ribosomes remain associated with the mRNA and continue scanning. The re-initiation at a downstream start codon is dependent on both mRNA cis-elements and trans-acting factors. The process of re-initiation depends strongly on the length of the short ORF, which is usually less than 30 codons and re-initiation cannot occur after translation of a long ORF. On the other hand, the re-initiation frequency increases with the distance between the short ORF and the main ORF. Re-initiation requires recruitment of the ternary complex (eIF2•GTP•Met-tRNA$_i$) and initiation factors by the scanning 40S ribosomes, which is the rate-limiting step for translation re-initiation. It has been suggested that after translation termination of the short ORF, some initiation factors remain associated with the translation machinery which are eventually used for re-initiation. This explains the decrease of re-initiation frequency with the increase of the length of short ORF. After translation of the short ORF, the 40S ribosome is not immediately competent for re-initiation. The 40S subunit scans for some distance during which it acquires certain initiation factors and the ternary complex, before re-initiation. This type of translation regulation can be seen in response to physiological and

environmental stresses for expression of stress-response genes (Hinnebusch, 2005, Sonenberg and Hinnebusch, 2009).

Expression of a yeast transcriptional activator, GCN4 mRNA is one the best studied examples of translation control via re-initiation. Under normal conditions, translation of GCN4 mRNA is repressed. During stress conditions such as amino acid deprivation, eIF2 phosphorylation is induced, which reduces availability of the ternary complex. Low levels of TC thereby inhibit cap-dependent translation initiation. However, these conditions induce the expression of GCN4 mRNA, which in turn stimulates the expression of other amino acid biosynthetic genes. Under low TC, a fraction of 40S subunits after termination re-initiate at the GCN4 start codon and overcomes the inhibitory effect of four upstream ORFs. The uORFs act as sensors of polyamine levels in mRNAs which are subjected to polyamine responsive translation regulation (Hinnebusch, 2005, Sonenberg and Hinnebusch, 2009).

1.4.4 INTERNAL INITIATION

Internal initiation is the process of direct binding of the 40S ribosome at or upstream of the AUG codon. This alternative translation initiation mechanism was first demonstrated in picornaviruses. This strategy of internal initiation is mostly used by viruses which shut down canonical initiation in host cells. This mechanism does not require mRNA 5'm^7G cap structure and hence does not require both eIF4E (cap-binding protein) and the N-terminal region of eIF4G containing the eIF4E-binding domain (Jackson et. al., 2010, Komar and Hatzoglou, 2005).

In cap-independent or internal initiation, ribosomes are internally recruited to the RNA without having to bind at the 5' m^7G cap structure. This alternative form of initiation also does not require 5' m^7G cap for the assembly of initiation factors or cap recognition by the eIF4F cap binding complex. The 40S ribosomal subunit recognizes an RNA sequence within the 5' non-coding region of the RNA and initiation occurs downstream from the 5'end of the mRNA at the start codon. Ribosomes are not able to scan through the 5'UTR if mRNAs have highly structured secondary structures. Hence cap-independent initiation involves features that are different from canonical cap-binding ribosome scanning model (Fitzgerald and Semler 2009) (Fig. 1.4).

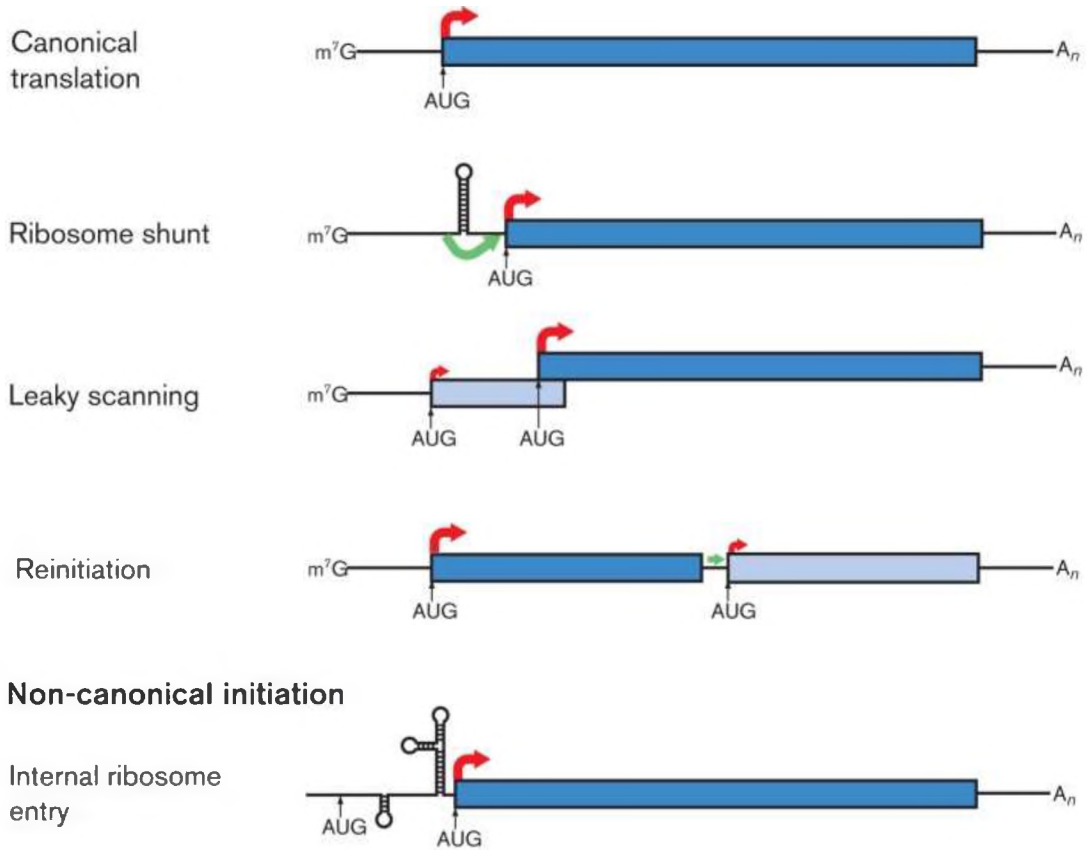


Fig 1.3. Modes of Translation Initiation. Canonical translation initiation: The 40S ribosomal subunit scans mRNA in a 3' direction to locate the AUG start codon, followed by 60S subunit joining and formation of a translationally active 80S ribosome; Ribosome shunting: The ribosome shunts over a large segment with extensive secondary structure; Leaky scanning. The first AUG start codon is by-passed, and translation starts at the next AUG codon; Reinitiation: After initiation at the first start codon, the 40S subunit remains bound to the mRNA and resumes scanning, and re-initiates from a downstream AUG codon; Internal initiation. The 40S ribosomal subunit is recruited directly in close vicinity of the AUG codon surpassing the scanning mechanism (Firth and Brierley, J Gen Virol, 2012).

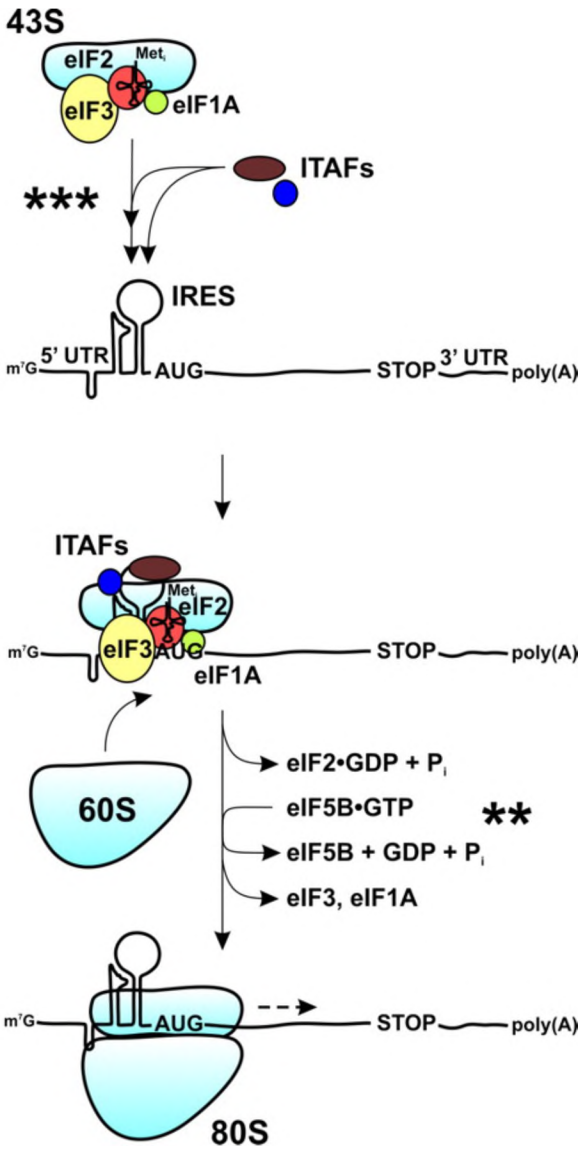


Fig 1.4. Model of internal initiation. This translation initiation mechanism is directed by an IRES element, which involves direct recruitment of the 40S ribosomal subunit to the vicinity of the initiation codon and is generally independent of the recognition of the 5′-mRNA end. Internal ribosome recruitment may require a subset of canonical initiation factors and ITAFs. The 40 S recruitment may either result in recognition of the initiation codon or the 40 S ribosome scans downstream of the internal landing site to locate the initiation codon (Komar and Hatzoglou, J. Bio. Chem, 2005).

1.5 IRES ELEMENTS IN VIRUSES

Internal initiation was first observed during picornaviral infection in viral RNAs. Picornaviruses like poliovirus (PV) and encephalomyocarditis virus (EMCV) were found to have a long and structured 5'UTR and a viral-encoded VPg protein covalently linked to the 5'end, instead of a m^7G cap. These features made the viral RNAs incompatible for cap-dependent initiation and mediated translation via internal binding of ribosomes. These RNA elements were called Internal Ribosome Entry Sites (IRESs). During picornaviral infections, cap-dependent translation of host cells was shut down and the virus RNA operated through IRES-mediated translation initiation to synthesize viral proteins (Fitzgerald and Semler 2009).

Soon after the discovery of IRES elements in Picornaviruses, they were also found in other RNA viruses such as Foot-and-mouth disease virus (FMDV), Hepatitis C virus (HCV), Cricket paralysis virus (CrPV), Human immunodeficiency virus (HIV) and DNA viruses. Most viral IRESs operate via cap-independent initiation mechanism which can proceed in the presence of viral proteases. During viral infection, expression of viral proteases leads to proteolysis of eIF4G and PABP, which inhibits cap-dependent translation of host proteins and leads to protein synthesis shut down. Despite performing the same functions, viral IRESs differ in their nucleotide sequence, RNA secondary structure, initiation factor and trans-acting factor requirements (Martinez-Salas, et. al, 2012).

1.6 IRES TRANS-ACTIVATING FACTORS (ITAFs)

IRES trans-activating factors (ITAFs) are non-canonical factors which bind to IRES elements and function in IRES-mediated translation. These unique RNA-binding proteins play a role in other cellular processes like pre-mRNA processing, alternative splicing, ribosome biogenesis, mRNA export, localization and mRNA stability (Komar and Hatzoglou, 2011). Some notable examples of ITAFs include- polypyrimidine tract binding protein (PTB), heteronuclear ribonucleoprotein (hnRNP), upstream of N-ras (unr), lupus autoantigen (La) etc. A remarkable property of ITAF is that they are nuclear proteins that shuttle from nucleus to cytoplasm to participate in IRES-led translation (Fitzgerald and Semler 2009) (Fig. 1.5).

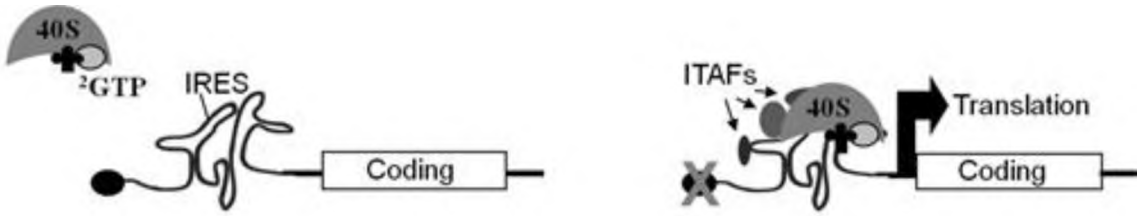


Fig 1.5 IRES trans-acting factors. ITAFs are specific proteins that are required for the recruitment of 43S initiation complex in a cap-independent manner to structurally complex IRES-containing 5'UTR (King et. al., Biochem Soc Trans, 2010).

1.7 CLASSIFICATION OF VIRAL IRES

Viral IRESs function via multiple RNA-protein interactions for ribosome assembly due to unique secondary/tertiary structure in viral RNAs. Viral IRESs are classified into four classes, termed Type I, II, III and IV according to their RNA structure organization. Each class of IRES is characterized by a distinct secondary structure, initiation factors and ITAF requirement (Lee et.al, 2017) (Table 1, Fig. 1.6).

Type I IRES

Type 1 IRES includes picornaviruses like Enteroviruses (e.g., Poliovirus, Coxsackievirus B3) and Rhinovirus (e.g. Human Rhinovirus). Picornaviruses are known to cause a range of diseases in humans like the common cold, poliomyelitis etc. The 5'UTR of type I IRESs are unusually long (around 450 nt) and contains six stem-loops (named domain I-VI). Domain I is critical for viral RNA replication, while domains II to VI function in IRES activity (Lee et. al., 2017, Martinez-Salas, et. al, 2018). During picornaviral infection, cap-dependent translation in host cells is shut down. The RNA virus takes advantage of the IRES elements to highjack the translation machinery and hence evades translation inhibition. These virus RNAs internally recruit the 40S ribosomal subunit by process directed by RNA structural motif, subset of initiation factors and a number of RNA-binding proteins. Type 1 IRESs require almost all initiation factors except eIF4E and N-terminal of eIF4G for IRES activity. During viral infection, eIF4G is cleaved by virus-encoded 2A protease, which results in loss of eIF4E-binding domain (N-terminal of eIF4G). However, type 1 IRESs require C-terminal of eIF4G as

2A proteolysis does not affect eIF3/eIF4A- binding ability. (Martinez-Salas, et. al, 2012, Lee et. al., 2017, Lozano and Martinez-Salas, 2015)

Type 1 IRESs usually have a long 5' non-translated region and include several upstream AUG triplets before the start codon. Domain IV and V are conserved, while domains III and VI are variable. Domain IV forms a cruciform structure which includes two motifs- an internal C-rich loop and a GNRA (N=any nucleotide, R=purine) tetraloop. The C-rich loop of domain IV can recruit PCBP2 to perform its role in translation initiation during viral infection. GNRA tetraloop serves as a primer for RNA-RNA interaction during tertiary structure formation (Lee et. al., 2017, Martinez-Salas, et. al, 2018).

Domain V consists of a hairpin and an internal loop, which provides binding site for eIF4G and for polypyrimidine tract-binding protein (PTB). In most type 1 IRES, the 48S ribosome complex scans some distance within the 5'UTR to locate the start codon. In addition to initiation factors, auxiliary factors termed ITAFs were also needed for viral IRES activity. PTB and PCBP2 are two ITAFs associated to type 1 IRES. PTB is known to recognize U/C rich sequences in viral RNA and facilitates IRES interaction with ribosomal subunits. PCBP2 enhances type 1 viral IRES activity and is absolutely required to assemble 48S initiation complex on IRES (Lee et. al., 2017, Martinez-Salas, et. al, 2018).

Type II IRES

Type 2 IRESs include Cardiovirus (e.g., Encephalomyocarditis virus) and Aphthovirus (e.g., Food-and-mouth disease virus). For the assembly of 48S complex and for translation initiation, type 2 IRESs require eIF4A, eIF2, eIF3 and the C-terminal of eIF4G. Translation initiation complex formation is however independent of cap-binding protein eIF4E. During viral infection, eIF4G is cleaved by virus-encoded L protease, which results in loss of eIF4E-binding domain (N-terminal of eIF4G) (Martinez-Salas, et. al, 2012, Lee et. al., 2017, Lozano and Martinez-Salas, 2015).

The 5'UTR of type 2 IRES is about 450 nt long and contains a conserved pyrimidine tract upstream of the start AUG codon. The RNA structure of type 2 IRES assembles into a complicated secondary structure, arranged into twelve stem-loop structures (A-L) and the IRES element is contained within modular domains (II-V). Domain A functions as cis-acting replication element. Domain II contains a conserved pyrimidine tract which acts as binding site for PTB protein. Domain III adopts a cruciform structure which harbors GNRA tetraloop, RAAA and a C-rich loop. The GNRA tetraloop participates in RNA-RNA interaction and is essential for IRES activity. Domain IV is organized into two hairpin loops (stem loop J and K) which forms a Y-shape RNA essential for eIF4G binding. Domain V consists of a hairpin structure followed by a conserved pyrimidine tract, which provides a binding site for both eIF4B and PTB (Lee et. al., 2017, Martinez-Salas, et. al, 2018).

An interesting feature of type 2 viral IRES is the existence of more than one AUG codon on the initiation zone. Start codon selection and initiation of translation occurs through direct ribosome transfer at the initiation site. Due to long and complex structure

of type 2 IRES, they require several ITAFs to maintain a proper IRES conformation for ribosome assembly (Lee et. al., 2017, Martinez-Salas, et. al, 2018).

Type III IRES

This category includes members of Flaviviridae (e.g., Hepatitis C Virus and Classical swine flu virus). The genome of HCV contains an IRES which is about 340 nucleotide long. Type 3 viral IRESs, have the ability to bind to the 40S ribosome subunits directly, independent of most canonical initiation factors and the ribosomal P site is placed in the immediate vicinity of the initiation codon. For the assembly of a stable 48S complex, HCV-like IRES requires the ternary complex (eIF2•GTP•Met-tRNA$_i$) and eIF3. HCV-like IRESs have no requirement for canonical initiation factors like those of the eIF4F family, eIF4A, eIF4B or ATP hydrolysis. Some studies show that eIF3 binds to the IRES-40S complex and stabilizes it but may not be required for the formation of the 48S complex (Hellen and Sarnow, 2001).

The RNA structural organization of type 3 IRESs differ from IRESs belonging to type 1 and 2. The 5'UTR of HCV possess a poly(U) tract and a complex structure (pseudoknot) upstream of the AUG codon and is divided into four domains (I-IV). Domain II is involved in eIF2 GTP hydrolysis, 60S subunit joining and helps to accommodate the mRNA in the tRNA exit site of the ribosome. Domain III binds to the 40S subunit and eIF3, domain IV harbors the AUG codon and the pseudoknot facilitates AUG recognition by the 40S subunit. Type 3 IRESs require a number of ITAFs to induce IRES conformational changes. These include PTB, PCBP2, La protein, NSAP1, hnRNP D and hnRNP L, Nucleolin, Unr and several others (Lee et. al., 2017, Martinez-Salas, et. al., 2012).

Type IV IRES

This group includes Dicisroviruses e.g. Cricket Paralysis virus. Type 4 IRESs are short in length and use a uniquely distinct mechanism to promote internal initiation. 48S complex assembly takes place without the requirement of any canonical initiation factors, ternary complex or the initiator tRNA. Initiation begins at a non-AUG codon (e.g. GCU, CCU, CUC, CUU codons) instead of an AUG codon. The genome of CrPV contains two large ORFs, the first encoding the non-structural proteins and the second encoding the capsid proteins. An intergenic region (IGR) of 190 nucleotides present between these two coding regions contains an IRES. The IGR is arranged into three pseudoknots (PKI, PKII and PKIII) which substitutes for the initiator Met-$tRNA_i$ directing translation initiation at a non-AUG triplet. PKI occupies the ribosomal A-site and forms a structure that mimics tRNA/mRNA interaction and PKII plays a role in 60S association. The IGR IRESs binds with ribosomes in an elongation-competent state and begin translation with Alanine-tRNA instead of Methionine-$tRNA_i$ (Lee et. al., 2017, Martinez-Salas, et. al, 2012).

A current model suggests that PKI binds to the A-site of the 40S subunit and eEF2 triggers the translocation of PKI to the P-site, leaving an empty A-site. The elongation factor eEF1A brings Ala-tRNA to the A-site. After this, the 80S ribosome undergoes a pseudo-translocation (without peptide bond formation) and eEF2 promotes pseudo-translocation, moving the codon (GCU) to the ribosomal P-site. Type 4 IRESs mimic the elongation step and bypasses the initiation step, hence exploiting the dynamic properties of the ribosomes (Lee et. al., 2017, Martinez-Salas, et. al, 2012, Lozano and Martinez-Salas, 2015).

HEPATITIS A VIRUS IRES

HAV contains an IRES in its 5'UTR but is not categorized in any of the four classes of viral IRES due to its unique characteristics distinct from other viral IRESs. HAV lack of sequence relatedness with other viral IRESs and is not able to shut down host cell protein synthesis. Unlike other viral IRES, HAV IRES activity requires most initiation factors (eIF2, eIF4E, eIF4G, eIF4A) (Lopez-Lastra et. al., 2005).

Although HAV mRNA does not have a 5'cap structure, it requires the cap-binding protein eIF4E to activate HAV IRES-mediated translation. Unlike type 1 and type 2 viral IRES, HAV IRES activity is strongly inhibited if eIF4G is cleaved by viral proteases (2A protease or L protease). The translation initiation driven by HAV IRES is dependent on eIF4E and intact eIF4F complex. It has been demonstrated that HAV IRES has initiation factor requirements similar to those of capped mRNAs for translation initiation by ribosome scanning mechanism. Like type 1 and type 2 viral IRES, HAV IRES activity is found to be stimulated by ITAFs- PTB and PCBP2 (Sadahiro et. al., 2018, Redondo et. al., 2012, Ali et. al., 2001).

VIRAL IRES TYPE	INITIATION FACTORS REQUIRED	VIRUS SPECIES
Type 1	eIF3, eIF1, eIF1A, eIF5, eIF2, eIF4G, eIF4A, eIF4B, and eIF5B	Poliovirus Human Rhinovirus Human Coxsackievirus
Type 2	eIF3, eIF1, eIF1A, eIF5, eIF2, eIF4G, eIF4A, eIF4B, and eIF5B	Encephalomyocarditis virus Foot-and-mouth disease virus
Type 3	eIF2, eIF3, and eIF5	Hepatitis C Virus
Type 4	None	Cricket Paralysis Virus
HAV-type	All eIFs	Hepatitis A virus

Table 1. Viral IRES Classification and initiation factor requirements. Adapted from (Jackson et. al., Nat Rev Mol Cell Biol, 2010; Ali et. al, 2001).

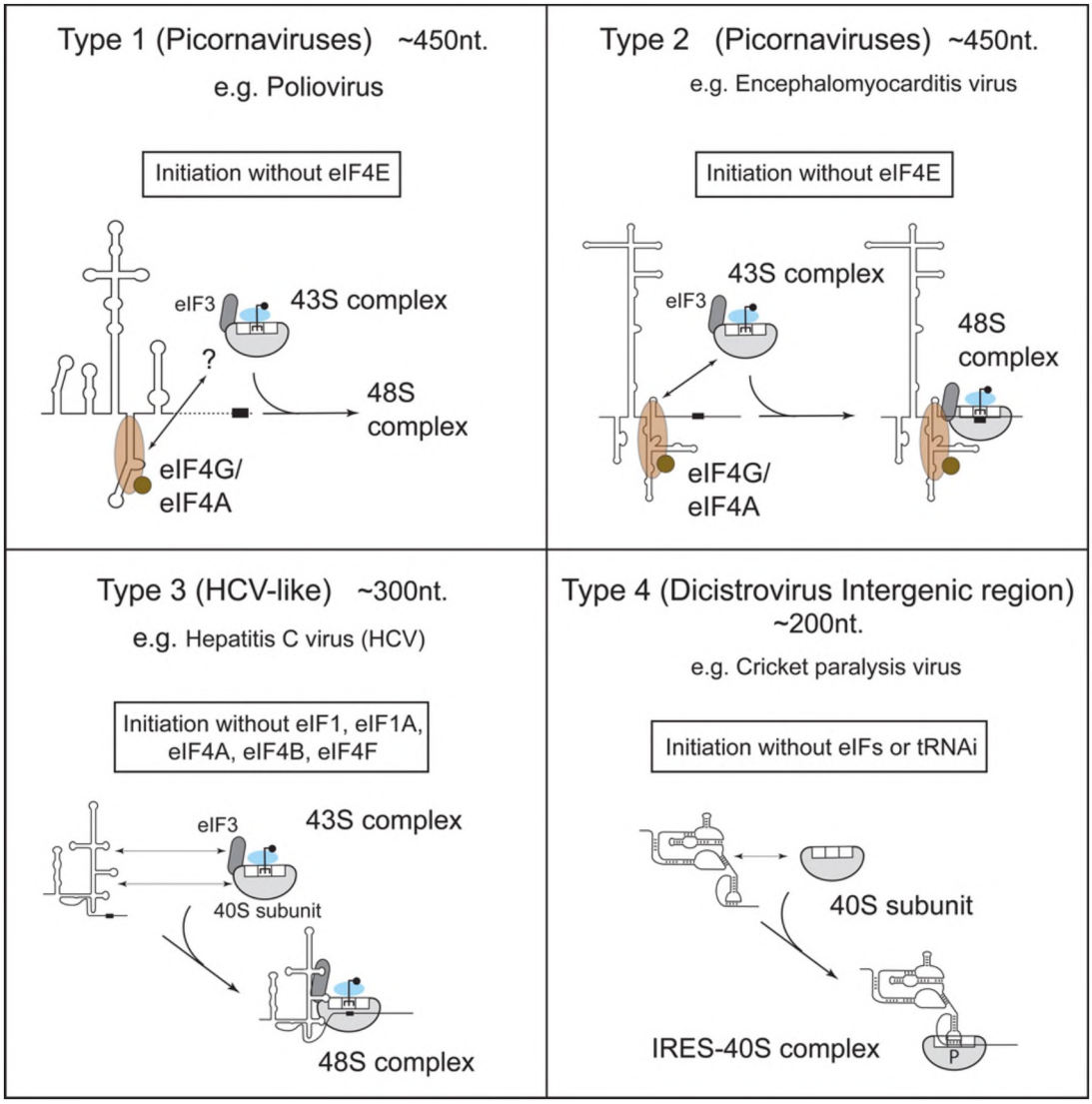


Fig 1.6. Major Classes of IRES in RNA Viruses. The diagrams show the secondary structure of 5′ UTRs and initiation factor requirements for (A) picornavirus type I, (B) picornavirus type II, (C) Type III HCV (hepatitis C virus), and (D) Type IV CrPV IGR IRES (Jackson et. al., Nat Rev Mol Cell Biol, 2010).

1.8 CELLULAR IRES

Unlike viral mRNAs, all cellular mRNAs are capped and undergo cap-dependent translation initiation. However, in conditions of stress, such as nutrient deprivation, hypoxia, mitosis or apoptosis, global cap-dependent translation is diminished. Cellular mRNAs which contain IRES elements in their 5'UTR sustain protein expression by internal ribosome recruitment, bypassing 5'cap usage. The first cellular IRES was discovered in mRNA encoding immunoglobulin heavy chain-binding protein (BiP), which is a stress induced chaperone protein. BiP IRES was found to sustain translation and was activated during viral infection (Leppek et. al., 2018).

Approximately 3-5% of mammalian mRNAs are predicted to harbor IRES element. These mRNAs mostly encode oncogenes, growth factors, apoptotic proteins, transcription factors and transporters. Some of the well-known and widely studies mammalian IRESs include- Apaf-1, XIAP, c-Myc, CAT-1, VEGF, FGF2 and several others. Cellular IRESs are less structure than viral IRES and have few structural similarities. The action mechanism of most cellular IRESs are unknown (Komar and Hatzoglou, 2011, Martinez-Salas, et. al, 2012).

Cellular IRESs are known for two major physiological functions-

a) To support low levels of translation initiation under normal physiological conditions for mRNAs having highly structured 5'UTR.
b) To support elevated levels of translation under stress-related physiological conditions when cap-dependent translation is hampered.

Most IRES-containing cellular mRNAs have long, GC rich and highly structured 5'UTR which may contain several upstream AUG codons, hence incapable of mediating efficient cap-dependent translation. Many physiological and stress conditions (e.g. ER stress, hypoxia, nutrient deprivation, apoptosis, mitosis, cell differentiation) result in inhibition of cap-dependent and increase in IRES-mediated translation. It is interesting to note that many IRES-containing cellular mRNAs encode stress-related proteins. Under these conditions, most canonical initiation factors (eIF2, eIF4E, eIF4G) are inactivated or degraded. Hence like viral IRES, most cellular IRESs can also function without participation of canonical initiation factors (Komar and Hatzoglou, 2011).

1.8.1 SIGNIFICANCE OF IRES-MEDIATED TRANSLATION

The role of IRES-mediated translation has been studied in several physiological and pathological stress conditions. A number of cellular responses like growth, proliferation, nutrition and environmental signals lead to IRES-mediated translation in certain genes. Physiological stress-related conditions like apoptosis, heat shock, radiation, amino acid starvation, mitosis may allow IRES-containing genes to be translated. Most of these genes help the cells to cope during stress conditions and are mostly regulators of cell survival, death or proliferation. Different IRESs may be regulated by different conditions, for example, VEGF IRES is regulated by hypoxia, IRES of c-Myc is regulated following genotoxic stress and XIAP IRES is regulated by γ-irradiation or anoxia. (Komar and Hatzoglou, 2011, Ozretić et. al., 2012).

GROWTH FACTORS

A widely studies cellular IRES-containing gene is Fibroblast growth factor-2 (FGF2) which promotes epithelial proliferation and angiogenesis. FGF2 5'UTR is long and structured and contains an IRES element. Studies in breast carcinomas have found translational deregulation of FGF2 (Ozretić et. al., 2012).

Proteins of the VEGF family play a key role in angiogenesis and stimulates endothelial cell proliferation, migration and their proteolytic activity. Another well studies cellular IRES-containing gene is vascular endothelial growth factor A (VEGF-A), which is important for pathological angiogenesis in tumors and is upregulated in tumorigenesis. VEGF-A mRNA contains two IRES sequences, synthesizing two different protein isoforms. IRES-dependent translation of VEGF-A was found to be enhanced in breast cancer cells with increased levels of eIF4G and 4E-BP-1 (Komar and Hatzoglou, 2011, Ozretić et. al., 2012).

Human insulin-like growth factor 2 (IGF2) mRNA contains an IRES element in its 5'UTR which supports cap-independent translation. IGF2 functions to regulate fetal development and growth and has been associated with a number of cancers, lung, prostate, colon and hepatocellular carcinomas (Ozretić et. al., 2012).

ONCOGENES AND TUMOR SUPPRESSORS

The tumor suppressor gene p53 is a well-studied cellular IRES, which functions in controlling cell-cycle progression and cell survival (Yang et. al., 2006). p53 mRNA contains two IRES, one in its 5'UTR and the other extends into the protein-coding region. p53 IRES located in the 5'UTR directs translation of full-length p53 protein and this

IRES is activated at the G2-M transition stage. The other IRES mediates translation of ΔN-p53 isoform and is highly active at the G1-S transition stage (Ozretić et. al., 2012).

Myc family members (c-Myc, N-Myc, L-Myc) are known to contain IRES elements in their 5'UTR and have been extensively studied. Members of the myc family are potent oncogenes and their deregulation is frequently found in cancers. The c-Myc mRNA is capped at its 5'terminal and is able to translate via both cap-dependent and cap-independent mechanisms (Ozretić et. al., 2012).

APOPTOTIC PROTEINS

During apoptosis, many initiation factors (eIF4G, eIF2α, eIF4B) are degraded by caspases, which disables the protein synthesis machinery and halts protein synthesis (Clemens et. al., 2000). A number of apoptosis regulating genes are found to be translated by IRES-dependent mechanism, which determines fate of the cell during transient apoptotic stress leading to cell survival or cell death.

BCL2-associated athanogene (BAG1) encodes a multifunctional protein BAG-1 which functions to regulate signal transduction, protein folding and cell survival. BAG-1 is found to be translated by IRES-driven mechanism when cells are subjected to heat shock (Ozretić et. al., 2012).

Apoptotic protease-activating factor (Apaf-1) encodes Apaf-1 protein which is an essential component of the apoptosome. Apaf-1interacts with inactive procaspase-9 and facilitates its proteolytic cleavage and activation. It contains an IRES element in its 5'UTR which functions to induce apoptosis (Ozretić et. al., 2012).

X-linked inhibitor of apoptosis (XIAP) is an intrinsic inhibitor of apoptosis, which inhibits caspase-3, caspase-7 and caspase-9. XIAP mRNA contains an IRES element in its unusually long 5'UTR which supports IRES-mediated translation when cells are subjected to serum starvation, anorexia or irradiation. IRES-mediated translation of XIAP under stress conditions may therefore enhance the survival of cancer cells and may lead to cancer progression (Ozretić et. al., 2012).

TRANSPORTER PROTEINS

Cationic amino acid transporter (Cat-1) contains an IRES element which was found to be activated during amino acid deprivation (Fernandez et. al., 2001). Mammalian cells subjected to amino acid starvation result in eIF2α phosphorylation and dephosphorylation of eIF4E. Phosphorylation of eIF2α reduces the availability of active eIF2α which results in reduced ternary complex formation and dephosphorylation of eIF4E results in reduced concentration of active eIF4F. These events stalls host cell protein synthesis. The starved cells respond to these stressful conditions by synthesizing proteins involved in amino acid biosynthesis and amino acid transporters (such as Cat-1), which mediates lysine and arginine uptake (Fernandez et. al., 2002, Hellen and Sarnow, 2001).

SNAT2 is a ubiquitously expressed member of the Sodium coupled Neutral Amino acid Transporter family. Amino acid starvation causes nutritional stress in cells, leading to a decrease in cap-dependent translation initiation and reduced global protein synthesis. Under such conditions, translation is promoted for proteins that are essential for cellular stress response. Amino acid starvation was found to induce SNAT2 mRNA

and protein levels. The 5'UTR of SNAT2 was reported to contain an IRES element that is active under amino acid fed and starvation conditions. Amino acid starvation caused an increase in mRNA and protein expressions by 2.5 folds. Amino acid starvation causes dephosphorylation of 4E-BP1, reducing eIF4F activity. Amino acid starvation also caused eIF2α phosphorylation, reducing the availability of ternary complex. The increase in SNAT2 expression was correlated with eIF2α phosphorylation (Gaccioli et al., 2006).

1.8.2. STRUCTURE OF CELLULAR IRES

Most IRES elements are located in the 5'UTR region of mRNAs upstream of the initiation codon, although some may also be located in the mRNA coding region. Cellular IRESs have not been classified into groups due to the lack of similarities in primary sequence or common structural motif. Cellular IRESs have complex secondary structures which include stem loops and pseudoknots. These structures have been identified for a number of cellular IRESs using chemical and enzymatic probing. These include c-Myc, FGF-2, Apaf-1, Bag-1, Cat-1 and other transcripts. Computational analysis of the 5'UTR of BiP and FGF-2 identified a common Y-stem loop structure, which shared some similarities with the structural motif found in picornaviruses, pestiviruses and HCV IRES. However only some IRESs displayed structure preservation in order to retain complete IRES activity (Fitzgerald and Semler 2009, Komar and Hatzoglou, 2011).

It has been proposed that cellular IRESs may not have well-defined overall structure and may perhaps depend upon short motifs and ITAFs for their functions. Studies have proposed that RNA-binding proteins unwind complex RNA structure of the IRES, and a single-stranded RNA is made available for other ITAFs or 40S subunit to

bind to. A well-known example is Bag-1 IRES, which involves the activity of PCBP1 to open Bag-1 IRES structure to further allow binding of PTB-1 (Fig. 1.7). Another example includes Apaf-1 IRES, where Unr opens up IRES secondary structure which exposes recognition sequences and allows PTB to bind. The Unr protein binds to the highly structured Apaf-1 IRES at a purine-rich region and opens stem-loop structures to allow PTB binding (Baird et al., 2006, Leppek et. al., 2018).

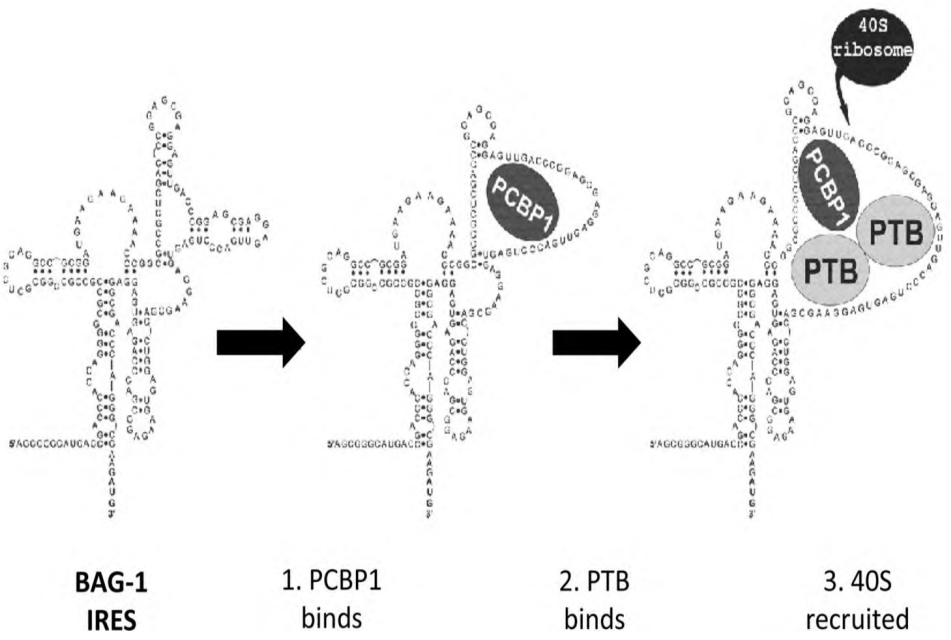
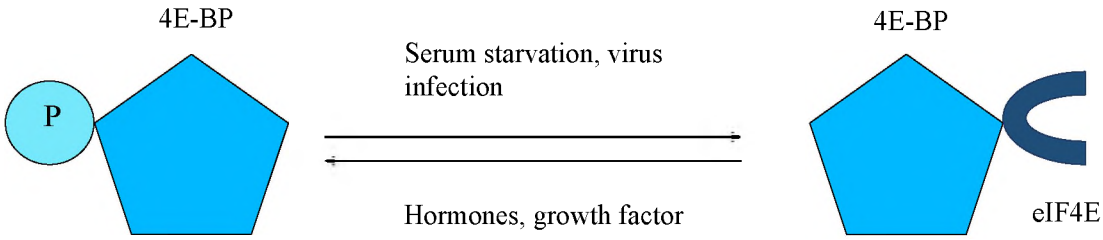


Fig 1.7. ITAF binding to Bag-1 IRES changes its structural conformation. Bag-1 mRNA contains an IRES which has been shown to require both PCBP1 and PTB for its activity. Initially, the protein PCBP1 binds to a stem–loop in the IRES, which modifies the loop structure in Bag-1 IRES. This allows two PTB proteins to bind Bag-1 IRES, which in turn allows recruitment of the 40S ribosomal subunit. In this example, the ITAFs are behaving as RNA chaperones (Pickering et. al., Molecular and Cellular Biology, 2004).

1.8.3. INITIATION FACTORS REQUIRED FOR CELLULAR IRESs

Cellular IRESs like viral IRESs are known to exhibit multiple interactions with components of the translation machinery (40S ribosomal subunit, initiation factors and ITAFs) (Godet et. al., 2019). Different cellular IRESs may require specific set of canonical initiation factors and ITAFs for proper positioning of the initiation codon at the ribosomal P-site, with or without ribosomal scanning from the 5'end. Cellular stress conditions induce a switch from cap-dependent translation to IRES-mediated translation. During cellular stress conditions or apoptosis, canonical initiation factors become inactivated or are degraded. IRESs of the same family of proteins can also have different eIF requirements. Cellular IRES-containing mRNAs like c-myc and N-myc require eIF3 and eIF4A for their activity but may function without eIF4E or intact eIF4G. On the other hand, another member of the myc family, L-myc requires both eIF4E and intact eIF4G for translation. A number of cellular mRNAs are known to have reduced requirement for eIF2α and are to be insensitive to eIF2α phosphorylation, a translation inhibiting factor. Some examples that are widely studied include c-Src, N-myc, Cat-1, etc (Komar and Hatzoglou, 2011).

The cap-binding protein eIF4E of the eIF4F cap-binding complex is a major target for regulation of protein synthesis in mammalian cells. The activity of eIF4E is regulated by altering its phosphorylation state (Proud, 2005). Phosphorylation of eIF4E is increased when cells are stimulated with mitogens, serums or growth factors, which leads to increased translation in cells. On the other hand, phosphorylation of eIF4E decreases when cells are exposed to heat shock, serum depletion, during mitosis or when cells are infected with virus, which leads to translation inhibition in cells (Fig. 1.8). The activity of eIF4E is also regulated by physical sequestration with a protein named 4E-binding protein (4E-BP). 4E-BP binds to eIF4E and prevents the formation of eIF4F complex, hence inhibiting cap-dependent translation (Proud, 2005). Studies have indicated that eIF4E bound to 4E-BP is unable to form the eIF4F complex. Heat shock and serum depletion like stress conditions induces dephosphorylation of 4E-BP, which causes it to bind to eIF4E, hence competing with eIF4F complex formation. This eventually results in inhibition of protein synthesis in cells. However, in the presence of growth factors or insulin, 4E-BP becomes heavily phosphorylated and releases eIF4E, which allows it to interact with eIF4A and eIF4G and assemble the eIF4F complex. This eventually stimulates translation in cells (Feigenblum and Schneider, 1996).



Cap-dependent translation

Cap-dependent translation inhibition

Fig 1.8. Cellular stress conditions induce eIF4E inactivation. Serum starvation or picornavirus infection induces hypo-phosphorylation of 4E-BP, allowing it to compete for binding to eIF4E with eIF4G, causing eIF4F levels to become limiting. During growth conditions, 4E-BP is hyper-phosphorylated and is no longer able to bind eIF4E (King et. al., Biochem Soc Trans, 2010).

Phosphorylation of eIF2α is another mechanism involved in regulation of translation initiation. During high nutrient availability and low stress conditions, eIF4F and eIF2 ternary complex is abundantly available, which promotes high levels of cap-dependent translation. Nutritional stresses like glucose depletion or amino acid deprivation induces phosphorylation of eIF2α and results in reduction in global translation. Increased eIF2α phosphorylation leads to eIF2B inhibition and insufficient exchange of eIF2•GDP to eIF2•GTP. During nutrient deprivation conditions, serine 51 of eIF2α is phosphorylated, which engages with eIF2B and competes with its GDP exchanging capacity (Fig. 1.9). Other stress conditions like heat shock, hypoxia, ER stress, UV irradiation and viral infection can also lead to eIF2α phosphorylation and downregulate eIF2 activity (Komar and Hatzoglou, 2005). eIF2α can be phosphorylated by four different kinases, each of which monitors different exogenous and endogenous stresses. GCN2 is an eIF2α kinase induced by nutrition stress, ER stress signals PKR-like endoplasmic reticulum kinase (PERK), heme deprivation in erythroid cells activates heme-regulated eIF2α kinase (HRI) and protein kinase R (PKR) which is involved in antiviral defense. Increased eIF2α phosphorylation reduces eIF2•GTP recycling and hence reduces levels of eIF2-ternary complex (Baird and Wek, 2012).

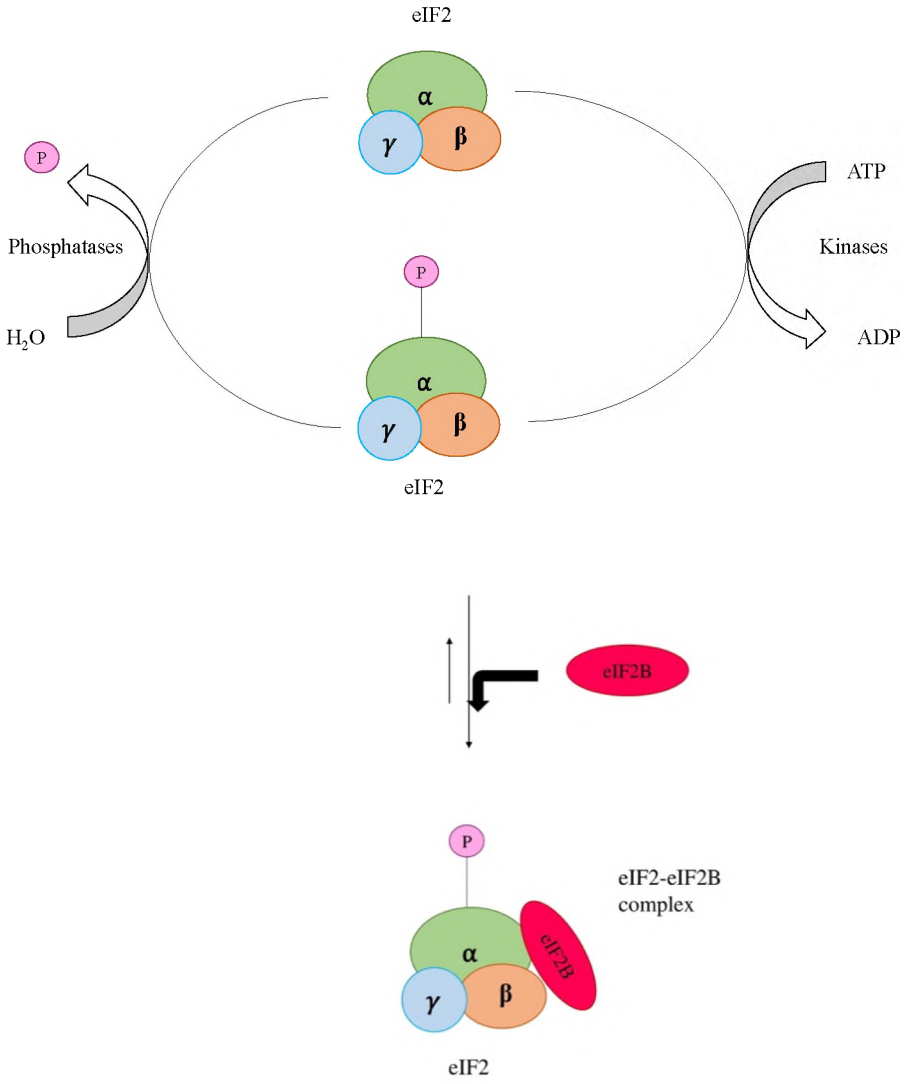


Fig 1.9. Cellular stress conditions induce eIF2 inactivation. Kinases such as PKR leads to phosphorylation of the α subunit of eIF2. Phosphorylated eIF2 binds with stronger affinity to guanine-nucleotide-exchange factor eIF2B, resulting in reduced levels of ternary complex (King et. al., Biochem Soc Trans, 2010).

Cap-dependent translation initiation may also be compromised as a result of degradation of eIF4G during cellular stress situations like viral infection or apoptosis. Viruses like rhinovirus, polioviris, aphthovirus encode proteases (2A protease or L protease) which cleaves eIF4GI and eIF4GII producing N-terminal and C-terminal fragment (Fig. 1.10). Retrovirus like HIV encodes PR protease which cleaves eIF4GI into three fragments- N-terminal, central domain and C-terminal domain (Morley et. al., 2005). eIF4G is an adaptor protein which binds to eIF4E at N-terminal, and with eIF3 & eIF4A at the C-terminal moiety. These interactions are essential for ribosome binding at the 5'terminal and are compromised by eIF4G degradation. The N-terminal fragment bound to eIF4E mediates cap recognition and the C-terminal fragment mediates mRNA ribosome interaction through eIF3. Degradation of eIF4G by viral protease uncouples eIF4G activities, leading to loss of cap-dependent translation initiation (Haghighat et. al., 1996, Ventoso et. al., 2001). Studies have also shown that eIF4G is susceptible to degradation during apoptosis induced by serum depletion, etoposide or cycloheximide treatment in different cell lines. eIF4G is cleaved by the action of caspase-3 leading protein synthesis inhibition. Reports have found that eIF4G is degraded as a result of cellular apoptotic response and it can be blocked by caspase inhibitors (Clemens et al., 1998, Bushell et al., 1999).

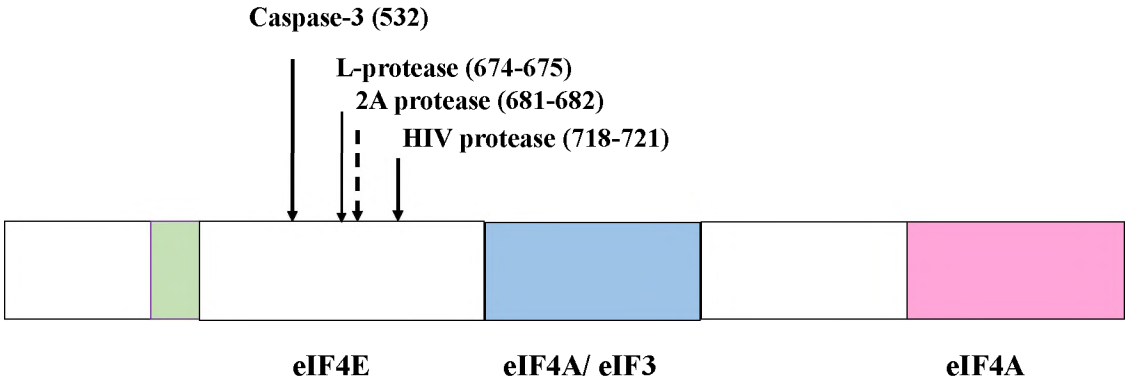


Fig 1.10. Cleavage of eIF4G: eIF4G is cleaved by Caspase-3, viral L-protease, 2A protease and HIV protease at different sites. eIF4G is cleaved in such a way which separates eIF4E binding domain from the eIF4A/eIF3 binding domain (Adapted from Morley et. al., Cell Death and Differentiation, 2005).

1.8.4 ROLE OF ITAFs IN CELLULAR IRES-MEDIATED TRANSLATION

IRES trans-acting factors (ITAFs) are RNA-binding proteins that are normally not utilized in canonical translation initiation. ITAFs act as auxiliary proteins that enable cellular IRESs for maximum activity (Godet et. al., 2019). ITAFs are proteins that have the ability to make multiple interactions with the IRES and modulate IRES conformation. ITAFs may possess chaperone activity which helps the IRES to fold into a conformation that is appropriate for translation initiation. Some of the most common ITAFs known for cellular IRESs include- PTB, PCBP2, Unr, hnRNP C, hnRNP K, hnRNP P, La protein and several others. Some cellular IRESs may require not just a single ITAF, but a combination of two or three ITAFs to function efficiently. For example, La and hnRNP C are required for the activity of XIAP IRES. Activation of c-myc IRES involves PCBP1, PCBP2, hnRNP C and hnRNP K. PTB and Unr proteins are required for Apaf-1 IRES activity (Godet et. al., 2019) (Table 2).

Most known ITAFs are confined to the nucleus where they have a role in nuclear RNA metabolism. These factors diffuse into the cytoplasm under specific cellular stress conditions to participate in cap-independent translation. The cytoplasmic enrichment of ITAFs is believed to be responsible for increased IRES activity for some IRESs (Lewis and Holcik, 2008). However, some ITAFs have also demonstrated to act negatively and inhibit IRES activity. For example, PTB inhibits BiP IRES activity and acts as a negative ITAF (Kim et al., 2000). RNA-binding protein HuR is known to interact with AU-rich elements and decreases p27 IRES activity (Kullmann et. al., 2002). Many cellular IRESs have also found to exhibit cell type-specific variations. IRESs of a particular gene may be more active in certain cells and under specific physiological conditions. This may be

governed by positive or negative ITAF regulation. Studies have demonstrated that an ITAF can function differently for different IRESs. For example, during HRV2 infection or after UV irradiation, hnRNP A1 relocalizes from nucleus to cytoplasm. hnRNP A1 in the cytoplasm enhances HRV-2 translation following infection but inhibits Apaf-1 IRES activation following UV irradiation (Hellen and Sarnow, 2001, Lopez-Lastra et. al., 2005, Fitzgerald and Semler 2009). There is also evidence of an IRES being regulated by different ITAFs (positive or negative regulator) depending of cell environmental and physiological conditions. For example, p53 IRES, which is induced by genotoxic or cytotoxic stress, is acted upon by negative ITAFs nucleolin and PDCD4 to inhibit p53 IRES activity in non-stressed cells. Alternatively, during stress, positive ITAFs RPL26 and hnRNP Q binds to p53 IRES to facilitate secondary structure unwinding and leads to IRES activation. IRES activity is also found to be tissue specific in some cases. An example includes FGF family of proteins, where FGF1 IRES is strongly controls the FGF1 expression during myoblast differentiation and muscle regeneration. In contrast, FGF2 IRES activity was found to be strongest in brain and testis during spermatogenesis and during the formation of synaptic networks between neurons for translational induction (Godet et. al., 2019).

Cellular IRES-containing mRNA	Encoded Protein Function	ITAFs required
Apaf-1	Pro-apoptotic protein	PTB, UNR
Bag-1	Anti-apoptotic protein	PTB, PCBP1
BiP	ER chaperone	PTB, hnRNPQ
Cat-1	Amino acid transporter	PTB, hnRNPL
C-myc	Transcription factor	PTB, PCBP1, PCBP2, hnRNPA1, hnRNPK
FGF2	Growth factor	hnRNPA1
HIF-1	Transcription factor	PTB
p53	DNA damage response	PTB
XIAP	Anti-apoptotic protein	PTB, hnRNPA1, hnRNPC1/C2, La,

Table 2. Cellular IRESs and identified ITAFs. Adapted from King et. al., Biochem Soc Trans, 2010; Pichon et. al., 2012.

1.9 PUMA (P53 UPREGULATED MODULATOR OF APOPTOSIS)

PUMA belongs to the Bcl2 homology 3 (BH3) only Bcl2 family and is an essential mediator of p53 dependent and independent apoptosis. PUMA was identified in cells as a gene activated by p53 (Nakano and Vousden, 2001). During genotoxic stress such as DNA damage, PUMA is transactivated by p53. PUMA is responsible for all proapoptotic activity of p53. PUMA may also be activated by p53-independent mechanism in response to non-genotoxic stimuli like ER stress, growth factor or cytokine depletion (Yu and Zhang, 2008). PUMA is shown to induce apoptosis in a number of cell types including fibroblasts, lymphocytes, neurons and cardiomyocytes. Like other BH3 proteins, PUMA acts as a signaling molecule which transduces death signals to the mitochondria. PUMA acts through multidomain Bcl2 family members in order to induce mitochondrial dysfunction and caspase activation. The BH3 domain of PUMA is involved in interactions with other anti-apoptotic Bcl-2 proteins like Bcl-2 and Bcl-X_L. PUMA interacts with Bcl-2 and Bcl-X_L proteins and inhibits their activity as well as relieves inhibition of pro-apoptotic proteins Bax and/or Bak leading to their activation (Yu and Zhang, 2008) (Fig. 1.11).

Structural analysis of PUMA domains has found that PUMA contains two functional domains- the BH3 domain and the mitochondrial localization signal (MLS). PUMA BH3 domain forms an amphipathic α-helical structure that binds directly to Bcl-2 family anti-apoptotic proteins (Yu and Zhang, 2008). The C-terminal region of PUMA contains a hydrophobic domain (MLS) that is essential for its mitochondrial localization. PUMA protein localizes to the mitochondria to interact with anti-apoptotic Bcl-2 family members to inhibit their suppression of pro-apoptotic proteins (Bax and Bak). PUMA

heterodimerizes with Bcl-2 and Bcl-X_L, antagonizing their function by dissociating them from Bax. This induces cytochrome c release resulting in caspase 3 and caspase 9 activation. PUMA nucleotide and protein structure is highly conserved between mouse and human, sharing over 90% sequence identity. The genomic region of PUMA includes the promoter region, exon 1a and intron 1, which contains a high percentage of guanine and cytosine nucleotides and has a tendency to form strong secondary structure. The basal expression levels of PUMA in unstressed cells has been found to be low which may be an outcome of its genomic secondary structure that leads to reduced PUMA expression (Nakano and Vousden, 2001, Yu and Zhang, 2008).

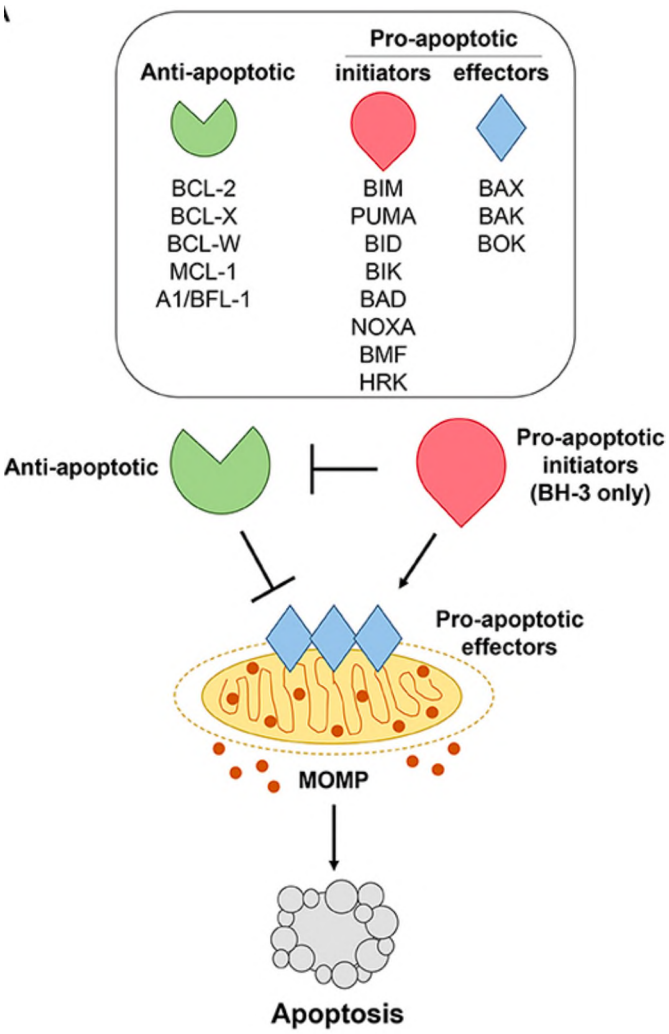


Fig 1.11. Apoptotic pathway. In response to cytotoxic stimuli, pro-apoptotic BH3-only proteins-BIM, BID, NOXA, and PUMA, interact with the BH3 domain of anti-apoptotic members- BCL-X_L, and BCL-2 and inactivates them. Inactivation of anti-apoptotic members changes the inactive form of BAX/BAK to the active form, resulting in oligomerization of BAX/BAK in the mitochondrial outer membrane. Oligomerized BAX/BAK form a pore to release mitochondrial protein cytochrome c into the cytoplasm, enabling the release of apoptotic factors and caspase activation, resulting in apoptosis induction (Adams et. al., Front Oncol, 2019).

1.9.1 PUMA IS REQUIRED FOR APOPTOSIS DURING SKELETAL MYOBLAST DIFFERENTIATION

Many cell types undergo differentiation and apoptosis, which are regulated throughout development to maintain homeostasis. Apoptosis during myoblast differentiation is considered to be essential for removing excess myoblasts during muscle regeneration and myogenesis. Previously, it was identified that during skeletal myoblast differentiation, approximately 30% of myoblast cells undergo apoptosis. 23A2 myoblast cells cultured in differentiating media resulted in activation of caspase 9 and caspase 3. DM induced apoptosis in myoblast cells and this was confirmed by cytochrome c release into the cytosol when cells are switched from growth media to differentiating media (Dee et. al., 2002; Shaltouki et. al., 2007). These experimental evidences led to the identification of pro-apoptotic protein PUMA whose expression increased on culturing skeletal myoblast cells in differentiating media. Studies by Weyman and co-workers have reported the role of a muscle regulatory transcription factor MyoD in apoptosis induced by culturing myoblasts and fibroblasts in DM. MyoD is reported to modulate apoptosis by inducing PUMA and hence plays an apoptotic role during cellular differentiation (Harford et. al., 2010). Furthermore, silencing of MyoD was found to abrogate apoptosis in myoblasts cultured in DM and ectopic expression of MyoD correlated with PUMA induction (Harford et. al., 2017).

1.9.2 TRANSLATIONAL REGULATION OF PUMA

PUMA translation was found to be involved in apoptosis when myoblast cells were induced to differentiate in differentiating media. PUMA was also found to play a critical role in apoptosis that occurs in response to DNA damaging agents like etoposide. Weyman and co-workers in collaboration with our group have reported the identification of an IRES element in PUMA 5'non translated region (Shaltouki et. al., 2013). This IRES element was found to facilitate PUMA translation in skeletal myoblast cells when cultured in differentiating media or when treated with etoposide. In differentiating media, there was a decrease in total protein synthesis and apoptosis in a fraction of cells. Apoptosis results in caspase-dependent cleavage of a number of canonical initiation factors which are essential for cap-dependent translation. They found reduced global translation which correlated with hypo-phosphorylation of 4E-BP. 4E-BP is phosphorylated in response to serum induction, which prevents its association with eIF4E and allows translation initiation. In the absence of serum (differentiating media conditions), 4E-BP is hypo-phosphorylated which leads to its association with eIF4E, resulting in eIF4E inactivation and loss of cap-dependent translation initiation. Decrease in global translation also correlated with phosphorylation of eIF2α, which leads to its inactivation and inability to participate in translation initiation.

Weyman and colleagues were the first to report the presence of an IRES element in PUMA 5'UTR which participates in IRES-mediated translation when differentiation is induced in skeletal myoblast cells (Shaltouki et. al., 2013). PUMA IRES activity increased when cells were switched from growth media to differentiating media. PUMA mRNA consists of a 261 bp 5'UTR which has high GC content and is predicted to form a

highly stable structure (using mFold) with a ΔG value of -91.74 kcal/mol. RNA structures with a ΔG value of -60 to -80 kcal/mol are known to completely inhibit ribosome scanning in mammalian systems. Therefore, ΔG value of -91.74 kcal/mol would inhibit the scanning mode of translation. Several monocistronic and bicistronic assays were performed using different constructs of PUMA to understand translation driven by PUMA IRES element in growth media vs in differentiating media. A hairpin structure was inserted upstream of a reporter construct, which was found to inhibit expression of the reporter gene. Surprisingly, the hairpin structure was not able to inhibit expression of the reporter gene driven by PUMA IRES element. In addition to the above experiments, PUMA IRES was verified for its RNA integrity, i.e., absence of alternative splicing in differentiating media and lack of cryptic promoters in bicistronic constructs (Shaltouki et. al., 2013). However, molecular mechanisms governing PUMA translation in DM conditions remains to be explored. In this study, we investigated initiation factor requirements for PUMA translation and identified specific ITAF necessary for PUMA translation. Finally, we also analyzed the mode of recruitment of 40S ribosomal subunit to PUMA IRES.

CHAPTER II

MECHANISM OF 40S RECUITMENT TO PUMA IRES AND INITIATION FACTOR REQUIREMENTS FOR TRANSLATION

2.1 ABSTRACT

Previous study by Weyman and colleagues have identified the presence of an IRES element in PUMA 5'UTR region (Shaltouki et. al., 2013). IRES-driven translation of PUMA was activated when cells were cultured in differentiating media. Differentiating media conditions causes cellular stress and results in down regulation of protein synthesis. Differentiation-led cellular stress in cells also resulted in inactivation of canonical initiation factors like eIF4E. Under DM conditions, 4E-BP was hypo-phosphorylated, which led to eIF4E inactivation. In addition, differentiation of cells also led to inactivation of another canonical initiation factor, eIF2. eIF2 was modified by phosphorylation at its α subunit. Phosphorylated eIF2α loses its ability to participate in ternary complex formation and hence is unable to facilitate translation initiation. In this study, we analyzed the requirement of other canonical initiation factors (eIF4A and eIF4G) for PUMA translation. We used specific inhibitors for these proteins

(Hippuristanol is a specific inhibitor of eIF4A; purified human rhinovirus 2A protease cleaves eIF4G and inhibits its activity) to analyze if they were essential for PUMA translation. We also studied 40S recruitment to PUMA IRES. Our aim was to determine if the 40S ribosomal subunit is recruited directly near the AUG codon or upstream of the AUG codon.

2.2 MATERIALS and METHODS

ANTIBODIES AND REAGENTS

Enzymes EcoRI HF, SacI HF and RNase inhibitor were purchased from NEB. MEGAscript T7 Transcription kit and mMESSAGE T7 Transcription kit was purchased from ThermoFisher Scientific. Translation grade [^{35}S]-methionine and transcription grade α-[^{32}P]UTP were purchased from Perkin Elmer. Rabbit Reticulocyte Lysate System Nuclease Treated (Promega) was purchased from ThermoFisher Scientific. HRV 2A protease plasmid was a kind gift from Dr. Tim Skern. eIF4G C-terminal antibody (Cat # B 983.10) was purchased from ThermoFisher Scientific. Hsp70 Antibody (Cat # 610607) was purchased from BD Biosciences. Purified recombinant Hsp70 protein was purchased from Boston Biochem, R&D Systems (Cat # AP-100-050). Streptavidin Agarose beads and Protein A/G Agarose beads were purchased from ThermoFisher Scientific. Biotin RNA labeling mix 10X (Roche) was purchased from ThermoFisher Scientific.

PLASMIDS

The following four PUMA plasmids were used for this study- PUMA 5'UTR, 5'UTR +AUG 5'UTR + 50 ntd, & full-length PUMA cDNA. These fragments were PCR amplified using PUMA cDNA (Life Technologies) and the following primers:

Sac1 forward: 5'-AAAAAGAGCTCCTGAGACGCGGCATAGAGCC-3'

EcoRI reverse: 5'AAAAAGAATTCGGCGCTCCCTGGAGCC-3'

EcoRI reverse: 5'AAAAAGAATTCCATGGCGCTCCCTGGAGC-3'

EcoRI reverse: 5'AAAAAGAATTCGGCTAGACCCTCTACG-3'

EcoRI reverse: 5'AAAAAGAATTCTCACTGTTCAATCTGATTTTATTGAAAAG-3'

The resulting PCR product was run on Agarose gel to confirm the size of PCR product PUMA 5'UTR - 261 nt, PUMA 5'UTR +AUG - 264 nt, PUMA 5'UTR+50 nt - 311 nt, PUMA full length - 1823 nt). Purified PCR product was digested with *EcoRI* and *Sac1* (PCR-added restriction sites) and cloned into pBSKSII(+) vector digested with Sac1/EcoRI. All PUMA constructs were verified by DNA sequencing.

IN VITRO TRANSCRIPTION

PUMA plasmids were linearized using *EcoRI* and purified by Phenol/Chloroform extraction method. The corresponding PUMA RNA transcript was synthesized using the MEGAscript T7 kit to synthesize uncapped RNA or mMESSAGE T7 Transcription kit (Ambion) to synthesize capped mRNA, according to the protocol provided by the manufacturer. The transcription reaction mixtures were incubated at 37°C for 3 hrs. Reactions were further incubated for 20 min with 1 U of DNase at 37°C. This was followed by RNA purification using MEGAclear Transcription Clean-Up Kit (Ambion, Life Technologies). RNA was suspended in 20 μL RNase-free water. The RNA constructs were analyzed on 1% formaldehyde agarose gel to confirm size and determine RNA quality.

IN VITRO TRANSLATION

The *in vitro* translation experiments were done using Rabbit Reticulocyte Lysate cell free System, Nuclease Treated (Promega). In vitro transcribed and purified PUMA mRNA (1 μg) in the presence of 35 μl RRL, 1mM amino acid mixture (without Methionine), RNAse inhibitor (40U/μl) and 10 mCi/ml [^{35}S]-methionine (Perkin Elmer) was incubated in a final volume of 50 μl reaction for 90 min at 30°C. The reaction was stopped with SDS protein loading buffer. Reactions were resolved on a 12% SDS-PAGE gel. The gel was fixed, dried and visualized using Typhoon Phosphor Imager (GE Healthcare). Each experiment was repeated independently three times using different RNA preparations.

HRV2 2A PROTEASE: PROTEIN EXPRESSION AND PURIFICATION

HRV2 2A protease protein expression and purification was performed as described (Skern *et al.*, 1993). The expression vector pET8c/HRV2 2A was transformed into *E.coli* BL21(DE3) pLysE cells. Plasmid was cultured in one-liter fresh medium and induced with 0.3 mM IPTG. Bacteria were harvested by centrifugation and the cell pellets were suspended in buffer (50mM NaCl, 50mM Tris-HCl pH 8, 1mM EDTA, 5 mM DTT, 5% glycerol), followed by sonication. Protein was precipitated by saturated 4M ammonium sulfate solution. Precipitated protein was collected by centrifugation and pellet was dissolved in buffer.

This suspension was loaded onto FPLC Mono-Q column using AKTA purifier protein purification system. Fractions containing proteinase was identified by SDS-PAGE, pooled and applied directly to Superdex 75 column for gel filtration. Fractions

containing 2A proteinase were pooled and concentrated using an Amicon Ultra Centrifugal filter unit.

Purified HRV2 2A protease was used to cleave eIF4G in RRL extract. RRL extract (100 μg) was incubated with varying amounts (2 μg and 4 μg) of 2A protease at 30°C for 10 min. HRV2 2A protease catalyzed cleavage of eIF4G was analyzed by Western blot using eIF4G C-terminal monoclonal antibody. The 2A protease treated RRL extract was used for in vitro translation of PUMA mRNA.

2.3 eIF4A IS ESSENTIAL FOR PUMA TRANSLATION

2.3.1 INTRODUCTION

The cap-binding multi-subunit protein complex eIF4F comprises 3 subunits: eIF4E (which binds the mRNA cap structure), eIF4A (RNA helicase that exhibits ATP-dependent RNA binding activity), and eIF4G (a scaffolding protein that mediates 43S preinitiation complex binding to the mRNA through interactions with eIF3) (Bordeleau et. al., 2005). The eIF4A in eIF4F complex functions to unwind RNA secondary structure to facilitate binding of the 40S ribosomal subunit. The helicase activity of eIF4A plays a key role to unwind the secondary structure of 5'UTR of mRNAs. This generates an unstructured RNA region which facilitates ribosome recruitment for translation initiation (Rogers et. al., 1999). eIF4A is an RNA helicase which is required for RNA secondary structure remodeling in order to create ribosome landing pads. The initiation factor possesses both RNA-binding and ATPase activity. Several studies have demonstrated the role of eIF4A in translation of mRNAs having secondary structures. It was found that in the presence of an inactive eIF4A (mutant eIF4A lacking RNA unwinding activity) cap-dependent translation of structured mRNAs was more susceptible to inhibition. The mutant eIF4A was found to prevent mRNA association with the 43S ribosomal subunit and led to inhibition of initiation complex formation. IRES-dependent translation in Picornaviruses was inhibited by dominant negative eIF4A mutant (Svitkin et. al., 2001).

Hippuristanol is a small molecule specific inhibitor of RNA helicase eIF4A, known to inhibit cap-dependent and eIF4A-dependent translation (Cencic and Pelletier, 2016). Hippuristanol is a selective inhibitor of eIF4A which prevents eIF4A from interacting with RNA and inhibits RNA-binding activity of eIF4A. Hippuristanol also

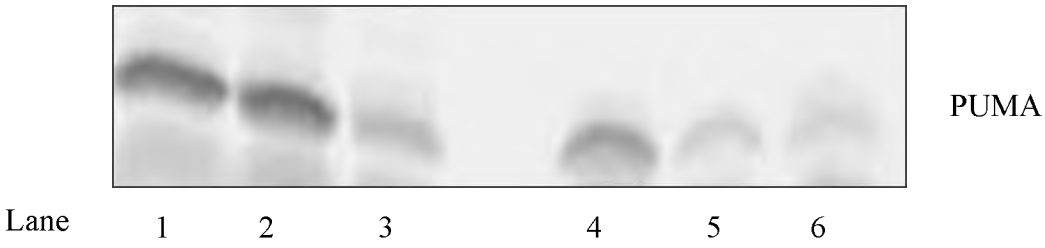
inhibits RNA-stimulated ATPase activity of eIF4A by locking eIF4A in a closed conformation preventing it from participating in initiation by inhibiting RNA unwinding (Cencic and Pelletier, 2016). Hippuristanol inhibits the ATPase activity and RNA-binding ability of eIF4A but does not prevent ATP binding to eIF4A. Hippuristanol impairs the ability of eIF4A to be recruited to the 5'cap structure and eventually disrupts the 48S complex formation. Hippuristanol binds at the C-terminal domain of eIF4A but does not interfere with ATP binding at the N-terminal domain (Bordeleau et. al., 2006, Lindqvist et. al., 2008). Hippuristanol has been used in vitro and ex vivo to characterize the eIF4A-dependency of cellular IRES containing mRNAs (Olson et. al., 2013). Studies have investigated the effect of hippuristanol on translation driven by poliovirus, encephalomyocarditis virus and cricket paralysis virus. Hippuristanol was found to inhibit translation in poliovirus and encephalomyocarditis virus, but not in cricket paralysis virus, suggesting that the poliovirus and encephalomyocarditis virus requires eIF4A for translation but cricket paralysis virus can translate without eIF4A (Bordeleau et. al., 2006). Experimental evidences have indicated that eIF4A is indispensable for mRNAs forming secondary structures. Several studies have used hippuristanol to assess the requirement of eIF4A for translation of IRES-containing mRNAs (Bordeleau et. al., 2006, Lindqvist et. al., 2008). Regulation of eIF4A activity may be essential for translation initiation in cap-dependent mRNAs and eIF4A-dependent IRESs. We carried out similar experiments to determine the requirement of eIF4A in PUMA translation.

Previous study has determined the initiation factors that were not essential for cap-independent translation of PUMA. PUMA translation in differentiating media conditions proceeded via its IRES sequence. Enhanced PUMA expression in

differentiating media conditions also did not require canonical initiation factors like eIF4E (Shaltouki et. al., 2013). In this study, we attempted to identify other initiation factors that were necessary for cap-independent translation of PUMA mRNA.

2.3.2 RESULT

The plasmid pBSKS/PUMA was linearized by enzyme *EcoRI* digestion and in vitro transcribed with T7 RNA polymerase to generate PUMA mRNA. We performed in vitro translation experiments in hippuristanol-treated rabbit reticulocyte lysate. Reactions were performed in the presence of [^{35}S]-methionine and separated on a 10% SDS polyacrylamide gel. We found that under conditions of inactive eIF4A, PUMA translation was severely affected in a dose-dependent manner, both for capped and uncapped mRNAs. PUMA translation product decreased significantly with increasing hippuristanol concentrations (1 and 2 μM) in both mRNAs (Fig. 2.1). Our data indicates PUMA expression for capped and uncapped transcripts reduced by more than 2 folds at 2 μM Hippuristanol. For control, RRL was supplemented with DMSO instead of Hippuristanol. Previous study has reported translation of PUMA being cap-independent and PUMA IRES activation during cellular differentiation (Shaltouki et. al., 2013). However, our findings suggest that in vitro translation of PUMA may not be eIF4A-independent. This can be explained by the fact that PUMA 5'UTR forms a strong secondary structure; therefore, helix unwinding may be a prerequisite for PUMA translation in in vitro system. eIF4A may be required to unwind the secondary structure in the 5'UTR of PUMA and for efficient initiation.



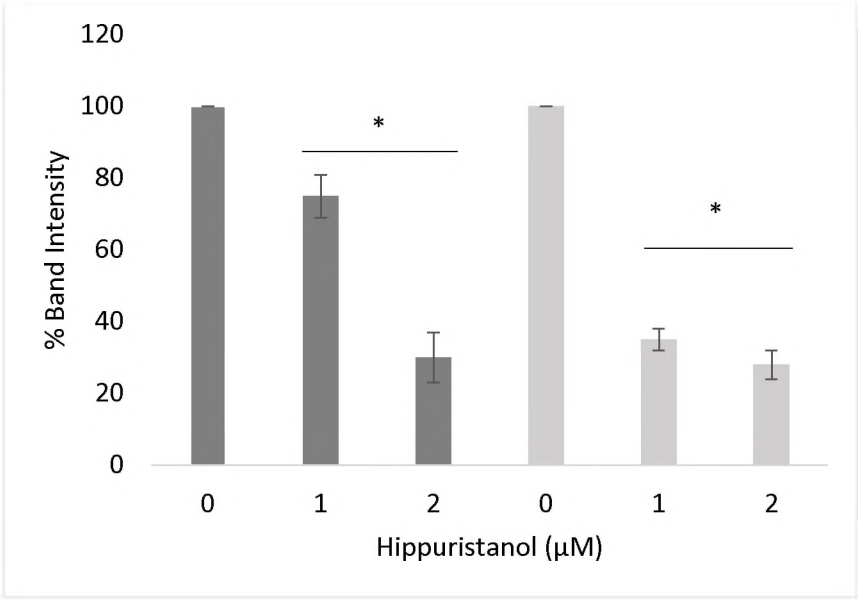


Fig. 2.1 Hippuristanol inhibits in vitro translation of PUMA mRNA in a cell-free system. A. Capped and Uncapped PUMA mRNAs were subjected to translation in a Rabbit Reticulocyte Lysate cell-free system in the presence or absence of Hippuristanol. In vitro translation of PUMA mRNAs performed in RRL extracts in the presence of [^{35}S]-methionine and eIF4A inhibitor Hippuristanol were analyzed by SDS- PAGE. Lane 1 represents expression of capped PUMA mRNA in RRL, lanes 2-3 represents expression of capped PUMA mRNA in RRL treated with Hippuristanol (1 and 2 μM). Lane 4 represents expression of uncapped PUMA mRNA in RRL, lanes 5-6 represents expression of uncapped PUMA mRNA in RRL treated with Hippuristanol (1 and 2 μM). Shown are results from one representative experiment.

B. Quantification of the translation products using a Typhoon phosphor imager. The results shown are the mean ± SEM value of translation efficiencies from three independent experiments. P value: *<0.05, **<0.001

2.4 INTACT eIF4G IS REQUIRED FOR PUMA TRANSLATION

2.4.1 INTRODUCTION

eIF4F is a cap-binding multi-subunit complex, composed of three polypeptides: eIF4E, eIF4A and eIF4G and functions in facilitating mRNA unwinding and ribosome binding to the mRNA. eIF4G is a scaffolding protein which interacts with both eIF4E and eIF4A to promote ribosome binding at the 5'end of mRNA. The N-terminal of eIF4G interacts with cap-binding protein eIF4E, while the C-terminal binds to eIF4A and eIF3. Several viral IRESs express proteases which cleaves eIF4G at specific sites, separating the N-terminal domain from the C-terminal domain. This proteolytic cleavage is a strategy adopted by viruses to inhibit cap-dependent translation in host cells and favor IRES-dependent translation of viral RNAs that function without intact eIF4G. eIF4G is found to be cleaved by proteases during viral infection and during apoptosis by caspase-3 (Haghighat et.al., 1996, Wilfred et. al., 1998). These conditions result in drastic reduction of cap-dependent translation. However, several viral and cellular IRESs were found to be translated efficiently despite eIF4G cleavage. Some viral and cellular IRESs require intact eIF4G, whereas others show enhanced activity when eIF4G is cleaved. It has been experimentally demonstrated that cellular IRESs like BiP and c-Myc are immune to proteolytic eIF4G cleavage. In fact, the activity of theses IRESs was found to be enhanced in the presence of cleaved eIF4G (Thoma et. al., 2004).

Viral proteases like 2A protease and Lb protease are known to cleave eIF4G, separating its C-terminal and N-terminal subunits. Cleavage of eIF4G by 2A and Lb protease results in inhibition of cap-dependent translation initiation due to reduced capacity of capped mRNAs to recruit 40S ribosomal subunits. On the other hand,

cleavage of eIF4G was found to stimulate translation initiation on uncapped mRNAs and poliovirus and human rhinovirus IRESs, but not in EMCV or FMDV IRESs. The C-terminal of eIF4G (which binds to eIF4A) was sufficient to mediate translation initiation in viral IRESs and uncapped mRNAs. It has been postulated that cleavage of eIF4G generates a more active form of eIF4A which readily stimulates IRES activity (Borman et. al., 1997).

2.4.2 RESULT

The plasmid pBSKS/PUMA was linearized by digesting it with enzyme *EcoRI* and in vitro transcribed with T7 RNA polymerase to generate PUMA mRNA. We performed in vitro translation of PUMA mRNA in human rhinovirus 2A protease treated RRL extract (Promega). Reactions were performed in the presence of [^{35}S]-methionine and resolved on a 10% SDS polyacrylamide gel. We checked the cleavage of eIF4G in RRL by Western Blot using C-terminal eIF4G antibody. HRV 2A protease cleaves eIF4G into 110 kDa C-terminal and 72 kDa N-terminal fragments (Fig. 2.2). We found that under conditions of cleaved eIF4G, PUMA translation was severely affected, both for capped and uncapped mRNAs. PUMA translation product was found to decrease in the presence of HRV2 2A protease (2 and 4 μg). PUMA expression for capped and uncapped transcripts reduced by approximately two-folds in the presence of 2 and 4 μg 2A protease (Fig. 2.3). For control, RRL was supplemented with equal volume of buffer instead of HRV 2A protease. These results suggest that translation of PUMA in an in vitro system requires active and intact eIF4G. Hence, it can be concluded that PUMA translation is not eIF4G-independent in in vitro system and an intact eIF4G protein is needed for efficient PUMA translation.

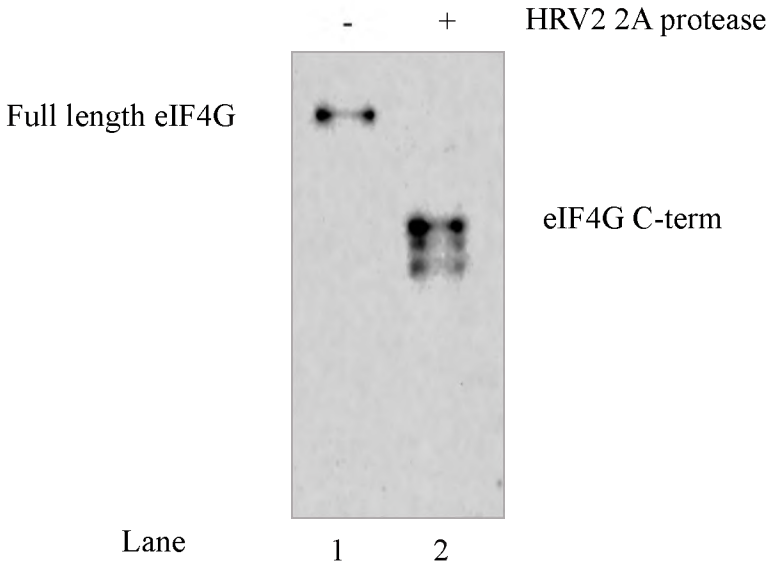


Fig. 2.2. Cleavage of eIF4G by 2A protease. RRL (100 μg) was incubated with purified HRV 2A protease (4 μg) for 10 min at 30°C. eIF4G cleavage was analyzed by Western Blot analysis using eIF4G C-terminal antibody. Lane 1 represents full-length eIF4G in untreated RRL, lane 2 represents cleaved eIF4G in 2A protease treated RRL.

Lane 1 2 3 4 5 6

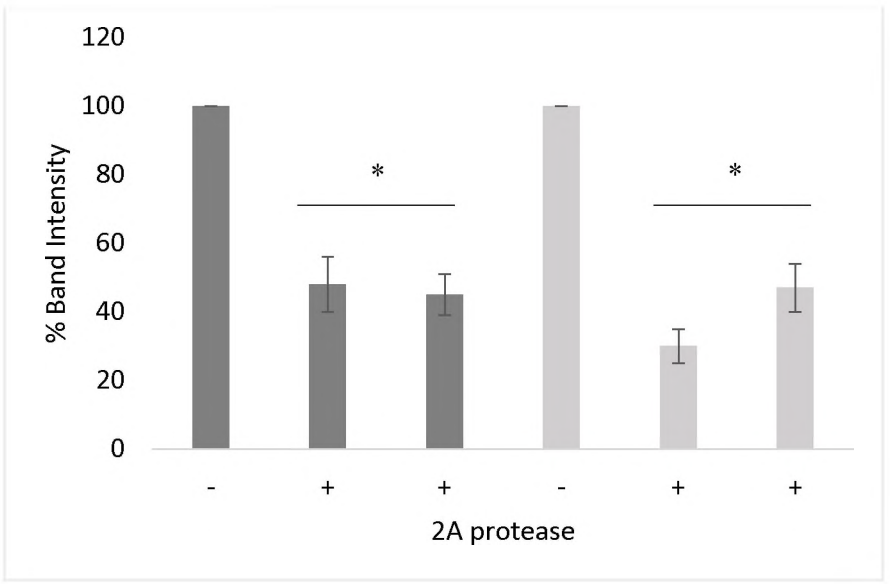


Fig. 2.3. eIF4G cleavage affects PUMA translation in vitro. A. Capped and Uncapped PUMA mRNAs were subjected to translation in a Rabbit Reticulocyte Lysate cell-free system pre-treated with HRV2 2A protease. In vitro translation of PUMA mRNAs performed in RRL extracts in the presence of [^{35}S]-methionine and HRV 2A protease were analyzed by SDS- PAGE. Lane 1 represents expression of capped PUMA mRNA in RRL, lanes 2-3 represents expression of capped PUMA mRNA in RRL treated with HRV 2A protease (2 & 4 μg). Lane 4 represents expression of uncapped PUMA mRNA in RRL, lanes 5-6 represents expression of uncapped PUMA mRNA in RRL treated with HRV 2A protease (2 & 4 μg). Shown are results from one representative experiment.
B. Quantification of the translation products using a Typhoon phosphor imager. The results shown are the mean ± SEM value of translation efficiencies from three independent experiments. P value: *<0.05, **<0.001

2.5 43S RIBOSOMAL COMPLEX RECRUITMENT TO PUMA IRES

2.5.1 INTRODUCTION

All viral and cellular IRESs require specific set of canonical and non-canonical initiation factors for their efficient activities. Different viral and cellular IRES exhibit distinct mode of the assembly of preinitiation complex (Balvay et. al., 2009). Type 1 IRES like poliovirus recruit the 48S pre-initiation complex upstream of the start site and involves scanning of the complex to locate the start AUG codon. On the other hand, EMCV IRES recruits the 48S pre-initiation complex at the initiation AUG site and does not involve complex scanning (Balvay et. al., 2009). Finally, in HCV and CrPV IRES, the 40S ribosomal subunit binds directly to the IRES, without critical initiation factors like eIF4A, eIF4E, eIF1 and makes multiple contacts with the 40S ribosomal subunit (Balvay et. al., 2009, Jackson et. al., 2010). Among cellular IRESs, c-Src IRES was found to exhibit many attributes that are similar to the characteristics of HCV IRES. Like HCV IRES, c-Src IRES activity was stimulated when eIF4E and eIF2α were inactivated (Allam and Ali, 2010). Similar to Hepatitis C virus IRES, c-Src IRES also does not require the scanning mechanism to recruit the 43S preinitiation complex at the initiation site AUG codon. The c-Src IRES was found to bind directly to the 40S subunit and the 43S complex does not require scanning to locate the initiation codon (Allam and Ali, 2010). Additional nucleotides downstream of the initiator AUG codon which form structural motifs are required for efficient function for c-Src IRES activity (Allam and Ali, 2010). On the other hand, viral IRESs such as Poliovirus, Hepatitis A virus IRES and cellular IRESs like Bag-1, Apaf-1, SHMT1 and IRES of the Myc family (c-Myc and L-Myc) have been demonstrated to recruit the preinitiation complex upstream of the

initiation site followed by the complex scanning along the 5'UTR region in search of the initiator AUG codon (Spriggs et. al., 2009, Mitchell et. al., 2003, Pickering et. al, 2004, Fox et. al., 2009).

Previous study by Weyman and colleagues demonstrated that during DNA damage and under cellular differentiating media conditions, IRES-mediated translation in PUMA is activated (Shaltouki et. al., 2013). DNA damage and cellular differentiation are stress inducing conditions which results in inactivation of cap-binding protein eIF4E and eIF2α by eIF2α phosphorylation and 4E-BP hypo-phosphorylation. Cap-independent translation of PUMA was further assessed by analyzing the effect of a competing cap analog on in vitro translation of PUMA. Presence of a cap analog did not affect translation of PUMA mRNA, suggesting that internal initiation of PUMA mRNA occurs independent of the 5' m^7G cap structure (Shaltouki et. al., 2013). In order to understand the sequence of events involved in internal initiation of PUMA, it was also important to study the mechanism of 40S ribosomal subunit recruitment to PUMA IRES.

2.5.2 MATERIAL and METHODS

IN VITRO TRANSLATION ASSAY

PUMA 5'UTR and PUMA 5'UTR + AUG plasmid were linearized by digestion with enzyme *EcoRI* and purified by Phenol/Chloroform extraction method. Uncapped PUMA RNA transcripts were synthesized using the MEGAscript T7 kit, as per manufacturer's instructions. Transcription was performed using T7 RNA Polymerase, in the presence of 1μg of linearized DNA template, mM ATP, CTP, GTP and [α^{32}P]-UTP (800 Ci/mmol). The reaction mixture was incubated for 3 hrs at 37 °C, followed by incubation with 1 U DNase for 20 min at 37 °C. Unincorporated [α^{32}P]-UTP was eliminated by passing the transcription mixtures through NucAway Spin Columns and RNA was eluted in 20 μL RNase-free water.

SUCROSE DENISTY GRADIENT ANALYSIS

^{32}P-labelled PUMA constructs (5'UTR and 5'UTR + AUG) were synthesized using MegaScript T7 Transcription kit (Ambion, Life Technologies) and α-[^{32}P]UTP (800 Ci/mmol). Radiolabelled transcripts were purified using Nucaway Spin Columns (Ambion, Life Technologies) as per the manual instructions. 25 μl of translation reactions (17.5 μl nuclease-treated RRL, 20 μM amino acids, 4U RNase inhibitor) was incubated with ^{32}P-labelled PUMA transcript (50,000 rpm) in the presence of 2 mM GMP-PNP for 30 min at 30 °C. Reactions were stopped on ice and layered on to 10-30% sucrose gradient in buffer containing 20mM Hepes KOH pH 7.8, 100mM Potassium Acetate, 5mM Magnesium chloride, 1mM DTT. The gradient tubes were centrifuged in SW55 Ti rotor at 40,000 rpm for 3 hrs at 4°C. ISCO Programmable Density Gradient System was

used to collect the gradient fractions. Scintillation counting was done to determine the radioactivity of gradient fractions.

2.5.3 RESULT

We investigated the mechanism of 43S initiation complex recruitment to PUMA IRES using a classical approach, Sucrose Density Gradient Analysis. Sucrose density gradient polyribosomal analysis is a method to separate the ribosome fractions of different densities. The 43S pre-initiation complex was allowed to assemble on PUMA IRES in cell-free rabbit reticulocyte lysate. Uncapped PUMA mRNA constructs (5'UTR only and 5'UTR + AUG) were synthesized in vitro using ^{32}P-UTP. Rabbit Reticulocyte lysate was pre-treated with 2mM GMP-PNP. Guanosine-imidotriphosphate is a non-hydrolysable analog of GTP which causes stalling the 43S complex at the initiation codon and results in its accumulation on the messenger RNA. It allows recruitment of the 40S subunit to the mRNA and its migration towards the initiation site but inhibits GTP-hydrolysis and joining of the 60S large ribosomal subunit. This results in accumulation of 48S complex (43S pre-initiation complex and mRNA). ^{32}P-labelled uncapped PUMA mRNA constructs were incubated with Rabbit Reticulocyte lysate cell-free translation extract containing 2mM GMP-PNP for 30 minutes. The reaction mixtures were centrifuged, and ribosome fractions of different densities were separated by sucrose density gradient ribosome profiling. The gradient fractions were then analyzed by radioactive scintillation counting to determine the ribosome-bound mRNA (48S complex assembly) and incorporation of ^{32}P labeled RNA probe into the 48S ribosomal complex. Our results indicate that the 40S ribosomal subunit bound strongly to the PUMA 5'UTR + AUG (Fig. 2.4). The 48S complex assembly was more efficient with the PUMA RNA probe containing the initiation AUG codon. In contrast, without the AUG codon, 40S ribosomal subunit binding to the PUMA 5'UTR was weak/ practically absent. These

results also indicate that the 40S ribosomal subunit is not recruited directly to the IRES sequence in case of PUMA 5'UTR. Our results suggest that there might be a scanning mechanism required in case of PUMA IRES in order to locate the start codon by the pre-initiation complex. It can therefore be asserted that although internal initiation of PUMA mRNA is cap-independent and may proceed without the cap-binding protein eIF4E and under reduced requirement for eIF2α, the 43S pre-initiation complex most likely lands upstream of the start AUG codon and scans along the 5'UTR. The initiation complex does not land directly at the AUG codon and scanning is required in order to find the start codon.

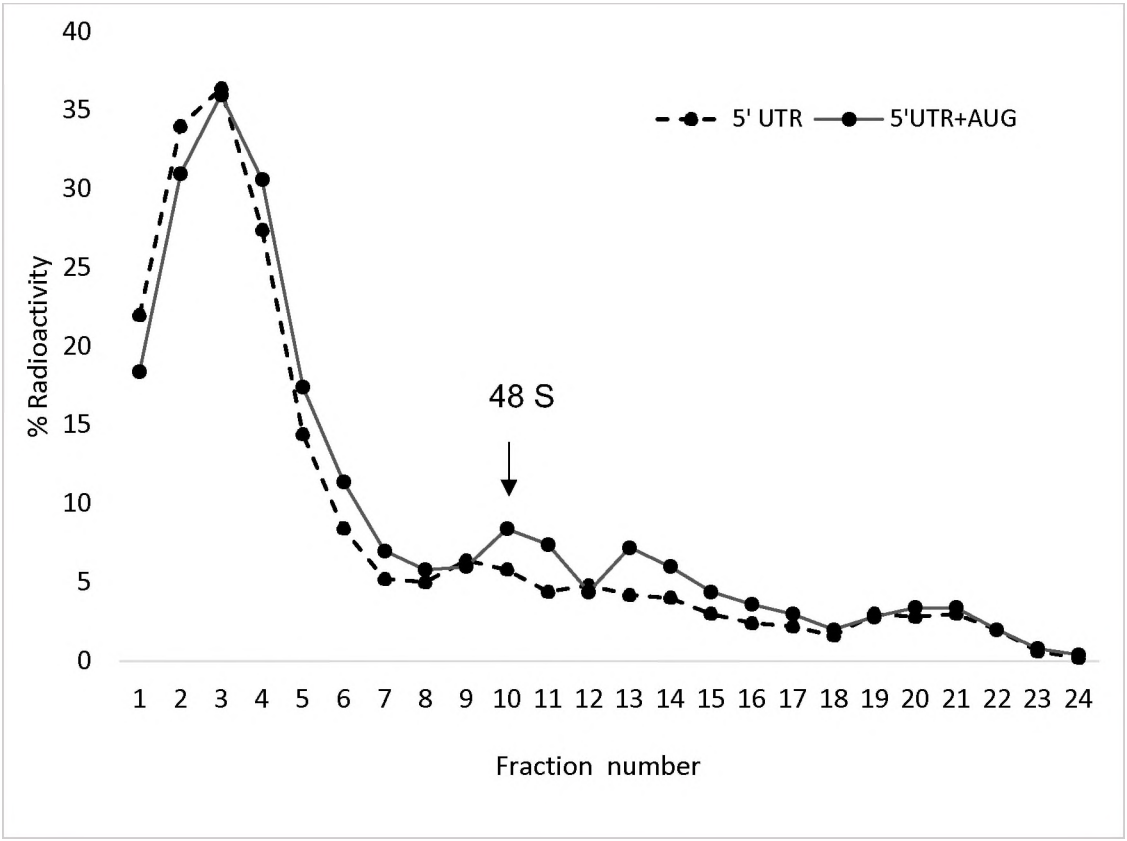


Fig. 2.4. Assembly of Translation initiation complexes on PUMA IRES in Rabbit reticulocyte lysate cell-free extract and analysis by sucrose density gradient centrifugation. ^{32}P-labeled uncapped RNA probes PUMA 5'UTR and PUMA 5'UTR + AUG were reconstituted in RRL in the presence of 2mM GMP-PNP and incubated for 30 min at 30°C. The lysates were layered onto 10-30% sucrose gradient, followed by centrifugation at 40,000 rpm for 3 hours. The fractions were separated by density gradient fractionation and the radioactivity of the gradient fractions was determined by scintillation counting. The peak positions of 48S complex are indicated.

2.6 DISCUSSION

It has been demonstrated previously that PUMA translation adopts an alternative mode of initiation (IRES-mediated) when cells were subjected to stress conditions such as differentiation (Shaltouki et. al., 2013). Cellular stress conditions signal the cells to stop translational activities and results in a global protein synthesis shut down. Loss of cellular translation is a result of inactivation of the key players involved in translation e.g. the translation initiation factors (eIF4E, eIF2, eIF4G). As mentioned earlier, differentiation conditions in cells induced phosphorylation of eIF2α and hypo-phosphorylation of 4E-BP, resulting in eIF2α and eIF4E inactivation respectively (Shaltouki et. al., 2013). Studies using chemical inhibitor hippuristanol have identified eIF4A requirement of several cellular IRESs. For example, c-myc and N-myc IRES require eIF4A for their activation (Spriggs et. al, 2009). Hippuristanol has been used previously for functional characterization of different IRESs, in order to determine eIF4A dependency in vitro and ex vivo. Translation efficiencies of Poliovirus, EMCV and CrPV IRES was tested in the presence of hippuristanol. It was found that the poliovirus and EMCV IRES expression was inhibited in the presence of hippuristanol, hence these IRESs required eIF4A. However, hippuristanol had no effect on CrPV IRES-driven translation, confirming that CrPV did not require eIF4A (Bordeleau et. al., 2006).

Previous studies have shown that the 5'cap structure protects the mRNA from inactivation and also enhances the efficiency of translation. It has been suggested that the mechanism of translation initiation might be different for capped and uncapped mRNAs. Studies have demonstrated the translational abilities of different in vitro transcribed mRNAs where it was found that certain uncapped mRNAs, for example, alfalfa mosaic

virus and β-Globin may be less active than their capped forms. The uncapped mRNAs degraded more rapidly than the capped mRNAs. It was also demonstrated that the capped mRNA has reduced requirement for eIF4F for efficient translation initiation. (Fletcher et al., 1990). Hence, we performed in vitro translation analysis for both capped and uncapped PUMA mRNAs.

To increase our understanding of PUMA translation, we investigated the requirement of other two members of the eIF4F family- eIF4A and eIF4G. In this study, we used specific inhibitors for each factor (Hippuristanol and 2A protease), we found that both eIF4A and eIF4G are essential for PUMA translation in an in vitro system. Hippuristanol is a specific inhibitor of eIF4A which inhibits RNA-binding activity and ATPase activity of eIF4A. eIF4A fails to perform RNA helicase functions in the presence of hippuristanol, hence inhibiting cap-dependent translation. Based on our results, we report that in vitro translation of PUMA mRNA (capped and uncapped) was disrupted by eIF4A inactivation (Fig 2.1). Our experimental analysis shows that PUMA expression decreased significantly in the presence of hippuristanol. PUMA translation reduced by more than two-folds with increasing hippuristanol concentrations (1 and 2 μM). It can therefore be suggested that eIF4A may be required for PUMA IRES structure remodeling to unwind its complex secondary structure.

HRV 2A protease is a protease that cleaves eIF4G separating the eIF4E-binding domain and the eIF4A-binding domain. Most canonical translations require intact eIF4G and hence are disrupted by viral 2A protease. IRES-mediated translation in some cellular mRNAs may require intact eIF4G for efficient activity. A well-known example includes L-myc IRES, which requires full-length eIF4G for its function, unlike c-myc IRES which

requires only C-terminal of eIF4G (Spriggs et. al., 2009). Cleavage of eIF4G eventually results in loss of eIF4F complex formation and therefore 40S recruitment to the mRNA 5' terminal. Using purified HRV 2A protease, we were able to cleave eIF4G present in rabbit reticulocyte lysate, separating the C-terminal and N-terminal eIF4G domains (Fig 2.2). Based on our in vitro translation assays of PUMA mRNA with 2A protease, we report that translation of PUMA mRNA (capped and uncapped) was disrupted by eIF4G cleavage. Translation of PUMA under cleaved eIF4G conditions led to reduced protein expression. PUMA expression was found to decrease by almost two folds in the presence of 2 and 4 μg concentrations of 2A protease (Fig 2.3).

Next, we wanted to determine 40S ribosomal subunit recruitment to PUMA RNA. Previous studies have reported "land and start" mechanism in some IRESs, where the 40S initiation complex is recruited in close proximity of the AUG codon (Balvay et. al., 2009). A well-studied example includes the c-Src IRES. This unique cellular IRES exhibited properties similar to HCV IRES wherein scanning by the 43S complex was not needed. The initiation complex was recruited directly at the initiation site (Allam and Ali, 2010). On the other hand, cellular IRES like Apaf-1, c-Myc and L-Myc were demonstrated to exhibit "land and scan" mechanism, where the 40S complex is recruited upstream of the AUG site and scans until it locates the start codon (Spriggs et. al., 2009, Mitchell et. al., 2003). To understand the mechanism of 40S ribosome assembly in PUMA IRES, we performed sucrose density gradient analysis. We analyzed the assembly of 43S pre-initiation complex on PUMA IRES in rabbit reticulocyte lysate. The 43S pre-initiation complex was allowed to assemble on uncapped ^{32}P-labeled PUMA probes (5'UTR or 5'UTR+AUG). Radioactivity from each fraction was determined by

scintillation counting. Our results indicate that in case of PUMA 5'UTR, the 43S complex is not recruited directly to bind at the initiation site. 43S complex recruitment is more efficient with PUMA 5'UTR + AUG sequence (Fig 2.4). It is possible that the initiation complex binds to the 5'UTR upstream of the AUG codon and scans in search of the initiation site. Hence, our results suggest "land and scan" translation initiation mechanism in PUMA IRES. Several reports have demonstrated that the mechanism of ribosome recruitment may be governed by the dependence of IRES on certain initiation factors. It can therefore be predicted that IRESs which involve "land and scan" mechanism have a greater requirement for eIF4A as compared to IRESs which involve "land and start" mechanism. As mentioned previously, 5'UTR of PUMA has a high GC% and forms complex secondary structure. Hence, recruitment of the 43S complex upstream of the start site may necessitate the requirement of RNA helicase eIF4A in order to unwind RNA secondary structures for efficient translation initiation.

For future studies, sucrose density gradient analysis can be done with PUMA constructs in growth media and differentiating media. It is likely that the assembly of the 43S pre-initiation complex on PUMA IRES will be more efficient in DM as compared to GM.

CHAPTER III

IDENTIFICATION OF SPECIFIC ITAFs INVOLVED IN UPREGULATED PUMA EXPRESSION IN DIFFERENTIATING MEDIA

3.1 INTRODUCTION

Cap-independent or IRES-dependent translation initiation proceeds under cellular stress conditions such as mitosis, heat shock, apoptosis, nutrient deprivation and differentiation (Komar and Hatzoglou, 2011). Under these conditions, cap-dependent translation initiation is shut down for most genes due to reduced activity or inactivation of canonical initiation factors. In addition to canonical initiation factors, several RNA-binding proteins are involved in IRES-mediated translation. Cellular IRESs require a specific combination of canonical initiation factors and auxiliary trans acting factors for their function. These protein cofactors play a role in IRES structure stabilization and/or to promote ribosomal complex assembly. ITAFs may act as RNA chaperones to stabilize RNA secondary structure to allow efficient 40S ribosomal subunit binding. ITAFs may also function as an adaptor protein to anchor other proteins or the 40S subunit (King et. al., 2010, Komar and Hatzoglou, 2011).

In this study, we performed a detailed and comprehensive analysis to ascertain the contribution of any RNA-binding protein in PUMA IRES activity. To identify ITAFs involved in PUMA IRES activation, we performed RNA affinity pull down assay. Biotin labeled PUMA RNA was used to pull down RNA-binding proteins in growth media and differentiating media. Proteins bound to PUMA RNA in differentiating media but not in growth media was identified by mass spectrometric analysis. To confirm PUMA RNA and protein interaction, we performed RNA EMSA using radiolabeled PUMA RNA and purified protein. In addition, competition RNA EMSA was performed using unlabeled PUMA RNA as competitor.

3.2 MATERIALS AND METHODS

IN VITRO TRANSCRIPTION

PUMA plasmid (5'UTR + 50 nt) was linearized using restriction digestion enzyme *EcoRI* and purified by Phenol/Chloroform extraction method. Biotin-labeled RNA was synthesized in a 20 μL reaction mixture using 10X Biotin RNA labeling mix (Roche), according to the manufacturer protocol. The transcription reaction mixtures were incubated at 37°C for 3 hrs. Reactions were incubated for 20 min with 1 U of DNase at 37°C. This was followed by RNA purification using MEGAclear Transcription Clean-Up Kit (Ambion, Life Technologies). RNA was suspended in 20 μL RNase-free water. The RNA constructs were analyzed on 1% formaldehyde agarose gel to determine size and integrity of RNA probes.

PUMA plasmid (5'UTR + 50 nt) was linearized using restriction digestion enzyme *EcoRI* and purified by Phenol/Chloroform extraction method. Uncapped PUMA RNA transcripts were synthesized using the MEGAscript T7 kit, as per manufacturer's instructions. Transcription was performed using T7 RNA Polymerase, in the presence of 1μg of linearized DNA template, mM ATP, CTP, GTP and [α^{32}P]-UTP (800 Ci/mmol). The reaction mixture was incubated for 3 hrs at 37 °C, followed by incubation with 1 U DNase for 20 min at 37 °C. Unincorporated [α^{32}P]-UTP was eliminated by passing the transcription mixtures through NucAway Spin Columns and RNA was eluted in 20 μL RNase-free water.

CELL CULTURE AND PREPARATION OF 23A2 CELL LYSATES

23A2 cells were cultured in growth media and differentiating media as described previously (Shaltouki et al., 2013). Cells 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% P/S (10,000 I.U./ mL penicillin and 10,000 μg/ mL streptomycin). Cells were switched to differentiating media containing BME, 1% Penicillin/Streptomycin and no FBS. Cells were incubated at 37°C in 5% CO_2. After culturing cells for 24 hours in GM and 3 hours in DM, cells were washed with cold PBS and lysed by scraping in RIPA lysis buffer (50 mM Tris pH 7.6, 50 mM NaCl, 1 mM Phenymethylsulfonyl fluoride and 1 mM dithiothreitol). The suspension was subjected to three freeze-thaw cycles, passed multiple times through a 26-gauge needle and then centrifuged at 100,000 X g for 30 min. The protein concentration of the supernatant was measured by Bradford Assay and it was used for RNA affinity pull down assay and in vitro translation reaction.

RNA AFFINITY PULL DOWN ASSAY

23A2 skeletal myoblast cells were cultured in Growth media and Differentiating media and their protein lysates were prepared as previously described. For RNA affinity pull down assays, Biotin labeled mRNA (PUMA 5'UTR + 50 ntd) and pre-washed Streptavidin agarose beads were used. 10μg of in vitro transcribed biotinylated PUMA mRNA (5'UTR + 50 ntd) was incubated with 100 μl of prewashed Streptavidin Agarose beads in 100 μl RNA binding buffer (20mM Hepes KOH pH 7.8, 100 mM KCl, 1.5mM $MgCl_2$) for 1 hour at 4°C. Beads were washed with 100 μl binding buffer and incubated

with 500 μg protein lysates (23A2 skeletal myoblast cells grown in Growth media or Differentiating media). Protein-beads mixture was rotated for 1 hour at room temperature. The beads were then washed thrice with 1 ml RNA binding buffer. Equal volume of SDS buffer was added to the beads and boiled for 5 min at 95°C. the eluted proteins were separated by 10% SDS Polyacrylamide gel electrophoresis. The gel was stained with silver staining as per the manufacturer's instructions. Unique proteins bands visible in DM lysate were excised from the gel and identified by mass spectrometry.

RNA GEL SHIFT MOBILITY ASSAY and COMPETITION ASSAY

^{32}P- labeled PUMA RNAs (5'UTR and 5'UTR + 50 nts) was transcribed in vitro and purified using NucAway spin columns. A total of 50,000 cpm of ^{32}P- labeled PUMA RNA probes was heated at 70°C for 5 min, slowly cooled to room temperature and then incubated with the indicated amount of purified protein (Hsp70) in 20 μl binding buffer containing 10mM Tris-HCl pH 7.9, 50mM KCl, 5mM $MgCl_2$, 10% glycerol, 0.5mM EDTA, 0.5 mM DTT, supplemented with yeast tRNA. The reaction mixture was incubated for 30 min at 30 °C. Native gel electrophoresis was done to resolve the RNA-protein complexes using 6% polyacrylamide gel in 0.5X TBE buffer. The gel was dried, exposed to storage phosphor screen and analyzed using a Typhoon Phosphor Imager.

EMSA competition assays were done similar to gel shift assay, cold PUMA 5'UTR + 50 ntd (5, 10, 20, 50 fold molar excess) was allowed to incubate with Hsp70 protein in 20 μl binding buffer (10mM Tris-HCl pH 7.9, 50mM KCl, 5mM $MgCl_2$, 10% glycerol, 0.5mM EDTA, 0.5 mM DTT, supplemented with yeast tRNA) for 10 min at 30 °C. ^{32}P- labeled PUMA RNA probes (50,000 cpm) was then added to the reaction and

allowed to incubate at 30°C for another 30 minutes. RNA-protein complexes were resolved on 6% TBE polyacrylamide gels. The gel was dried, exposed to storage phosphor screen and analyzed using a Typhoon Phosphor Imager.

3.3 RESULTS

3.3.1 IDENTIFICATION OF RNA-BINDING PROTEINS BOUND TO PUMA IRES IN DM CONDITIONS

As reported in previous studies by Weyman and colleagues, PUMA IRES was found to be activated when 23A2 cells were allowed to differentiate by culturing them in serum-free differentiating media (Shaltouki et. al., 2013). Cells cultured in DM for 3 hours were found to have upregulated expression of PUMA protein, as compared to cells cultured in GM (Shaltouki et. al., 2013). This study also demonstrated PUMA protein synthesis switch to IRES-mediated / cap-independent mode of translation initiation during cellular differentiation, due to inactivation and/or reduced availability of initiation factors (eIF4E and eIF2α). To obtain a complete understanding of IRES-mediated translation of PUMA during differentiation, we explored the possibility of involvement of an ITAF (or ITAFs) in PUMA IRES activation. Therefore, in this study, we aimed to identify specific RNA-binding proteins (ITAFs) that would bind to PUMA IRES in differentiating media conditions when global protein synthesis is shut down, but not in growth media conditions. The study by Weyman and colleagues using CAT/LUC bicistronic reporter constructs had reported that PUMA 5'UTR element supports expression of the reporter gene in DM, however the expression of the reporter construct was further enhanced when 5'UTR + 50 nt construct was used (Shaltouki et al., 2013), thus suggesting an important regulatory role of the sequence elements downstream of the AUG codon. Therefore, we used in vitro transcribed biotin labeled uncapped PUMA construct (5'UTR + 50 nt) for RNA affinity pull down assay. Two different cytoplasmic extracts were prepared with 23A2 skeletal myoblast cells cultured either in Growth media

or Differentiating media. The protein eluate was separated by SDS-PAGE and stained with Silver stain. In accordance with our hypothesis, PUMA IRES was found to interact with different proteins in GM and DM (Table 3). We identified a protein band of approximately 70 kDa that appeared to bind strongly to PUMA IRES in differentiating media extract but showed comparatively weaker or no binding to PUMA IRES in the growth media extract. Mass spectrometric analysis was performed, and the protein was identified as heat shock protein Hsp70. We noted several other proteins preferentially bound to PUMA IRES in DM or GM (marked with arrows on Fig. 3.1) that may serve as positive or negative regulators of PUMA expression. One was identified as ribosomal protein L24 (Fig. 3.1) Identification of the nature of the other protein is pending. However, we didn't pursue the analysis of these proteins further and plan to do it in the future.

Sample name	Protein	Accession	Mass Da	Peptides #	Sequence Cov (%)	Mascot Score
518DM3 band1	actin, cytoplasmic 1	6671509	42052	6	17.1	328
	heat shock 70 kDa protein 1B	124339826	70418	4	7.9	191
	annexin A2	6996913	38937	4	14.7	149
	elongation factor 1-alpha 1	126032329	50424	2	5	143
	acetyl-CoA carboxylase 1	125656173	266685	3	1.4	142
518DM3 band 2	actin, cytoplasmic 1	6671509	42052	4	14.4	242
518DM3 band 3	not identified					
518DM3 band 4	actin, aortic smooth muscle	6671507	42381	3	9.8	144
	nitric oxide synthase, brain	6724321	161740	2	1.5	100
518DM3 band 5	60S ribosomal protein L24	18250296	17882	2	13.4	151

Table 3. Sequence analysis of proteins from a silver stained SDS PAGE gel.

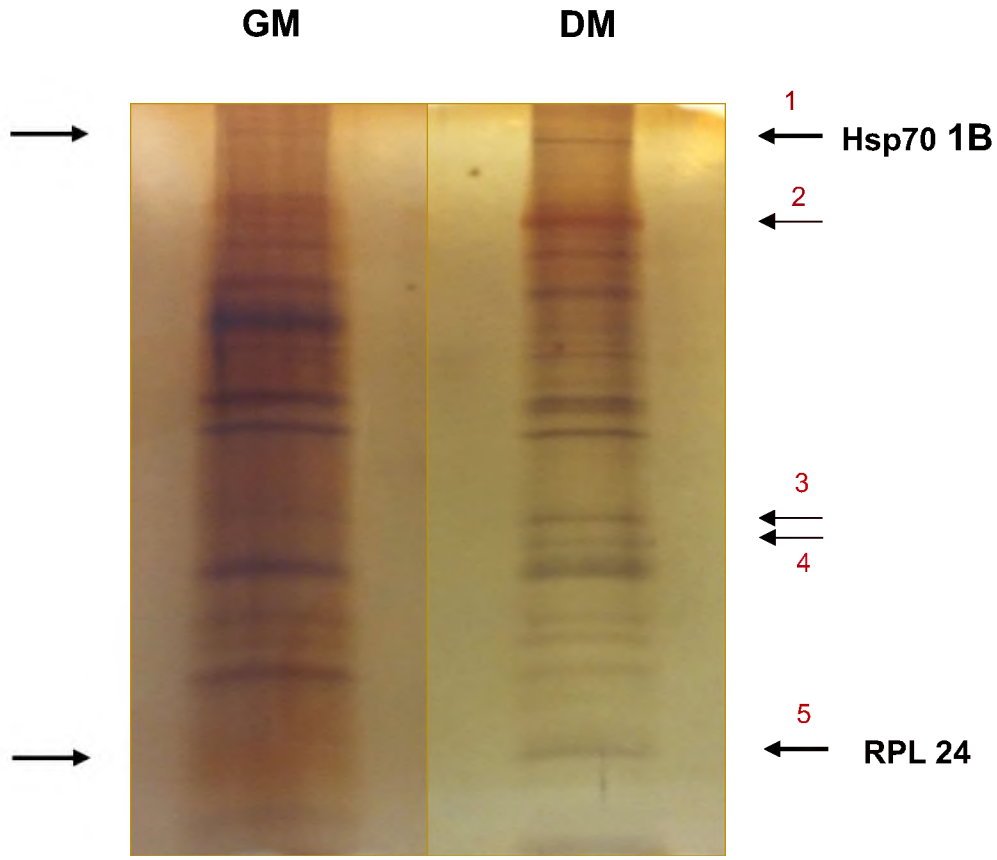


Fig. 3.1. Identification of RNA-binding protein in DM that binds to PUMA IRES. Cytoplasmic extracts from 23A2 skeletal myoblasts cells (cultured in Growth media or Differentiating media) were incubated with streptavidin beads coated with biotinylated PUMA 5'UTR+50 ntd RNA probe. The bound proteins were eluted and analyzed by SDS-PAGE and Silver Staining as described in Materials and Methods. Proteins that bound PUMA IRES in DM were identified by mass spectrometric analysis. The arrow indicates Hsp70 protein bound to PUMA IRES with higher affinity in DM as compared to GM extract.

3.3.2 PUMA IRES (5'UTR + 50 nt) BINDS TO HSP 70 PROTEIN IN VITRO

The RNA-binding properties of Hsp70 have been reported in several studies previously. Hsp70 was shown to bind both non-AU rich regions in 5'UTRs and AU-rich regions in 3'UTRs (Kishor et. al., 2013, Kishor et. al., 2017, Pavithra et. al., 2010). Our findings from biotinylated RNA pull down assay had identified Hsp70 protein binding to PUMA IRES in DM cellular lysate with higher affinity. We further performed gel mobility shift assays to investigate Hsp70 protein interaction with PUMA IRES, using in vitro transcribed ^{32}P-labeled PUMA RNA constructs and purified recombinant Hsp70 protein. As mentioned earlier, a fragment of PUMA mRNA comprising 5'UTR + 50 nt was found to demonstrate maximum IRES activity, therefore we used two different PUMA constructs to investigate PUMA IRES-Hsp70 interaction- uncapped ^{32}P-labeled PUMA 5'UTR (-261 to 0 nt) and uncapped ^{32}P-labeled PUMA 5'UTR+ 50 nt (-261 to +50 nt). Both PUMA RNA constructs were incubated with Hsp70 protein to allow protein-RNA complex formation. The migration of RNA alone and RNA-protein complexes was analyzed by running the reactions on a TBE gel. PUMA 5'UTR probe was incubated with Hsp70 protein and the migration of PUMA 5'UTR alone was the same as the migration of PUMA 5'UTR with Hsp70 protein. Our findings reveal that PUMA 5'UTR was not able to interact with Hsp70 protein (Fig. 3.2).

On the other hand, when PUMA 5'UTR + 50 nt probe was incubated with Hsp70 protein, it allowed the formation of RNA-protein complex (Fig. 3.2). PUMA IRES-Hsp70 protein complex migrated slower in the gel as compared to PUMA IRES without Hsp70 (Fig 3.2). Incubating PUMA IRES with 1-2 μM Hsp70 allowed us to see band shift or delayed migration. Our results confirm that PUMA 5'UTR+ 50 nt construct was

able to bind Hsp70 protein, whereas, PUMA 5'UTR failed to show this interaction, suggesting that the Hsp70 binding is likely confined to the 50 nt region downstream of the AUG codon.

In addition, we also performed EMSA-competition assay to confirm PUMA IRES-Hsp70 interaction. We incubated Hsp70 protein with increasing concentrations of unlabeled cold PUMA 5'UTR + 50 nt (5, 10, 20, 50-folds molar excess) before the addition of radiolabeled RNA to the reaction. Unlabeled PUMA probe acts as a competitor to bind with Hsp70. Our findings reveal that a 50-fold molar excess of cold PUMA IRES probe was able to effectively compete with ^{32}P-labeled PUMA IRES probe for binding with Hsp70 (Fig. 3.3). Based on our results, we confirm that PUMA IRES (5'UTR + 50 nt) binds to Hsp70 in vitro but not to PUMA 5'UTR.

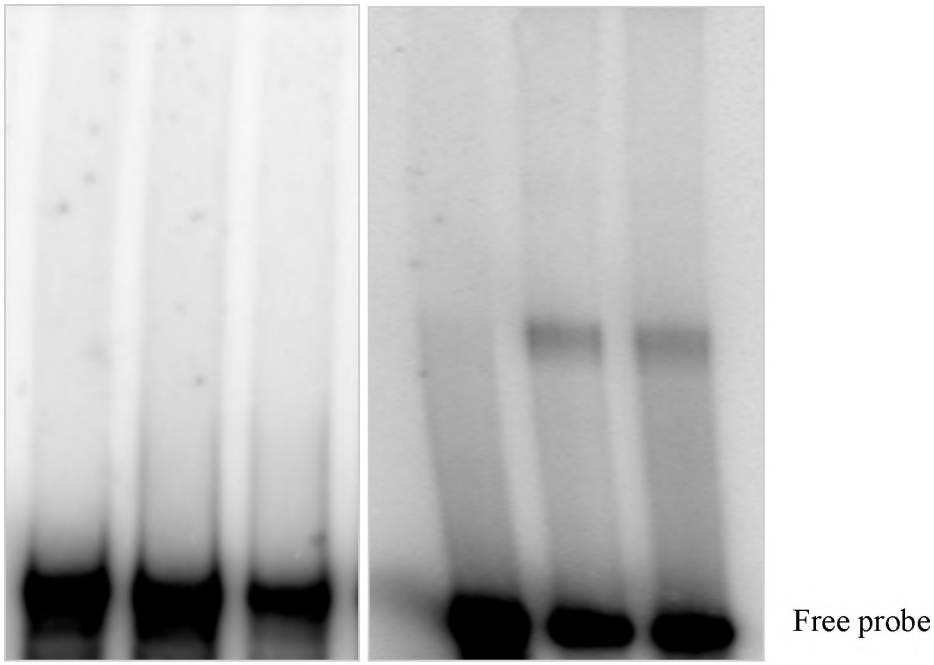


Fig. 3.2. Binding of Hsp70 to PUMA RNA sequence. Electrophoretic Mobility Shift Assay shows ^{32}P-labeled PUMA RNA probe (5'UTR & 5'UTR+50 nt) incubated with increasing concentrations of purified recombinant Hsp70 protein (1 and 2 μM). All samples were separated on 5% polyacrylamide gels. Lanes 1-3 shows PUMA 5'UTR incubated with buffer or Hsp70 protein (1 and 2 μM). Lanes 4-6 shows PUMA 5'UTR+50ntd incubated with buffer or Hsp70 protein (1 and 2 μM).

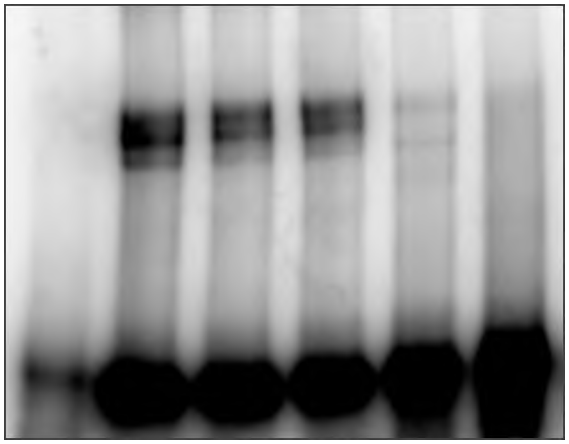


Fig. 3.3. Cold PUMA IRES (5'UTR + 50 nt) competes to bind with Hsp70. For competition assay, unlabeled or cold PUMA RNA probe was added (at 5, 10, 20, 50-fold excess) to the reaction 10 minutes before the addition of labeled PUMA RNA probe. Lane 1 shows PUMA IRES probe, lane 2 shows PUMA IRES bound to Hsp70 protein (2 μM), lane 3-6 shows ^{32}P- labeled PUMA IRES bound to Hsp70 protein (2 μM) in the presence of 5, 10, 20 and 50-fold molar excess cold PUMA IRES probe.

CONSERVED PUMA ORF sequence (1-50 ntd)

Fig. 3.4. Nucleotide sequence of PUMA ORF in mammals. Rat, Mouse and Human PUMA ORF (1-50 nt) shows 98% conservation and is approximately 80% GC rich.

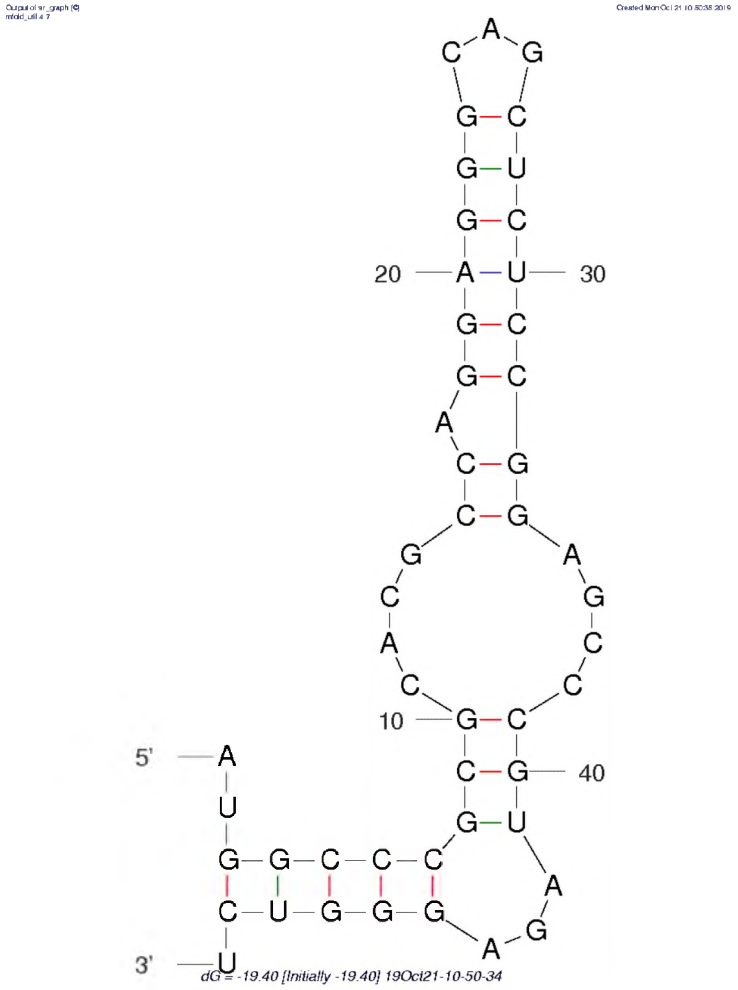


Fig. 3.5. Predicted secondary structure of PUMA ORF (1-50 nt) generated by MFOLD. PUMA ORF (1-50 nt) is predicted to form a strong secondary structure with hairpins and stem loops.

3.4 DISCUSSION

IRES-containing mRNAs have 5'UTRs that are usually long, have a high GC content and may contain upstream ORFs (Komar et. al., 2012). These characteristics are responsible for blockade of ribosome scanning at the time of translation initiation. IRES sequences present in the 5'UTR mediate ribosome binding directly to initiate cap-independent translation. To regulate IRES activity, specific auxiliary factors called IRES-trans acting factors (ITAFs) are required (Komar and Hatzoglou, 2011). ITAFs recognize specific sequences or secondary structures within the 5'UTR and bind to IRES sequences regulating its activity (Kim et. al., 2018). Several studies have reported cellular IRES activation of a number of genes during differentiation. These include FGF1, PDGF2, VEGF and c-Myc IRES (Caroline et. al., 2009; Gerlitz et. al., 2002).

To identify specific RNA-binding proteins that bound to PUMA IRES during differentiation and supported PUMA translation, we performed biotinylated RNA affinity pull down assay followed by mass spectrometric analysis. Previously it has been experimentally shown that 50 nucleotides downstream of the AUG codon are required for maximum PUMA IRES activity (Shaltouki et al., 2013). Therefore, in this study we allowed RNA-protein complex formation by incubating biotin labeled PUMA IRES sequence (5'UTR+50 nt) with cytoplasmic extracts from growth media or differentiating media cultured 23A2 cells. The bound proteins were analyzed by SDS-PAGE followed by silver staining. We found that PUMA IRES bound to different proteins in growth media and differentiating media (Table 3). Proteins that bound to PUMA IRES from DM lysate with higher affinity than GM lysate were identified by mass spectrometry (Fig 3.1). Hsp70 protein was identified as the RNA-binding protein associated with PUMA

IRES in differentiating media conditions with higher affinity as compared to growth media conditions. We performed further in vitro protein-RNA binding assays (gel shift assay) and found that Hsp70 binds to the 5'UTR + 50 nt region of PUMA transcript, but not PUMA 5'UTR (Fig 3.2). EMSA competition assay was performed which confirmed PUMA IRES (5'UTR + 50 nt) interaction with Hsp70 protein (Fig 3.3). It was interesting to find that Hsp70 did not bind to PUMA 5'UTR. Hence, our experiments confirmed that PUMA IRES binds with Hsp70 in vitro and also indicated the possibility of Hsp70 binding site being located within the coding region downstream of the AUG codon, i.e., nucleotides 1-50 of PUMA ORF. We compared mammalian PUMA ORF (1-50 nt) sequence and found it to be 98% conserved between mouse, rat and human (Fig 3.4). PUMA 5'UTR was predicted to form a strong secondary structure using MFOLD (Shaltouki et. al., 2013). We analyzed PUMA ORF (1-50 nt) which is almost 80% GC-rich and is predicted to form extensive stem-loop structure (Fig 3.5).

For a long time Hsp70 has been known to be a protein chaperone assisting in protein folding. However, it is evident from several reports that Hsp70 may also be involved in translation regulation. Studies have found that Hsp70 binds to the c-myc, VEGF and Cox-2 mRNAs (Kishor et. al., 2013). Hsp70 was found to interact with the AU-rich regions of these mRNAs in their 3'UTR resulting in mRNA stabilization. Reports suggests that the Hsp70 molecule binds to the RNA through its N-terminal ATP-binding region (Kishor et. al., 2013; Kishor et. al., 2017; Henics et. al., 1999). A recent study also demonstrated that Hsp70 can bind to non-AU rich 5'UTR of SMAR1 transcript (Pavithra et. al., 2010). The 5'UTR of SMAR1 comprises a stem-loop structure named SL1, to which Hsp70 interacts. The Hsp70-SMAR1 nucleoprotein binding was

found to increase SMAR1 protein levels as a result of SMAR1 transcript stabilization (Pavithra et. al., 2010). Together, these results and our results strongly indicate that Hsp70 may be an important regulator of cellular mRNA translation.

CHAPTER IV

Hsp70 IS ESSENTIAL FOR PUMA TRANSLATION

4.1 INTRODUCTION

Our previous data have shown that PUMA IRES binds to specific proteins in differentiating media but not in growth media. One of the proteins in differentiating media that bound to PUMA IRES with higher affinity was identified as Hsp70 protein by mass spectrometric analysis. We have also shown in vitro interaction between Hsp70 protein and PUMA IRES sequence (5'UTR+ 50 nt). We further attempted to determine the correlation between Hsp70 binding to PUMA RNA and its functional significance. To further understand the significance of Hsp70 for PUMA translation, we performed in vitro PUMA translation in Hsp70 depleted extract. Hsp70 was depleted from the translation extract using antibody depletion assay. The depleted extract was used to check PUMA protein synthesis. To follow up, we performed translation rescue experiment using Hsp70 depleted translation extract supplemented with purified Hsp70 protein. We also report the effects of GM and DM lysates on PUMA translation. As our previous assay found that PUMA IRES binds to different proteins in GM and DM protein lysates, we suspected that these lysates might have variable effect on PUMA translation. In order to determine the effect of RNA-binding proteins in GM and those in DM, we performed

in vitro PUMA translation in the presence of growth media or differentiating media cellular extracts.

4.2 MATERIALS AND METHODS

IMMUNO DEPLETION ASSAY

Protein agarose A/G beads were washed, pre-cleared and allowed to incubate with anti-Hsp70 monoclonal antibody for 3 hrs at 4°C. Nuclease treated Rabbit reticulocyte lysate (Promega) was added to the beads and antibody mixture and rotated overnight at 4°C. A mock control was set up with beads rotated with RRL extract without Hsp70 antibody. The supernatant was separated from the agarose beads by centrifuging at 6000 rpm at 4°C. Hsp70 protein levels were detected in the depleted extract by Western Blot. In vitro translation of PUMA mRNA was done using fresh RRL extract, mock-depleted RRL and Hsp70 depleted rabbit reticulocyte lysate. The same extracts were used for in vitro translation of control RNA Luciferase.

IN VITRO TRANSLATION ASSAY

In vitro translation experiments were done using Hsp70 depleted Rabbit Reticulocyte Lysate. In vitro transcribed and purified PUMA mRNA (1 μg) was incubated with 35 μl Hsp70 depleted RRL, 1mM amino acid mixture (without Methionine), RNAse inhibitor (40U/μl) and 10 mCi/ml [^{35}S] methionine (Perkin Elmer) in a final volume of 50 μl reaction for 90 min at 30°C. The reaction was stopped with SDS protein loading buffer. Reactions were resolved on a 12% SDS-PAGE gel. The gel was fixed, dried and visualized using Typhoon Phosphor Imager (GE Healthcare). Each experiment was repeated independently three times using different RNA preparations.

TRANSLATION RESCUE EXPERIMENT

In vitro translation rescue experiments were done as described above. In vitro transcribed and purified PUMA mRNA (1 μg), 35 μl Hsp70 depleted RRL supplemented with purified Hsp70 (0.2, 0.3, 0.4, 0.5 μM), 1mM amino acid mixture (without Methionine), RNAse inhibitor (40U/μl) and 10 mCi/ml [^{35}S]-methionine (Perkin Elmer) was incubated in a final volume of 50 μl reaction for 90 min at 30°C.

IN VITRO TRANSLATION ASSAY WITH GM/DM

In vitro translation experiments were done using Rabbit Reticulocyte Lysate supplemented with growth media lysate or differentiating media lysate. In vitro transcribed and purified PUMA mRNA (1 μg), 35 μl RRL supplemented with GM/DM lysate (5, 10, 15 μg), 1mM amino acid mixture (without Methionine), RNAse inhibitor (40U/μl) and 10 mCi/ml [^{35}S]-methionine (Perkin Elmer) was incubated in a final volume of 50 μl reaction for 90 min at 30°C. The reaction was stopped with SDS protein loading buffer. Reactions were resolved on a 12% SDS-PAGE gel. The gel was fixed, dried and visualized using Typhoon Phosphor Imager (GE Healthcare). Each experiment was repeated independently three times using different RNA preparations.

4.3 RESULTS

4.3.1 IN VITRO TRANSLATION OF PUMA REQUIRES Hsp70

Our findings indicated the functional significance of Hsp70 in PUMA translation. Interaction of PUMA IRES with Hsp70 protein with higher affinity in differentiating media suggested that Hsp70 may serve as an auxiliary factor for internal initiation of PUMA translation. To investigate the role of Hsp70 in PUMA translation directly, we performed in vitro translation of PUMA mRNA in the absence of Hsp70. We performed Immuno-depletion assay, using anti-Hsp70 monoclonal antibody to deplete Hsp70 from Rabbit reticulocyte lysate. Pre-cleared Protein A/G agarose beads were incubated with Hsp70 antibody for 5 hours at 4°C, followed by incubation with RRL overnight at 4°C. Immune complexes were pulled down by centrifugation. We also obtained a mock-depleted lysate, for which RRL was incubated with protein A/G agarose beads for the same period of time, without any antibody. Western blot was performed to detect protein levels in normal, and Hsp70 depleted lysates. Our results indicate almost 90% depletion of Hsp70 (Fig 4.1). We used normal RRL, mock-depleted RRL and Hsp70 depleted RRL to perform in vitro translation of uncapped PUMA mRNA. In vitro translation of control luciferase mRNA was performed using the same three extracts. We found that translation efficiency of PUMA was reduced substantially (approximately 2-folds) in Hsp-70 depleted translation extract (Fig. 4.2). On the other hand, there was no significant difference in the expression of luciferase when the same Hsp70 depleted extract was used for in vitro translation (Fig. 4.2). This assay confirmed that Hsp70 is a necessary component for enhanced PUMA protein synthesis.

In addition, we also performed translation rescue experiment to confirm that PUMA translation efficiency was reduced due to reduced availability of Hsp70 in the translation extract. In vitro translation rescue experiment was performed by supplementing Hsp70-depleted lysate with increasing concentrations of purified Hsp70 protein (0.2, 0.3, 0.4 and 0.5 μM). We found that addition of 0.5 μM purified Hsp70 restored PUMA expression by almost 3-folds (Fig. 4.3). Based on our findings, we can assert that Hsp70 is an essential factor which plays an important role in PUMA translation.

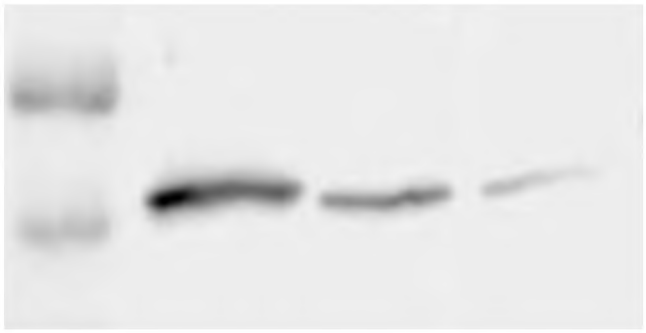
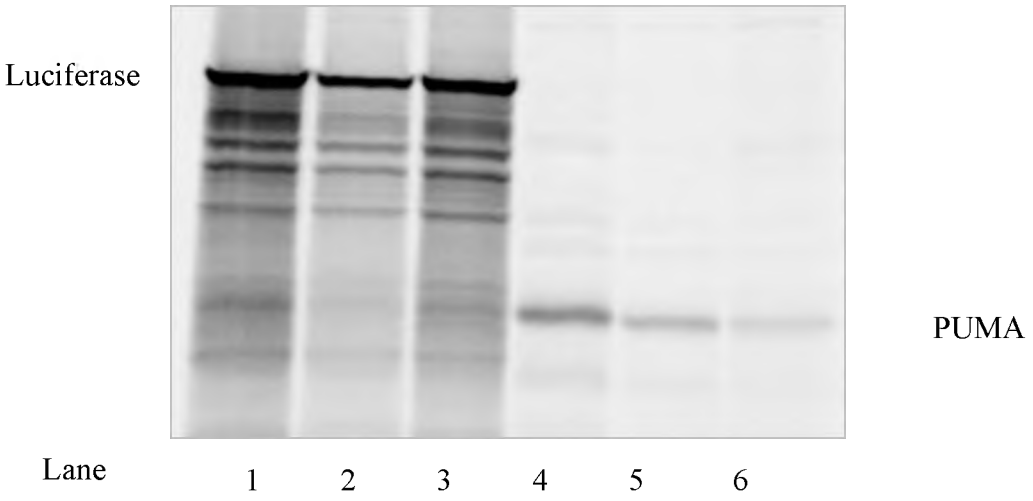


Fig. 4.1 Hsp70 Immuno-depletion. Hsp70 was depleted from RRL using monoclonal Hsp70 antibody and protein A/G agarose beads. Hsp70 expression was checked by western blot assay. Lane 1 represents Hsp70 levels in RRL, lane 2 represents Hsp70 levels in mock depleted RRL, lane 3 represents Hsp70 levels in Hsp70 depleted RRL,



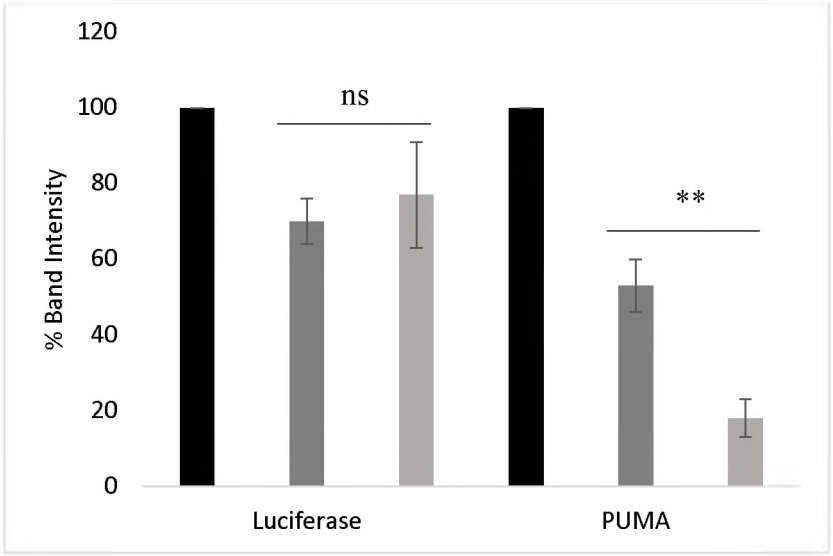


Fig. 4.2. In vitro translation of PUMA mRNA and control Luciferase mRNA in Hsp70 depleted RRL extract. In vitro translation reactions were performed for PUMA mRNA and control Luciferase mRNA using RRL extract (lanes 1 and 4), RRL extract incubated with beads only (lanes 2 and 5) and Hsp70 depleted RRL extract (lanes 3 and 6). Quantification of the translation products using a Typhoon phosphor imager. The results shown are the mean value of three independent experiments. The results shown are the mean ± SEM value of translation efficiencies from three independent experiments. P value: *<0.05, **<0.001

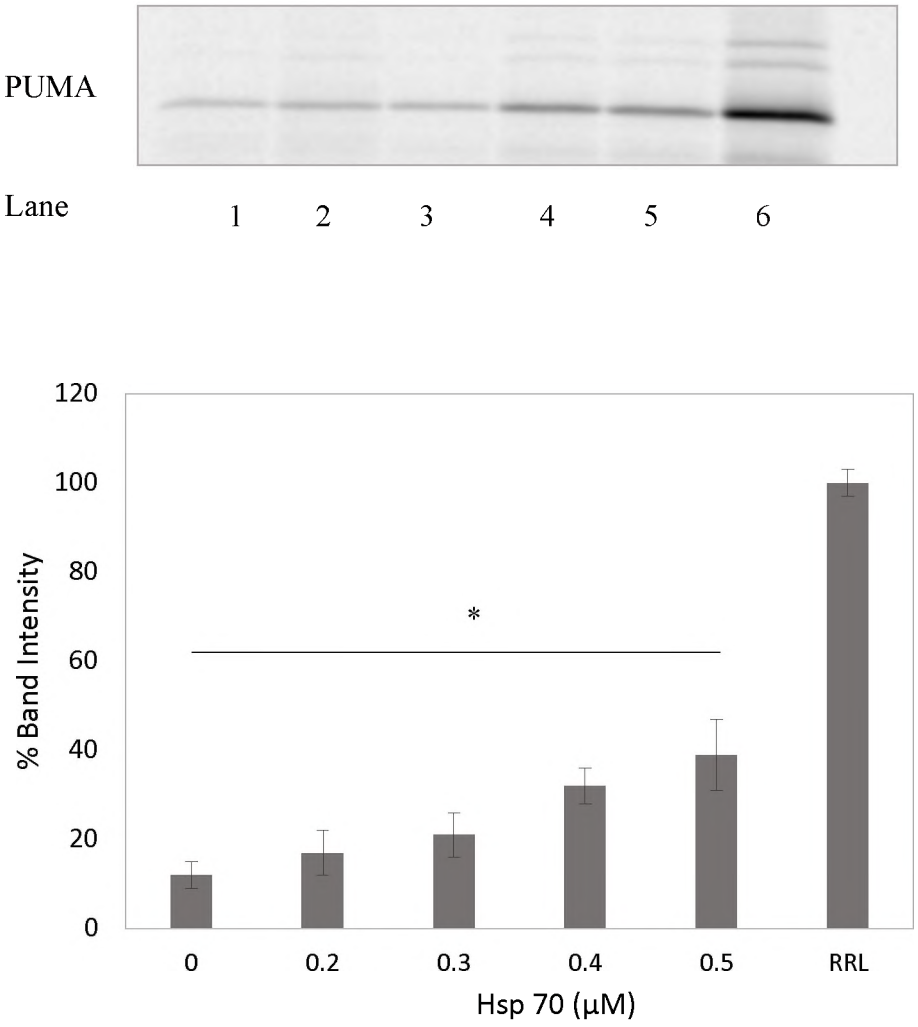
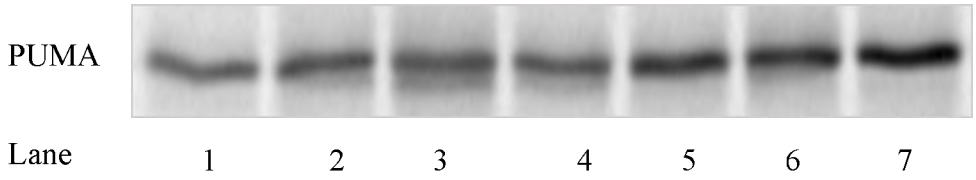


Fig. 4.3. In vitro translation of PUMA mRNA in Hsp70 depleted RRL, supplemented with Hsp70. Uncapped full-length PUMA mRNA was subjected to in vitro translation in Hsp70-depleted RRL containing [^{35}S]-methionine and purified Hsp70. Lane 1 represents PUMA expression in Hsp70-depleted RRL, lanes 2-5 represents PUMA expression in RRL supplemented with Hsp70 of increasing concentrations (0.2, 0.3, 0.4 and 0.5 μM) and lanes 6 represents PUMA translation product synthesized in RRL. Quantification of the translation products using a Typhoon phosphor imager. The results shown are the mean ± SEM value of translation efficiencies from three independent experiments. P value: *<0.05, **<0.001

4.3.2 PUMA TRANSLATION IS STIMULATED BY DM CELLULAR EXTRACT

Studies have reported previously that translation of several viral IRESs was stimulated by addition of certain factors. For example, translation of polioviral IRES was enhanced in the presence of HeLa extract and addition of cytoplasmic liver extract stimulated translation of Hepatitis A viral IRES (Brown et. al., 1979; Glass et. al., 1993). Our findings indicate that in differentiating media unique RNA binding proteins bind to PUMA IRES. As reported previously, increased PUMA expression in 23A2 myoblast cells cultured in DM may therefore be due to the activity of these unique RNA binding proteins. Hence it can be inferred that Growth media and Differentiating media cellular extracts may have distinct effects on PUMA translation. To investigate that, in vitro translation of uncapped PUMA mRNA was performed, in RRL supplemented with increasing concentrations of Growth media or Differentiating media lysates (5, 10 and 15 μg). We found that in the presence of DM cellular extract, PUMA expression was upregulated more as compared to PUMA expression in the presence of GM extract (Fig. 4.4). DM lysate had a more pronounced effect on in vitro PUMA translation than GM extract. Based on our findings, we confirm that PUMA translation is enhanced more by the addition of DM extract in RRL translation system. In conclusion, we believe that DM lysate contain additional factors that are able to enhance PUMA translation. These factors might be absent in GM lysate or low in abundance.



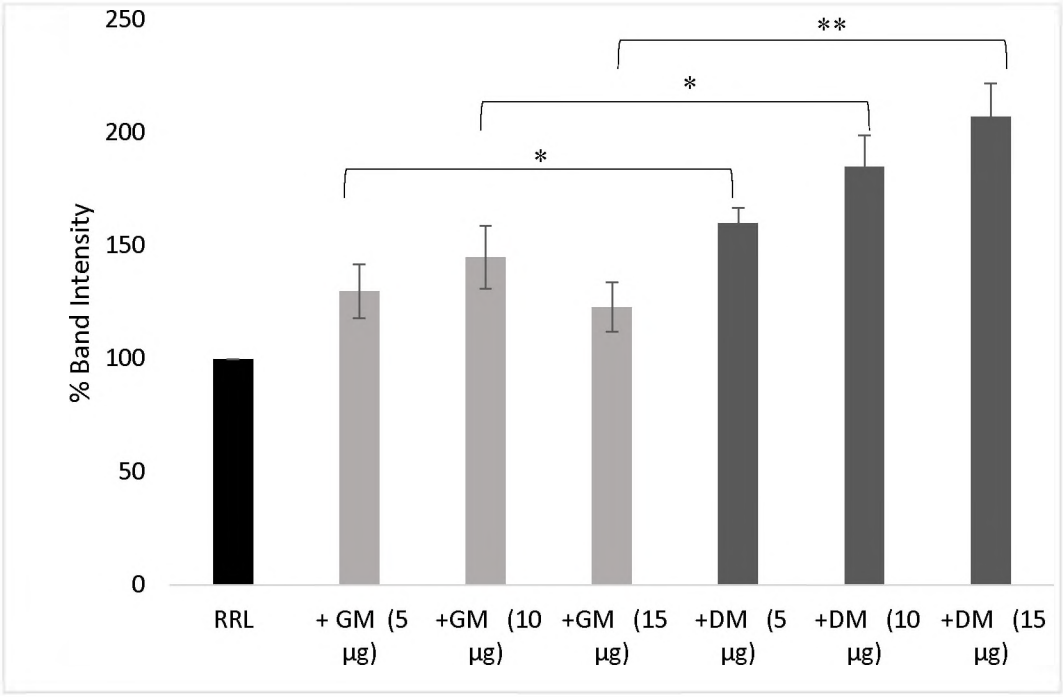


Fig. 4.4. In vitro translation of PUMA mRNA in a cell-free RRL system, supplemented with GM or DM extracts. Uncapped full-length PUMA mRNA was subjected to in vitro translation in a rabbit reticulocyte lysate cell-free system containing [^{35}S]-methionine and cytosolic extracts from 23A2 cells cultured in growth or differentiating media. Lane 1 represents PUMA translation product synthesized in RRL only, lanes 2-4 represents PUMA translation product synthesized in RRL supplemented with growth media lysate of increasing concentrations (5, 10, 15 µg) and lanes 5-7 represents PUMA translation product synthesized in RRL supplemented with differentiating media lysate of increasing concentrations (5, 10, 15 µg). Quantification of the translation products using a Typhoon phosphor imager. The results shown are the mean ± SEM value of translation efficiencies from three independent experiments. P value: *<0.05, **<0.001

4.4 DISCUSSION

Several studies have been reported which demonstrates the role of Hsp70 in viral life cycle of Hepatitis C virus, Rabies virus and Classical swine flu virus (Zhang et. al., 2015; Lahaye et. al., 2012). A recent study in Coxsackievirus have found Hsp70 to promote viral protein translation, affecting both initiation and elongation stages. The study confirmed Hsp70 mediated upregulation of an ITAF La protein, which facilitated recruitment of the translation machinery to the Coxsackievirus IRES and initiation complex formation (Wang et. al., 2017). This study also reported that Hsp70 activates Akt-mTORC1 signaling and induces hypo-phosphorylation of 4E-BP, resulting in binding of 4E-BP to eIF4E. Hsp70 suppressed cap-dependent translation and activated IRES-dependent translation initiation of Coxsackievirus during infection (Wang et. al., 2017). Previous studies have reported that PI3- kinase signaling through Akt leads to differentiation in 23A2 skeletal myoblast cells and increases hypo-phosphorylation of 4E-BP (Karasarides et.al., 2006). Hence, it can be suggested that Hsp70 plays a role during cellular differentiation which results in eIF4E inactivation and inhibition of cap-dependent translation.

To understand the functional significance of Hsp70, we aimed to determine the role of Hsp70 protein in PUMA translation. Using immune-depletion assay, we found that Hsp70 is essential for in vitro PUMA translation. Removal of Hsp70 from the translation extract resulted in inefficient PUMA protein synthesis. Translation of PUMA reduced by almost two-folds in the absence of Hsp70 (Fig 4.2). PUMA expression was restored by exogenous addition of Hsp70 (0.2, 0.3, 0.4, 0.5 μM) (Fig 4.3). Our findings indicate the functional significance of Hsp70 in PUMA translation, suggesting that Hsp70

may function as an ITAF for PUMA IRES and plays a significant role in PUMA IRES-mediated translation. We also performed in vitro translation of PUMA mRNA in RRL supplemented with purified Hsp70 protein. We did not see any effect on PUMA expression by the addition of Hsp70 (Data not shown). The results indicated that Hsp70 is not limiting in RRL, as further addition of Hsp70 protein did not enhance PUMA expression.

However, the exact mechanism of Hsp70 function is not clear. Hsp70 may have a role in recruiting the translation machinery (eIFs, 40S ribosome) at the initiation site, or Hsp70 may act as an RNA chaperone and stabilize PUMA mRNA. Another possibility is that Hsp70 may be involved in inducing conformational changes in PUMA IRES sequence in order to facilitate the interaction between PUMA RNA and translation machinery. Similar mechanism of action can be observed in other cellular IRES, for example, Bag-1 IRES, to which ITAFs PCBP1 and PTB binds to unwind the structural region near the start codon and facilitate ribosome recruitment (Pickering et. al, 2004). Another example can be found in Apaf-1 IRES, where ITAF UNR binds to a structural domain in Apaf-1 IRES and unwinds it, allowing recruitment of a second ITAF PTB and Apaf-1 IRES activation (Mitchell et. al., 2001).

In our study, we also report different effects of GM and DM lysates on PUMA translation. In vitro translation of PUMA in rabbit reticulocyte lysate in addition with DM extract resulted in more efficient PUMA protein synthesis. We found that PUMA translation product was further enhanced in the presence of DM lysate as compared to GM lysate of similar concentrations (Fig 4.4). These results validate and support our previous findings in which we observe different RNA-binding proteins bind to PUMA

IRES in GM and DM. We hypothesize that DM may express certain auxiliary factors which are either absent from GM lysate or present in low abundance, which explains their variable effect on PUMA translation.

Based on our findings, we suggest that Hsp70 may function as an auxiliary protein which assists PUMA protein synthesis under stress-related conditions such as differentiation when global protein synthesis is down regulated. During cellular differentiation when translation initiation of PUMA switches to IRES-dependent mechanism, Hsp70 may come into play and makes up for the unavailability of canonical initiation factors which compromises cap-dependent translation initiation.

With the help of RNA affinity pull down assay and mass spectrometric analysis, we identified several proteins binding to PUMA mRNA in DM conditions, but not in GM conditions. Hsp70 was one of them. In this study, we have shown that Hsp70 acts as a positive ITAF for PUMA expression in DM conditions. Similarly, a number of proteins were found to bind to PUMA mRNA in GM conditions but not in DM conditions (Table 3). For future studies, we will also identify proteins that bound to PUMA IRES in GM conditions. There is a possibility that these proteins might act as a negative ITAF for PUMA IRES, leading to reduced PUMA expression in GM conditions. Detailed experimental analysis will be done to investigate and identify negative ITAF/ITAFs involved in PUMA IRES regulation.

To determine the role of Hsp70 in translation initiation, sucrose density gradient analysis can be done to analyze 43S complex recruitment to PUMA IRES in Hsp70-depleted extracts. It is likely that the assembly of the 43S pre-initiation complex on

PUMA IRES will be less efficient in Hsp-70 depleted extracts as compared to non-depleted extracts.

During cellular stress conditions, ITAFs undergo posttranslational modifications which may affect their sub cellular localization and binding affinity for IRESs and overall activity (Komar and Hatzoglou, 2011). Hence, there is a possibility that switching of cells from GM to DM may influence activity and functions of ITAFs. To confirm that Hsp70 binds to PUMA mRNA with higher affinity in DM conditions only, we will check Hsp70-PUMA mRNA interaction ex vivo using RNA-protein co-immunoprecipitation (RIP). Immunoprecipitation will be done in 23A2 cells cultured in GM and DM using Hsp70 antibody, followed by RT-PCR using PUMA specific primers. To validate our immune-depletion results in cells, we will be performing si-RNA based silencing of Hsp70 in 23A2 myoblasts cells cultured in GM and DM. We expect to see reduced PUMA expression in Hsp70-silenced cells cultured in DM.

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