The role of eIF4AIII and 4E-T in mRNA decay

by

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DEDICATION

In loving memory of my father,
whose compassion and generosity were immeasurable,
hard work and integrity esteemed,
and whose courage and bravery were unwavering.
To my father, whose sincerity and kindness inspired me to want to make a difference and whom inspired this work.

To my mom, whose enduring strength and encouragement guided me through and kept me on my feet, and
for her infinite love and understanding which brings me peace.

and

To my husband, for believing that I can achieve anything, for regularly reminding me of it and supporting me,
and for taking this journey with me.

ABSTRACT

Translational control is crucial to balancing the cell's protein output and genetic expression. The substrate of the translational machinery – messenger RNA (mRNA) – is itself subject to regulation. The lifetime of an mRNA is limited and therefore mRNA decay is a critical step in the regulation of gene expression. Translation and mRNA decay are intimately related processes as they both handle the cell economy. mRNAs are generally in a balancing act between the translational and the repression/decay machinery, which ultimately decides the fate of an mRNA and its protein expression rate. In fact, translation affects the rate of mRNA decay. For instance, aberrant messages which contain a premature-termination codon (PTC) require ribosome scanning in order to read the message, discover the mistake, and essentially prompt its destruction. Here, a relationship between the nuclear translation-like factor - eIF4AIII, the nuclear import factor of eIF4E - 4E-T, and mRNA decay was discovered. eIF4AIII is a nuclear protein that interacts physically or functionally with translation initiation factors eIF4G and eIF4B, respectively, and shares strikingly high identity with the initiation factors eIF4AI/II. This work demonstrates that eIF4AIII but not eIF4AI is required for nonsense-mediated decay (NMD). NMD is a surveillance mechanism in eukaryotes which degrades the mRNA when a PTC is present. NMD is a splicing and translation-dependent event in mammalians. We show eIF4AIII is deposited at the exon-exon junction during splicing, is a shuttling protein, and is necessary for NMD. At steady state, 4E-T is predominantly cytoplasmic and is concentrated in bodies that conspicuously resemble the recently described Processing bodies (P-bodies), which are sites of mRNA decay. We demonstrate that 4E-T colocalizes with mRNA decapping

factors in *bona fide* P-bodies and that its binding partner, eIF4E, is tethered to P-bodies in a 4E-T dependent manner. Moreover, 4E-T controls mRNA half life. We demonstrate that 4E-T interaction with eIF4E represses translation, which is thought to be a prerequisite for targeting of mRNAs to P-bodies. Hence, analysis of prospective translation factors has led to elucidation of mRNA decay pathways.

RÉSUMÉ

Le contrôle de la traduction est important pour équilibrer la synthèse protéique et l'expression génétique. Le substrat de la machine traditionnelle, l'ARN messager (ARNm), est lui-même sujet à régulation. La durée de vie d'un ARNm est limitée, c'est pourquoi la dégradation de l'ARNm est une étape critique dans la régulation de l'expression des gènes. La traduction et la dégradation des ARNm sont des processus intimement lies car ils gèrent tous deux l'économie cellulaire. Les ARNm sont généralement dans un état d'équilibre entre la traduction et la répression ou dégradation, ce qui décidera ultimement de leur destin et du niveau d'expression de la protéine encodée par ces ARNm. En outre, la traduction d'un ARNm affecte son niveau de dégradation. Par exemple, des messages aberrants (contenant un codon primature (PTC)) nécessitent une lecture afin d'interpréter le message, de découvrir l'erreur, et éventuellement d'engendrer la dégradation. Nous avons découvert ici une relation entre le simili facteur de traduction nucléaire (eIF4AIII), le facteur d'import nucléaire de eIF4E (4E-T) et la dégradation des ARNm. eIF4AIII est une protéine nucléaire qui interagit physiquement et functionellement avec les facteurs d'initiation de la traduction eIF4G et eIF4B, respectivement, et est très similaire aux facteurs d'initiation eIF4AI et eIF4AII. Nous démontrons ici qu'eIF4AIII mais pas eIF4AI est impliqué dans la dégradation des ARNm non-sens (NMD). Le NMD est un mécanisme de surveillance eucaryote qui dégrade les ARNm contenant un PTC. Il s'agit d'un mécanisme dépendant de l'épiçage et de la traduction. Nous avons démontré qu'eIF4AIII est déposé au niveau de la junction interrois lors de l'épiçage, transite entre noyau et cytoplasme, et est nécessaire pour le NMD. A l'état stationnaire, 4E-T est majoritairement cytoplasmique et se concentre dans

des foci qui ressemblent aux « processing bodies » (P-bodies) décris récemment et qui seraientt des sites de dégradation des ARNm. Nous avons démontré d'une part que 4E-T colocalise avec les facteurs responsables de la perte de la coiffe des ARNm dans ces P-bodies, et d'autre part que son ligand eIF4E se retrouve dans ces P-bodies de façon 4E-T dépendante. De plus, 4E-T régule la demi-vie des ARNm. Nous avons aussi démontré que l'interaction entre 4E-T et eIF4E réprime la traduction, ce qui est supposé être nécessaire à l'adressage des ARNm aux P-bodies. Ainsi, l'analyse de facteurs de traduction putatifs a conduit à l'élucidation des voies de dégradation des ARNm.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Nahum Sonenberg, for allowing me the opportunity to work in such a reputable lab, under his guidance and expertise. I am grateful for the skills I've acquired over the years from being in such an enriching environment, an environment which is a reflection upon his proficiency in the scientific field.

I would like to thank past and present lab members for their help over the years, for their friendship, and for providing a pleasant working environment. I am grateful to Josée Dostie for teaching me laboratory techniques and training me as an undergraduate and summer student. I want to thank Lian Wee Ler, Mauro Costa-Mattioli and Sanjukta Basak with whom I worked very closely, for their help, and hard work. Thanks to Yaël Mamane and Maritza Jaramillo for critical reading of my thesis and providing their insightful comments, and thank you to Laurent Huck for his help with translating my abstract to French. Also, thank you to Maritza and Colin for their help with 4E-T alignments.

I want to thank Colin Lister, Pamela Kirk and Meena Vipparti for their commitment and hard work which eased our day-to-day workload. Meena, thank you for your thoughtfulness and the treats.

I'm grateful for the helpful scientific discussions I've had with various members of the department, students and professors alike, and for the friendships I've made over the years.

Thanks to Maureen for always having the answers regarding administrative matters readily available, but most of all for her friendship and her kindness. Thank you for the hugs and for all the yummy treats over the years.

Last, but not least, I want to express my extreme gratitude to my family. Thank you to my parents, my husband, my brothers and in-laws for their love and support, and encouraging words throughout the years. I am so grateful to my husband for being by my side to love and support me, for the enthusiasm and interest he showed in my work, and for urging me on; thank you for setting me up with all the computer equipment and my little office to do my work in, I share this with you. Finally, I am forever grateful to my parents who worked so hard and gave so much of themselves to provide for my brothers and me, so that we may have many opportunities. I am indebted to them for the education I received and a great deal more. This is as much yours as it is mine. Your unconditional love and support have been instrumental... Thank you. *Vi voglio bene assai. Grazie.*

PREFACE

Chapters 2 and 3 of this thesis include the entire text and figures of two published manuscripts, of which I am the first author. The contribution of the authors is acknowledged below.

Chapter 1

This chapter consists of a general overview and introduction of the topics pertaining to the work presented in Chapters 2 and 3. It presents a review of the recent literature in the subjects of translation and mRNA decay.

Chapter 2

Ferraiuolo, M.A., C.S. Lee, L.W. Ler, J.L. Hsu, M. Costa-Mattioli, M.J. Luo, R. Reed, and N. Sonenberg. 2004. A nuclear translation-like factor eIF4AIII is recruited to the mRNA during splicing and functions in nonsense-mediated decay. *Proc Natl Acad Sci U S A*. 101:4118-23.

This publication was the result of a fruitful collaboration with Lian Wee Ler and Mauro Costa-Mattioli as well as with Dr. Robin Reed at Harvard Medical School and her students Chung-Sheng Lee, Jeanne L. Hsu and Ming-juan Luo. In *Figure 1* of the published manuscript, RNAi and Northern blot was performed by me, and SDS-PAGE/Western blot by Lian Wee Ler; *Figure 2 and 3* were performed by Dr. Robin Reed's team; *Figure 4* was performed by me. In addition to the published figures, data not shown is included as an Appendix to Chapter 2. The translation assay in *Figure A1* was performed by Lian Wee Ler and me. The poliovirus experiment in *Figure A2* was performed by Mauro Costa-Mattioli and me, with the help of Lian Wee Ler. In addition to the authors, there are several people who provided their help and reagents, they are acknowledged at the end of this chapter. I would like to acknowledge the help of Nicolas Bottari, Lian Wee Ler and Avak Kahvejian for designing the various siRNAs.

Chapter 3

Reproduced from **The Journal of Cell Biology, 2005 Sep 12, 170(6), 913-24** by copyright permission of The Rockefeller University Press.

Ferraiuolo, M.A., S. Basak, J. Dostie, E.L. Murray, D.R. Schoenberg, and N. Sonenberg. 2005. A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay. *J Cell Biol*. 170:913-24.

All of the experiments in this publication were performed by me with the exception of *Figure 3.2,* which was performed by Josée Dostie. I would like to acknowledge the help of Sanjukta Basak who performed Western blot analysis for *Figures 3.4(d), 3.5(b), 3.6 (b) and 3.7(d)* and also assisted me with part of the Northern analysis in *Figure 7.* I want to acknowledge Dr. D.R. Schoenberg and his student Elizabeth L. Murray who constructed the Tet-regulated β-globin expression plasmids used in *Figure* 3.7 and with whom it was a pleasure collaborating with. I also would like to thank the confocal technician, Jacynthe Laliberté, for providing me with good technical training at the confocal facility and for her expertise and assistance with confocal imaging. Once again there are several people to thank for providing numerous reagents and their help; they are acknowledged at the end of this chapter.

Chapter 4

This chapter offers a general discussion of the data presented in Chapters 2 and 3 and also provides a current overview of the recent developments since their publication. Furthermore, it presents a discussion of data which is unpublished and offers suggestions for further experiments.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

eIF4AIII is essential for nonsense-mediated decay (NMD); siRNA against eIF4AIII, but not against eIF4AI and/or eIF4AII, inhibits NMD.

siRNA-mediated depletion of eIF4AIII has no effect on general translation *in vivo,* whereas depletion of eIF4AI inhibits general translation (~40% decrease) and poliovirus protein production.

eIF4AIII, but not eIF4AI, associates with spliced mRNA at the position of the exon-exon junction, *in vitro*. eIF4AIII associates with spliced mRNA *in vivo.*

eIF4AIII is a shuttling protein which exits the nucleus independently of the CRM-1 pathway.

4E-T and eIF4E colocalize with decapping factors in P-bodies.

Overexpression of 4E-T inhibits cap-dependent translation *in vivo,* only when bound to eIF4E.

Overexpression of 4E-T causes a marked increase of eIF4E in P-bodies.

eIF4E is dependent on 4E-T for its localization to P-bodies.

Other components of the eIF4F complex (eIF4A and eIF4GI) are excluded from P-bodies.

4E-T is required for P-body assembly; siRNA against 4E-T results in the disappearance of decapping factors from P-bodies.

The localization of 4E-T in P-bodies is dependent on mRNA.

4E-T localization to P-bodies is independent of its shuttling activity.

4E-T controls mRNA half-life; reduction in 4E-T levels results in an increase in ARE-mRNA stability.

LIST OF ABBREVIATIONS
In alphabetical order

ADP	adenosine diphosphate
Ago	argonaute
ARE	adenine/uridine rich element
ATP	adenosine triphosphate
BRF1	butyrate response factor 1
CBC	cap-binding complex
CBP	cap binding protein
CHX	cycloheximide
CRM-1	chromosome maintenance region 1
C-term	carboxy terminus
Dcp	decapping enzyme
DcpS	scavenger decapping enzyme
DRB	5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole
dsRNA	double-stranded RNA
4EHP	eIF4E homologous protein
4E-BP	eIF4E binding protein
Edc/p	enhancer of decapping
eIF	eukaryotic initiation factor
EJC	exon junction complex
eRF	eukaryotic release factor
4E-T	4E-Transporter
FRAP/ mTOR	FKBP12-rapamycin-associated protein/mammalian target of rapamycin
GDP	guanosine diphosphate
GFP	green fluorescent protein
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HA	hemagglutinin

HIT	histidine triad
IRES	internal ribosome entry site
LMB	leptomycin B
Lsm	Sm-like
MIF4G	middle internal fragment of eIF4G
miRISC	miRNA/RISC complex
miRNA	microRNA
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein
NES	nuclear export signal
NLS	nuclear localization signal
NMD	nonsense-mediated decay
NMR	nuclear magnetic resonance
NSD	nonstop decay
Nt	nucleotide
N-term	amino terminus
Nudix	nucleotide diphosphate linked to an X moiety
ODC	ornithine decarboxylase
ORF	open reading frame
PABP	poly(A) binding protein
PAIP	poly(A) binding protein interacting protein
PARN	poly(A) ribonuclease
P-bodies	processing bodies
PI3/K	phosphatidyl inositol 3-kinase
PP2A	protein phosphatase 2A
PRS	proline rich site
PTC	premature termination codon
RISC	RNA-induced silencing complex
RLP	ribosome landing pad
RNA	ribonucleic acid
RNAi	RNA interference

RRM	RNA recognition motif
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRISC	siRNA/RISC complex
siRNA	short interfering RNA
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SR	serine/arginine
ssRNA	single-stranded RNA
tRNA	transfer RNA
TCR-β	T-cell receptor β
TTP	tristetraprolin
UTR	untranslated region
VEGF	vascular endothelial growth factor
YFP	yellow fluorescent protein

CHAPTER 1

GENERAL INTRODUCTION

1.0 Overview

The life of a messenger RNA (mRNA) begins with its transcription in the nucleus by RNA Polymerase II. Following its birth in the nucleus, it will venture through nuclear processing events that result in its maturation. It will then journey across the nuclear envelope to a safe destination or will be immediately engaged in translation, during which the message contained in the RNA will be decoded into protein that will, in turn, effect the function encrypted by such a message. Along its journey, the mRNA will make many associations (with proteins and RNA) which modify its appearance, affect its function and its fate, and ultimately determine when it is terminated by decay enzymes. Because of its role as a key link in the chain from DNA to protein, mRNA metabolism profoundly influences gene expression. Thus, the stability of mRNA exerts a great impact upon cell growth, differentiation and responses to stress, to only name a few. Characterization of the mechanisms and regulation of mRNA turnover has revealed an affiliation to translation and, in recent years, increasing evidence has uncovered an intimate link between these two processes. The work presented here has shed some light on the relationship between translation and mRNA turnover.

At first, the general features of an mRNA, which play a crucial role in both mRNA stability and translation, will be described. Then, translation initiation, with emphasis on cap-dependent translation, will be summarized. Subsequently, the three main pathways of mRNA degradation will be addressed, giving particular prominence to general deadenylation-dependent decay and nonsense-mediated decay. This chapter will conclude with the description of the localization of mRNA degradation within the cell, the recently reported processing bodies (P-bodies). Altogether, the work presented in this

thesis has contributed to the elucidation of the nonsense-mediated decay pathway as well as the deadenylation-dependent general mRNA decay in P-bodies.

1.1 Features of the mRNA

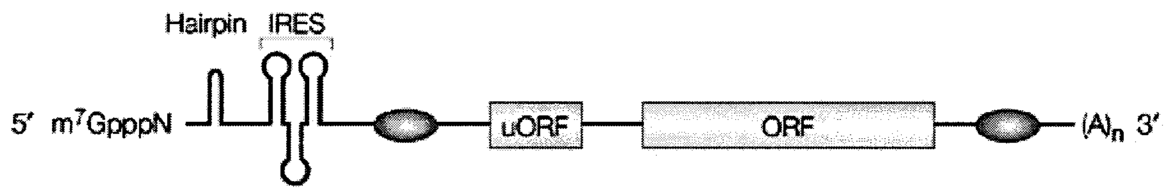
mRNA is composed of four general features: the cap structure at the 5'end, the open reading frame (ORF) which encodes for the effector protein, the poly(A) tail at the 3' terminus, and the untranslated regions upstream (5'UTR) and downstream (3'UTR) of the open reading frame (Fig. 1.1).

1.1.1 Cap structure

The cap (5'm^{7}GpppN) is added cotranscriptionally to all nuclear encoded mRNAs at the 5' terminus and consists of a methylated guanine (m^{7}G) at the 7' position bridged by a 5'-5' triphosphate to the penultimate base (when the 7-methylguanine is counted as the first base), which can be any nucleotide (N) (Shatkin, 1976). The cap influences mRNA metabolism on multiple levels. It affects RNA splicing, polyadenylation, mRNA export, mRNA stability and translation. The cap structure of most messages is affixed independently by two main cap-binding proteins, 1) the nuclear cap-binding complex composed of cap binding protein 20 and 80 (CBP 20/80), which participates in mRNA splicing (Izaurralde et al., 1994), polyadenylation (Cooke and Alwine, 1996), export (Izaurralde et al., 1992; Visa et al., 1996) and stability (Ishigaki et al., 2001; Lejeune et al., 2002), and 2) eukaryotic initiation factor 4E (eIF4E), whose main function is to recruit the translation initiation complex to the 5' end of the mRNA and promote protein synthesis (Shatkin, 1985). Notably, eIF4E has also been shown to influence mRNA transport (Cohen et al., 2001; Lai and Borden, 2000; Rosenwald et al., 1995; Rousseau et al., 1996; Topisirovic et al., 2003) and stability (von der Haar et al., 2004) (see section 1.2.3.1 and 1.2.4).

Figure 1.1. Features of the mRNA

The m^7GpppN cap structure at the 5' end of the mRNA, and the poly(A) tail ($(A)_n$ in the figure) at the 3' end, are canonical motifs that strongly promote translation initiation. Secondary structures, such as hairpins, block translation. Internal ribosome entry sequences (IRESs) mediate cap-independent translation. Upstream open reading frames (uORFs) normally function as negative regulators by reducing translation from the main ORF. Green ovals symbolize binding sites for proteins and/or RNA regulators, which usually inhibit, but occasionally promote, translation. (Adapted from (Gebauer and Hentze, 2004)



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1.1.2 5' and 3' UTRS

These are noncoding, regulatory elements at the extremities of the mRNA (Gebauer and Hentze, 2004). In the 5' UTR, these may include: hairpin structures, which are secondary or tertiary RNA structures which impede translation initiation (Koromilas et al., 1992); internal ribosome entry sites (IRES), which direct translation in a cap-independent fashion (Pelletier and Sonenberg, 1988); upstream open reading frames (ORF) which may decrease translation from the major reading frame (Morris and Geballe, 2000); and consensus sequence elements surrounding the initiator AUG which facilitate translation initiation (Kozak, 1991). Both the 5' and 3' UTRs may also contain sequence specific elements which recruit proteins and/or RNA which regulate translation efficiency and/or mRNA half life. For example, adenine/uridine rich elements (ARE) in the 3'UTR of messages influence mRNA stability and translation by ARE-binding proteins (ARE-BPs). They may either positively or negatively regulate these processes depending on which proteins are bound to these elements (Barreau et al., 2005).

1.1.3 Poly(A) tail

The poly(A) tail varies in length from 50 to 200 adenosine monophosphates at the 3' end of most eukaryotic mRNAs (Jacobson and Peltz, 1996). The poly(A) tail is a crucial determinant of both translation and mRNA stability (Kahvejian et al., 2001; Munroe and Jacobson, 1990; Wilusz et al., 2001b). It may also play a role in mRNA export (Huang and Carmichael, 1996). It is bound primarily by poly(A) binding protein (PABP). PABP interaction with the translation initiation factor eIF4G, bound at the 5'end of the message,

results in the circularization of the mRNA, which is believed to be a strong promoter of translation initiation (reviewed in (Kahvejian et al., 2001)).

1.2 Translation Initiation

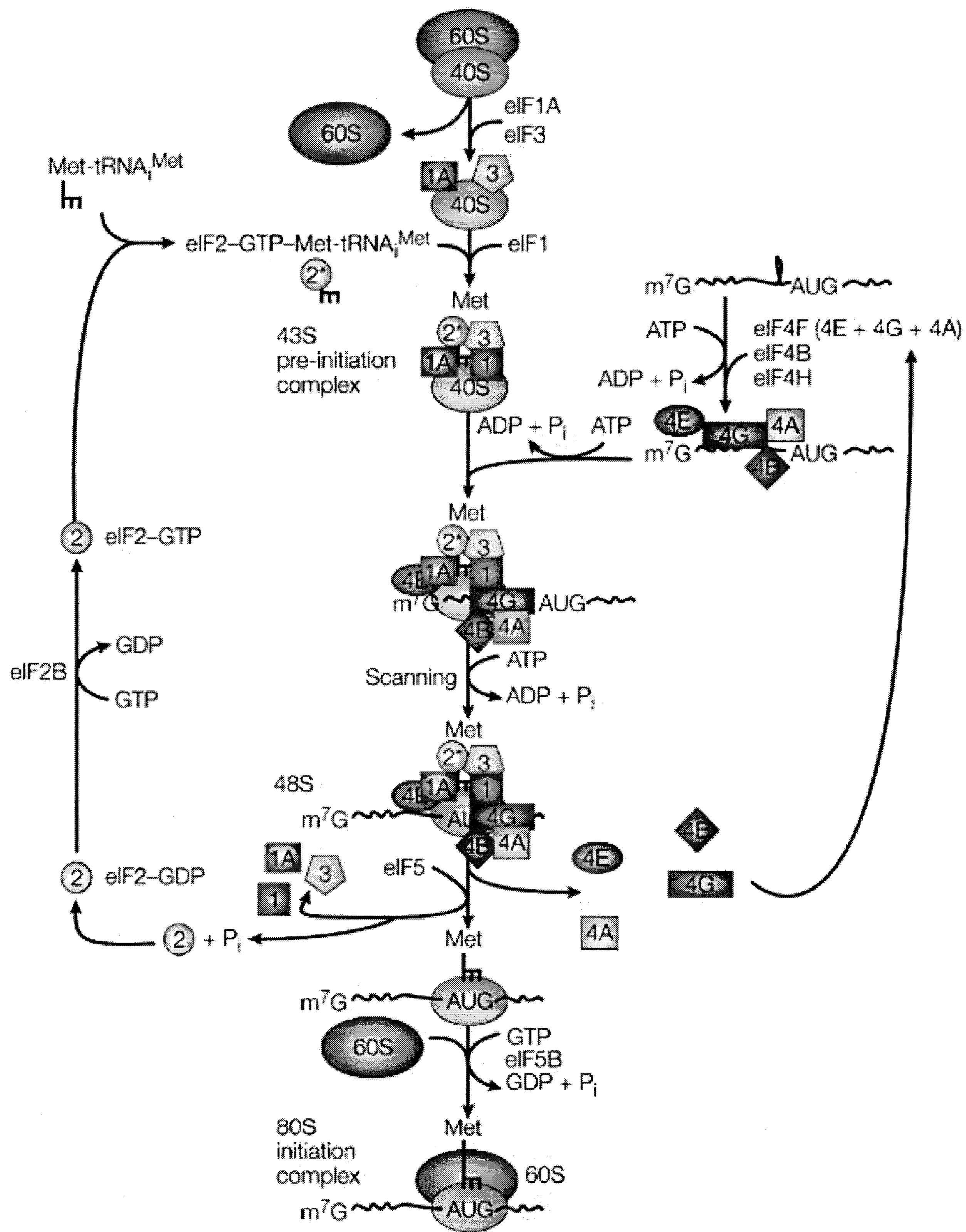
Recruitment of the small ribosomal subunit to the 5'end of the mRNA, recognition of the initiator AUG, and assembly of the 80S ribosome on the mRNA is referred to as the initiation step of translation. Translation initiation is the rate-limiting step of protein synthesis. The manner in which the ribosomal subunit encounters an AUG can occur through either a cap-dependent manner, which involves either scanning or shunting of the ribosome, or in a cap-independent fashion (Gingras et al., 1999; Holcik and Sonenberg, 2005).

1.2.1 Cap-dependent translation

Initiation begins with loading of the 43S pre-initiation complex onto the 5'end of the mRNA, and is achieved by the eIF4F complex (Fig. 1.2). The 43S preinitiation complex consists of a 40S small ribosomal subunit, which is maintained in its dissociated state by eIF1A and eIF3, and is also composed of eIF1, which is crucial for initiation codon selection and fidelity, and the methionine ternary complex. The ternary complex is composed of eIF2, which interacts with methionine tRNA and GTP; it is regulated by eIF2B, a guanine nucleotide exchange factor which replaces GDP with GTP. eIF4F is composed of three subunits: *i)* eIF4E, which interacts directly with the cap; *ii)* eIF4A, an ATP-binding RNA helicase, which is facilitated by eIF4B and eIF4H in unwinding secondary structure present in the 5'UTR; and *iii)* eIF4G, a scaffold protein which binds

Figure 1.2. Eukaryotic translation initiation

See text for details. Adapted from (Holcik and Sonenberg, 2005).



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to eIF4E, eIF4A, and the ribosome associated factor eIF3, therefore bridging the ribosome and the 5'end of the mRNA (Holcik and Sonenberg, 2005).

Once the pre-initiation complex is assembled onto the mRNA, the ribosome scans the mRNA in a 5'to 3'direction until it encounters the initiator AUG in a propitious context, a process that requires energy expenditure in the form of ATP hydrolysis. This is referred to as the scanning model of cap-dependent translation. Once an initiation codon is encountered by methionine tRNA in the form of the 48S preinitiation complex, eIF5 catalyses the dissociation of translation initiation factors by promoting eIF2 hydrolysis of GTP. The 60S large ribosomal subunit joins the small subunit to form the 80S initiation complex, which requires GTP hydrolysis by eIF5B. Subsequently, polypeptide synthesis ensues (reviewed in (Holcik and Sonenberg, 2005)).

An alternative mode of AUG recognition, to the scanning model just described, is referred to as ribosome shunting. In this case, ribosome binding to the mRNA also occurs in a cap-dependent fashion; however, the ribosome engages in discontinuous scanning by skipping or jumping a segment of mRNA, and therefore bypasses the need for melting secondary structures. This mechanism of translation initiation has mainly been documented for viruses and will not be described in detail here (reviewed in (Gingras et al., 1999)).

1.2.2 IRES mediated translation

Recruitment of the ribosome to the mRNA can occur through a mechanism which does not involve recognition of the cap. This mode of translation initiation entails the direct recruitment of the 40S ribosomal subunit to an internal structure of the mRNA within the

vicinity of the initiator AUG. It was first described for poliovirus and encephalomyocarditis virus (EMCV), picornavirus family members which lack a 5'cap structure (Jang et al., 1990; Pelletier and Sonenberg, 1988). These structures were coined ribosome landing pads (RLP), but are more commonly addressed as, IRES, for internal ribosome entry site.

However, IRES-mediated translation is not limited to uncapped RNAs since IRESes are present in capped viral mRNAs, such as those of hepatitis and cricket paralysis virus, and cellular mRNAs, which include ornithine decarboxylase (ODC) and vascular endothelial growth factor (VEGF). IRESes are generally characterized as being considerably long and containing a significant degree of secondary and tertiary structure; however, there is no communal sequence feature among all IRESes (reviewed in (Holcik and Sonenberg, 2005)). There is also a discrepancy in the requirement of canonical initiation factors amongst several viral IRESes, and it is thought that the tertiary structure of an IRES may impart specificity on the translation machinery (Holcik and Sonenberg, 2005).

Cellular IRES-containing mRNAs encode for proteins which function in cell growth, proliferation and differentiation. Cellular IRES-driven translation is particularly relevant to the cellular stress response during which global translation is shut off but translation of a subset of messages containing an IRES element, necessary for cell survival, takes place (Holcik and Sonenberg, 2005).

1.2.3 eIF4F Components

1.2.3.1. eIF4E

eIF4E was identified as a 24 KDa protein that specifically crosslinks to the cap (Sonenberg et al., 1978). The specificity of its interaction was affirmed by the failure to crosslink in the presence of cap analogs, m^7GMP and m^7GDP (Sonenberg et al., 1978). It was later purified on an agarose affinity column coupled to m^7GDP (Sonenberg et al., 1979) which heralded subsequent functional analysis of the protein. eIF4E is indispensable for cap-dependent translation and is the limiting factor in translation initiation in some systems (Gingras et al., 1999; Mamane et al., 2004). Its depletion from rabbit reticulocyte lysate resulted in the inhibition of translation initiation of capped messages, with no effect on IRES-mediated translation initiation (Svitkin et al., 1996). It was previously demonstrated that a reduction in eIF4E expression *in vivo*, via antisense RNA, leads to a decrease in general translation rates in HeLa cells and eventually to cell death (De Benedetti et al., 1991). Furthermore, its role as a crucial factor in translation initiation is underscored by the fact that disruption of the yeast eIF4E gene is lethal (Altmann et al., 1989). More recently, *in vivo* depletion of eIF4E by RNA interference (RNAi) in HeLa cells was also shown to negatively affect the rate of translation initiation by studying the polyribosome distribution. A larger 80S monosome peak and a shift to the lighter polysomes were observed in cells depleted of eIF4E versus control siRNA-treated cells (Svitkin et al., 2005).

Conversely, overexpression of eIF4E selectively elevates the synthesis of potent stimulatory proteins such as ODC, cyclin D1, c-myc and VEGF, and is associated with increased cellular proliferation (Mamane et al., 2004; Rousseau et al., 1996). The profound affect of eIF4E on cellular growth is emphasized by the fact that its overexpression induces oncogeneic transformation in NIH 3T3 and CHO (Lazaris-

Karatzas et al., 1990; Mamane et al., 2004) and promotes malignant growth in mice (Ruggero et al., 2004; Wendel et al., 2004). Moreover, serum starved fibroblasts overexpressing eIF4E are resistant to apoptosis (Mamane et al., 2004). In line with this experimental evidence, increased levels of eIF4E have been found to be associated with several human cancers including breast, colon, liver, head and neck, lung, and bladder (Mamane et al., 2004; Zimmer et al., 2000).

eIF4E is highly conserved across phylogeny (Fig. 1.3A). eIF4E is present in yeast, *Drosophila,* and vertebrates (reviewed in (Gingras et al., 1999). The yeast eIF4E protein bears 32% identity to human eIF4E (Altmann et al., 1989). Disruption of the yeast eIF4E gene causes lethality, which can be rescued by mammalian eIF4E (Altmann et al., 1989). Structural analysis of yeast eIF4E by NMR (Matsuo et al., 1997) and X-Ray crystallography analysis of murine eIF4E (Marcotrigiano et al., 1997) revealed that they share similar structures, which consist of one α/β domain that resembles a cupped hand (Fig. 1.3B). The co-crystal structure of murine eIF4E bound to 7-methyl GDP analog demonstrated that 7-methyl recognition takes place on the concave surface of eIF4E and is facilitated by three types of interactions: a) sandwiching of guanine between two tryptophan residues (W56 and W102) resulting in π-π stacking interactions between the base and the indole groups of the tryptophan residues; b) hydrogen bonds between eIF4E and the guanine base which include a hydrogen bond between o-6 of the guanine ring and W102 and hydrogen bonding between N-1 and N-2 of the guanine ring with E103 carboxyl group; c) a Van der Waals contact between N7 methyl group and residue W166 of eIF4E. All of the afore-mentioned residues of eIF4E responsible for cap

Figure 1.3 eIF4E alignment and structure

a) eIF4E Sequence Alignment Across Species. Black and grey boxes indicate similar residues conserved in 100%, or greater than 85%, of the sequences, respectively. Blue boxes indicate conservation of residues directly involved in recognition of the cap-structure by human and mouse eIF4E-1 and *S. cerevisiae* eIF4E. Yellow boxes indicate conservation of residues involved in recognition of eIF4Gs and 4E-BPs by mouse eIF4E-1. Green or red boxes indicate the presence of a Tyr or a Cys residue at a position equivalent to human eIF4E-1 Trp56. Positions of selected Trp residues and residues involved in the interaction of eIF4E-1 with eIF4G•4E-BP are indicated above the amino acid sequences, as is the location of the conserved consensus sequence (S/T)VXXFW. Adapted from (Joshi et al., 2004).

b) Crystal Structure of eIF4E. Three-dimensional structure of eIF4E (*blue*) bound to the m^7GDP cap analog (*yellow*). (*A*) Ribbon diagram of the mouse eIF4E structure determined by X-ray crystallography; (*B*) ribbon diagram of the yeast eIF4E structure as determined by NMR; (*C*) magnified view of the cap-binding area of mouse eIF4E. The amino acid side chains involved in the interaction are highlighted. Adapted from (Gingras et al., 1999).

A

B

(S/T)VxxFW

M1 E37 W43 W56 W73

W102 L131 W166

*H. sapiens*_eIF4E-1
*M. musculus*_eIF4E-1
*C. elegans*_IFE-3
*C. elegans*_IFE-1
*C. elegans*_IFE-2
*C. elegans*_IFE-5
*T. aestivum*_eIF4E_p26
*T. aestivum*_eIFiso4E_p28
*S. pombe*_eIF4E1
*S. cerevisiae*_eIF4E
*H. sapiens*_eIF4E-2
*M. musculus*_eIF4E-2
*C. elegans*_IFE-4
*A. thaliana*_nCBP
*M. musculus*_eIF4E-3

A

B

C

W102
W56
W166
R112
K162
R157

recognition are conserved from yeast to mammals (Marcotrigiano et al., 1997) (Fig. 1.3B).

eIF4E isoforms exist in nearly all species from C. elegans, *Drosophila,* and *S. cerevisiae* to mammals (reviewed in (Gingras et al., 1999)) (Fig. 1.3A). In mammalian cells, an isoform of eIF4E exists named eIF4E2 or 4E-HP (eIF4E Homologous Protein) (Rom et al., 1998). 4E-HP is also a cap-binding protein (Rom et al., 1998). eIF4E and 4E-HP are both ubiquitously expressed but have different requirements for interaction with eIF4G and 4E-BP. eIF4E interacts with eIF4G and 4E-BP whereas 4E-HP can interact with 4E-BP but does not interact with eIF4G (Joshi et al., 2004). The *Drosophila* homolog of 4E-HP has been characterized as a novel cap-binding repressor of translation. *Drosophila* 4E-HP represses translation of *caudal* mRNA, an anterior/posterior determinant in flies, through its interaction with Bicoid which binds a specific element in the 3'UTR (Cho et al., 2005).

Other cap-binding complexes: CBP20/80 and Dcp

A similar mode of cap recognition is mediated by the nuclear cap binding complex (CBC) which consists of CBP20/80 (Mazza et al., 2001; Mazza et al., 2002a; Mazza et al., 2002b). The CBP20 subunit makes direct contact with the cap structure; however, CBP20 cannot bind to the cap unaided, and requires CBP80 to facilitate the interaction presumably by evoking a conformational change in CBP20 (Izaurralde et al., 1995). Furthermore, CBP80 provides a platform for interaction with other proteins which are involved in the maturation of the RNA. Although this complex does not bear any structural similarities with eIF4E, cap recognition by CBP20 is mediated by base sandwiching via two tyrosine residues, in contrast to the two tryptophans in eIF4E

(Mazza et al., 2002b). CBP20 exhibits little homology (26% similarity at the amino acid level) to human eIF4E and is highly conserved across species (Mazza et al., 2001). CBP80 contains three domains similar to those found in the middle fragment of eIF4G (MIF4G), which is present in other proteins involved in RNA maturation (Marintchev and Wagner, 2005; Mazza et al., 2001). CBP80 interacts directly with eIF4G in yeast (Fortes et al., 1999) and mammals (Lejeune et al., 2004; McKendrick et al., 2001). It is therefore believed that the displacement of CBC for eIF4E occurs via simultaneous and transit interaction of eIF4G with CBP80 and eIF4E (Mazza et al., 2002b).

CBC plays a crucial role in mRNA maturation. It binds strongly to the cap and has been demonstrated to stimulate splicing (Izaurralde et al., 1994; Lewis et al., 1996a; Lewis et al., 1996b) and polyadenylation (Cooke and Alwine, 1996). Furthermore, it is exported with mRNA to the cytoplasm (Izaurralde et al., 1992; Shen et al., 2000; Visa et al., 1996). It is proposed that messages bound by CBC participate during the pioneer round of translation when the message is read for the first time by the ribosome (Ishigaki et al., 2001; Lejeune et al., 2002) (section 1.3.4). The pioneer round of translation is synonymous with a process called nonsense-mediated decay (NMD), an mRNA decay pathway which relies on translation to eliminate faulty messages containing a premature termination codon (PTC) (section 1.3.4). CBP bound messages have been shown to stimulate translation in yeast extracts, wherein eIF4G has been mutated and exhibits diminished interaction with eIF4E (Fortes et al., 2000).

It is expected that the decapping enzyme which hydrolyzes the cap structure when no longer bound by eIF4E, accesses the cap through a similar interaction as that of cap recognition by eIF4E and CBP80. Although the crystal structure of Dcp1p and Dcp2p

has been obtained, neither a model nor the residues mediating cap binding have been elucidated (She et al., 2006; She et al., 2004).

1.2.3.2. eIF4G

eIF4G is the scaffolding protein of the eIF4F complex. There are two isoforms of eIF4G in yeast (Goyer et al., 1993) and in humans termed, eIF4GI and eIF4GII (Gradi et al., 1998; Yan and Rhoads, 1995). The two isoforms are functionally interchangeable and differ in their level of expression among various tissues (Gradi et al., 1998).

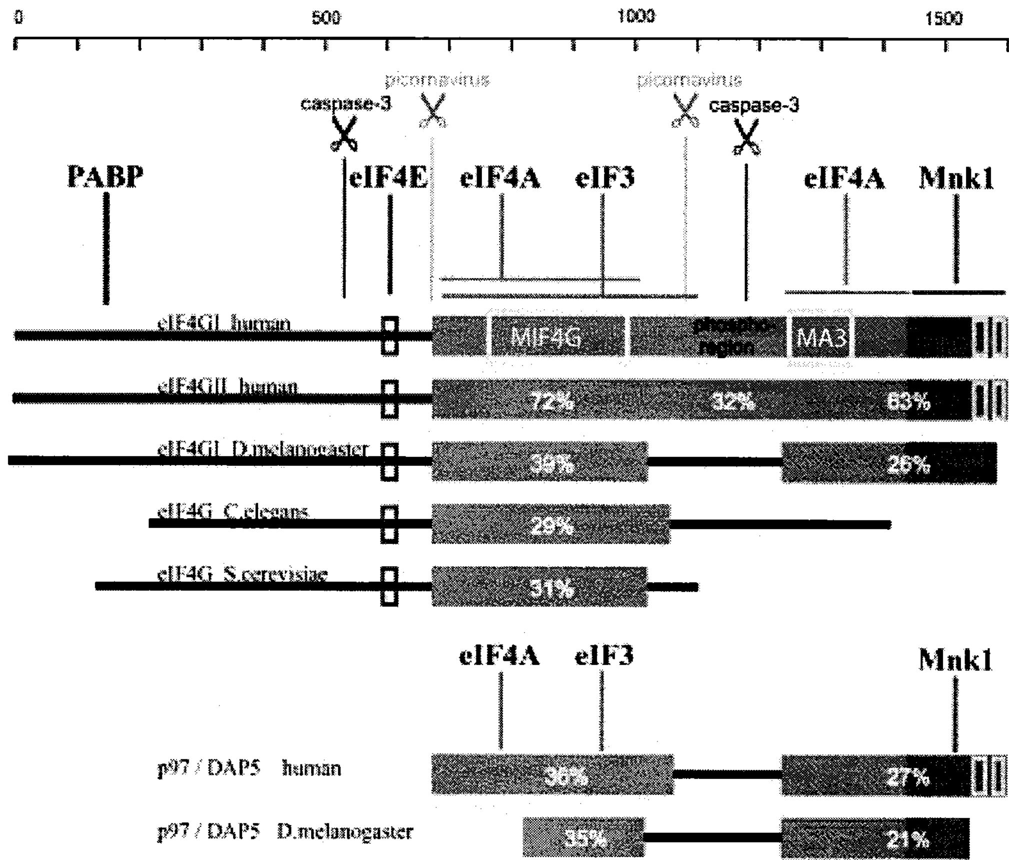
eIF4G domains

eIF4G is a large protein which binds to many translation initiation factors including eIF4E, eIF4A, eIF3, PABP, and to the eIF4E kinase, Mnk1/2 (reviewed in (Gingras et al., 1999)). It is therefore organized into many domains to mediate these interactions (Fig. 1.4) (Marintchev and Wagner, 2005; Ponting, 2000). eIF4G binds eIF4E at its N-terminus (Mader et al., 1995). Poliovirus infection causes the cleavage of eIF4G at its N-terminus and expulsion of the eIF4E binding site, which results in shutdown of host cell protein synthesis (Etchison et al., 1982; Lamphear et al., 1995). The binding site for eIF4E is mapped to an YXXXXLΦ motif (where X is any amino acid and Φ symbolizes a hydrophobic residue) which is shared among other eIF4E binding proteins (Mader et al., 1995) (see section 1.2.4.3). A peptide of eIF4GII containing this motif has been co-crystallized with eIF4E, and shown to contact the convex dorsal surface of eIF4E (Marcotrigiano et al., 1999). eIF4G also binds to PABP via its N-terminal region (Imataka et al., 1998; Tarun and Sachs, 1996). This region is upstream of the eIF4E

Figure 1.4. eIF4G domains

Comparison between eIF4G and p97/DAP5 domain/motif organization in different species. Colored blocks identify homologous regions with % sequence identity to human eIF4GI (4G/M, blue; phosphoregion, orange; 4G/C1, green; 4G/C2, red). The small purple box denotes the eIF4E binding site and the yellow boxes refer to the three HEAT domains, MIF4G, MA3 and AA-boxes. Cleavage sites and interaction regions identified for human eIF4GI and p97/DAP5 are also shown. The bars above them indicate the segments required for interaction with eIF4A and Mnk1, respectively.

Adapted from (Bellsolell et al., 2006) with modification.


0
500
1000
1500
caspase-3
picornavirus
picornavirus
caspase-3
PABP
eIF4E
eIF4A
eIF3
eIF4A
Mnk1
eIF4GI human
MIF4G
phospho-region
MA3
eIF4GII human
72%
32%
63%
eIF4GI D.melanogaster
39%
26%
eIF4G C.elegans
29%
eIF4G S.cerevisiae
31%
eIF4A
eIF3
Mnk1
p97 / DAP5 human
36%
27%
p97 / DAP5 D.melanogaster
35%
21%

binding site and is situated at the 5' proximal end. Interaction of eIF4G with PABP gives rise to the closed loop model of translation whereby the 5' and 3' ends of the mRNA are juxtaposed and thought to enhance translation efficiency by recycling ribosomes (Gallie, 1998; Kahvejian et al., 2001). Depletion of PABP from mammalian cell extracts hindered 40S and 80S ribosome formation and inhibited translation; accordingly, addition of PABP to depleted extracts stimulated translation in an eIF4G-dependent manner. Most importantly, PABP depletion reduced the efficiency with which eIF4E interacts with the cap of poly-adenylated mRNA, whereas its addition to extracts stimulated binding of eIF4F components to the cap (Kahvejian et al., 2005). This heralded the view of PABP as a canonical initiation factor.

The middle portion of eIF4G acts as a multi-adaptor region. The formally referred to as middle portion of eIF4G (MIF4G) is predicted to have secondary structure rich in α-helices and contains one binding site for eIF4A, eIF3, and a RNA recognition motif (RRM) (Gingras et al., 1999; Imataka and Sonenberg, 1997; Lamphear et al., 1995; Marintchev and Wagner, 2005; Ponting, 2000). Interestingly, homologs of the MIF4G domain are present in proteins which function in mRNA metabolism, i.e. CBP80 and Upf2 (Marintchev and Wagner, 2005; Ponting, 2000).

The C-terminal portion of eIF4G contains another binding site for eIF4A (Imataka and Sonenberg, 1997). This region of eIF4G harbours the MA3 domain, which is also abundantly α-helical, and is implicated in the binding to eIF4A (Ponting, 2000; Yang et al., 2004). The C-terminal end also contains the binding site for Mnk1/2 kinases, and brings the kinase within close proximity to its physiological target, eIF4E (Pyronnet et al., 1999). The Mnk-binding site is also composed of a α-helical domain which is named

AA (acidic/aromatic) box (Bellsolell et al., 2006; Marintchev and Wagner, 2005). The MIF4G, MA3 domains and the AA box are also referred to as HEAT domains (Bellsolell et al., 2006; Marintchev and Wagner, 2005). The C-terminal portion of eIF4G is not conserved in lower eukaryotes; it is not essential for cap-dependent translation but functions as a modulatory region (Morino et al., 2000).

Proteins which resemble eIF4G but are not functional homologs include p97 (Imataka et al., 1997) and poly(A) binding protein interacting protein 1 (Paip1) (Craig et al., 1998), Paip1 has homology to the middle portion of eIF4G and contains a C-terminal PABP binding domain (Craig et al., 1998); p97 resembles the C-terminal, two thirds of eIF4G (Imataka et al., 1997). These two proteins both lack a binding site for eIF4E and have antagonistic effects on translation rates (Craig et al., 1998; Imataka et al., 1997). p97 does not contain an eIF4E binding site, but does bind to eIF3, eIF4A and Mnk1, and therefore presumably inhibits translation by sequestering eIF4A and eIF3 in inactive complexes (Imataka et al., 1997). Incidentally, the C-terminal portion of p97, which contains an MA3 domain, does not bind to eIF4A (Imataka and Sonenberg, 1997). However, p97 promotes IRES-mediated translation of selective mRNAs during stress (Holcik and Sonenberg, 2005).

1.2.3.3. eIF4A

eIF4A is an ATP-binding, RNA-dependent helicase which is thought to unwind structured mRNAs at their 5' end and facilitate ribosome binding (Grifo et al., 1984; Ray et al., 1985; Rozen et al., 1990). It is the prototypic DEAD box protein. DEAD box proteins are a large family of proteins which exhibit ATPase activity and share nine

conserved sequence motifs that are required for helicase activity (Fig. 1.5A). Members of this family participate at all steps of RNA metabolism including splicing, ribosome biogenesis, mRNA export, mRNA decay, and translation (Cordin et al., 2006; Rocak and Linder, 2004).

The biochemical functions carried out by DEAD- box proteins are attributed to their conserved motifs. Mutational analysis of these motifs has contributed to knowledge of their function. To enumerate a few, Motif I, also known as Walker A motif, is needed for ATP binding. Motif II, otherwise known as the DEAD motif, is necessary for ATP hydrolysis. The Q motif, situated upstream of Motif I, is believed to regulate ATP binding and hydrolysis. The SAT motif (Motif III) is essential for unwinding activity. Motif VI is critical for RNA binding and ATP hydrolysis (Cordin et al., 2006; Pause et al., 1993; Pause and Sonenberg, 1992; Rocak and Linder, 2004)}(Fig. 1.5B).

The crystal structure of yeast eIF4A has been solved (Benz et al., 1999; Caruthers et al., 2000; Cordin et al., 2006; Johnson and McKay, 1999). eIF4A is composed of two domains joined by a linker region and is shaped as a "dumbbell." The two domains form a crevice for ATP positioning. The function of the other motifs (motifs Ia, Ib, IV, and V) have been deduced by analysis of the crystal structure of eIF4A, and are presumably involved in RNA binding by interactions with the sugar phosphate backbone (Cordin et al., 2006; Rocak and Linder, 2004) (Fig. 1.5B). eIF4A does not contain a canonical RNA binding motif; as most other helicases, it does not discriminate in the sequence that it binds to so that most mRNAs are substrates of the unwinding activity of this helicase.

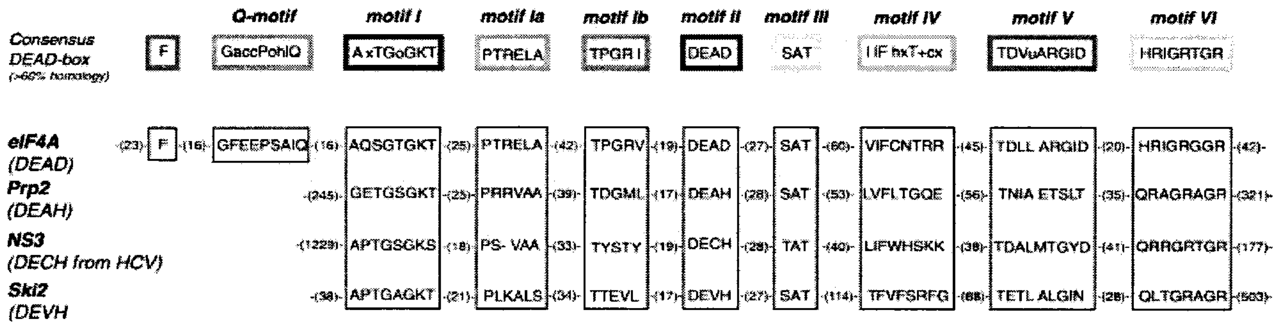
Several mutants were tested for their effect on translation *in vitro* (Pause et al., 1994b; Svitkin et al., 2001). Mutations within motif Ia (PTRELA to PRRVAA), the

Figure 1.5. eIF4A DEAD-box motifs

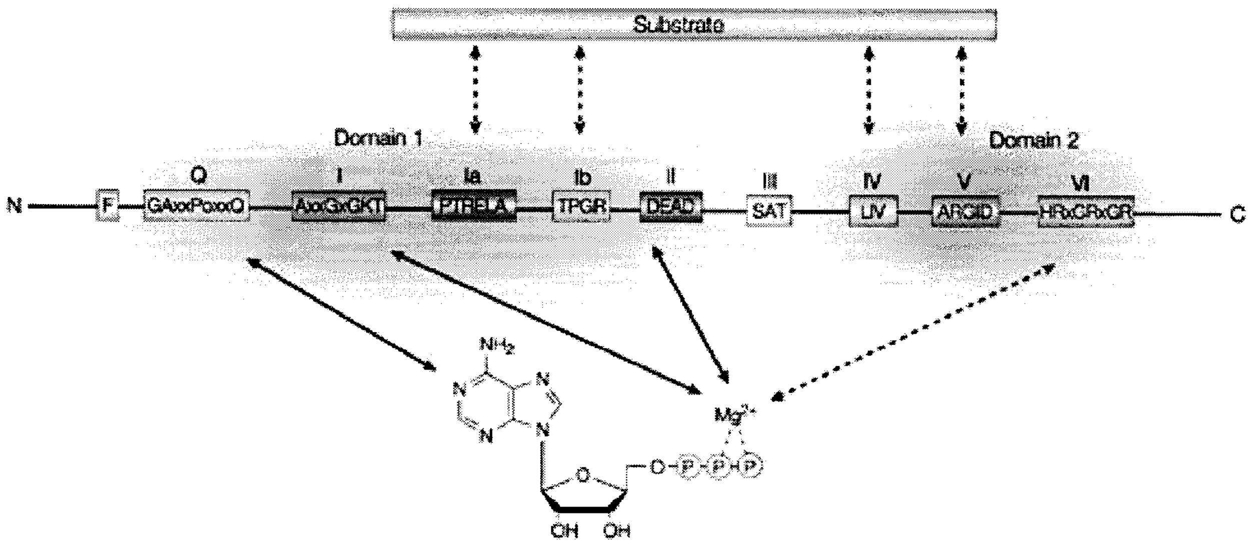
(A) Conserved motifs in the DEAD-box and related DExD/H-box families. Sequences of the conserved motifs from the yeast *S. cerevisiae* eIF4A (DEAD-box protein), Prp2 (DEAH-box protein), NS3 (DECH helicase from the hepatitis C virus) and Ski2 (DExH, Ski2 family). Symbols used are as follow: o: S,T; l: I, L, V; x: any residue; a: F, W, Y; c: D, E, H, K, R; h: A, F, G, I, L, M, P, V, W, Y; +: H, K, R; u: A, G. Adapted from (Cordin et al., 2006).

(B) The conserved motifs of DEAD-box proteins and their interaction with ATP. Mutational analysis of these motifs has led to identification of their functions which are schematically represented here. For more details see text. Adapted from (Rocak and Linder, 2004).

A



B



DEAD motif (**DEAD** to D**Q**AD) and Motif VI (H**R**IG**R**XX**R**; single mutants of all three R to Q/K) posed a potent dominant negative effect on both cap-dependent and cap-independent translation. eIF4A exerts its function in translation as a component of the eIF4F complex as well as unaccompanied as a single polypeptide (Pause et al., 1994b; Svitkin et al., 2001; Yoder-Hill et al., 1993). eIF4A recycles through the eIF4F complex which explains the abundance of eIF4A over eIF4F in cells. Evidence in support of recycling include the facts that: a) dominant negative mutants are impaired in their ability to recycle through eIF4F in kinetic experiments (Pause et al., 1994b), b) the helicase activity of eIF4F but not eIF4A is inhibited in the presence of dominant negative mutants (Pause et al., 1994b), c) a dominant negative mutant of eIF4A is more stably associated with the eIF4F complex but abolishes cap-binding activity of eIF4F and causes an inhibition in 48S ribosomal complex formation (Svitkin et al., 2001).

eIF4A helicase activity is increased twenty fold when it is part of the eIF4F complex (Ray et al., 1985; Rozen et al., 1990). NMR studies have defined the C-terminal fragment of eIF4A to be the main interaction face with the middle fragment of eIF4G (Oberer et al., 2005). This interaction occurs within the vicinity of the ATP and RNA binding regions. It is postulated that eIF4G acts as a clamp on eIF4A and causes the cooperative interaction of eIF4A with RNA and ATP, thus resulting in the enhancement of eIF4A helicase activity when it is part of the eIF4F complex (Oberer et al., 2005).

Isoforms of eIF4A

There are three isoforms of eIF4A in mammals, eIF4AI, eIF4AII, and eIF4AIII. Murine eIF4AI and eIF4AII are ~90% identical at the amino acid level (as are human eIF4AI and eIF4AII); they are functionally interchangeable and differ in their expression levels

among various tissues (Nielsen et al., 1985; Nielsen and Trachsel, 1988; Yoder-Hill et al., 1993). Genetic disruption of both yeast isoforms, TIF1 and TIF2, results in lethality (Linder and Slonimski, 1989). eIF4AIII is more distantly related to eIF4AI (Li et al., 1999). Human eIF4AIII bears ~65% identity to human eIF4AI and is ubiquitously expressed. It contains all the conserved DEAD box motifs and differs from eIF4AI and eIF4AII primarily in its N-terminal region (Fig. 1.6). eIF4AIII contains, in addition to the conserved DEAD-box motifs, eight highly conserved regions (A-H) which are not preserved in eIF4AI and eIF4AII (Fig. 1.6). In contrast to eIF4AI and eIF4AII, human eIF4AIII cannot form a 48S pre-initiation complex in a ribosome binding assay. Furthermore, eIF4AIII binds only to the middle fragment of eIF4G, unlike eIF4AI which possesses two binding sites for eIF4GI (Li et al., 1999).

eIF4AIII is phylogenetically conserved; its closest homolog is from *Xenopus* which shares ~93% identity to human eIF4AIII (Li et al., 1999; Weinstein et al., 1997). In contrast, there does not appear to be an ortholog of eIF4AIII in yeast. In *Xenopus*, eIF4AIII was found to stimulate translation of specific messages involved in epidermal formation in embryos (Weinstein et al., 1997).

Interestingly, human eIF4AIII exhibits a nuclear staining, in contrast to eIF4AI and eIF4AII, which are cytoplasmic (Bohnsack et al., 2002; Holzmann et al., 2000; Lejbkowicz et al., 1992)). In the nucleus, it colocalizes with splicing factors in speckles. Recently, eIF4AIII was identified by mass spectrometry analysis as a component of the human splicesomal C complex (Jurica et al., 2002; Rappsilber et al., 2002; Zhou et al., 2002).

Figure 1.6. eIF4AIII sequence alignment

ClustalW amino acid sequence alignments of human eIF4AI, eIF4AII, and eIF4AIII (Hs-eIF4AIII, ENSG00000141543, accession #P38919) and eIF4AIII homologs from *Xenopus laevis* (Xl-eIF4AIII, #O42226), *Drosophila melanogaster* (Dm-eIF4AIII, #CG7483), *Caenorhabditis elegans* (Ce-eIF4AIII, #O44781), *Nicotiana plumbaginifolia* (Np-eIF4AIII, #P41380), and *Schizosaccharomyces pombe* (Sp-eIF4AIII, #Q10055). Identical amino acids are highlighted in black. Positions of eIF4A family consensus motifs (Q–VI) and eIF4AIII-specific motifs (A–H) are indicated by black and white bars, respectively. Adapted from (Shibuya et al., 2006).

A Q I

Sequence	Alignment	End
Hs-eIF4AIII:	[illegible]	91
Xl-eIF4AIII:	[illegible]	94
Dm-eIF4AIII:	[illegible]	79
Ce-eIF4AIII:	[illegible]	79
Np-eIF4AIII:	[illegible]	71
Sp-eIF4AIII:	[illegible]	74
Hs-eIF4AI :	[illegible]	85
Hs-eIF4AII :	[illegible]	86

Ia B C Ib D

Sequence	Alignment	End
Hs-eIF4AIII:	[illegible]	184
Xl-eIF4AIII:	[illegible]	187
Dm-eIF4AIII:	[illegible]	172
Ce-eIF4AIII:	[illegible]	172
Np-eIF4AIII:	[illegible]	164
Sp-eIF4AIII:	[illegible]	167
Hs-eIF4AI :	[illegible]	179
Hs-eIF4AII :	[illegible]	180

II E III F

Sequence	Alignment	End
Hs-eIF4AIII:	[illegible]	278
Xl-eIF4AIII:	[illegible]	281
Dm-eIF4AIII:	[illegible]	266
Ce-eIF4AIII:	[illegible]	266
Np-eIF4AIII:	[illegible]	258
Sp-eIF4AIII:	[illegible]	261
Hs-eIF4AI :	[illegible]	273
Hs-eIF4AII :	[illegible]	274

IV G V VI

Sequence	Alignment	End
Hs-eIF4AIII:	[illegible]	372
Xl-eIF4AIII:	[illegible]	375
Dm-eIF4AIII:	[illegible]	360
Ce-eIF4AIII:	[illegible]	360
Np-eIF4AIII:	[illegible]	352
Sp-eIF4AIII:	[illegible]	355
Hs-eIF4AI :	[illegible]	367
Hs-eIF4AII :	[illegible]	368

H

Sequence	Alignment	End
Hs-eIF4AIII:	[illegible]	411
Xl-eIF4AIII:	[illegible]	414
Dm-eIF4AIII:	[illegible]	399
Ce-eIF4AIII:	[illegible]	399
Np-eIF4AIII:	[illegible]	391
Sp-eIF4AIII:	[illegible]	394
Hs-eIF4AI :	[illegible]	406
Hs-eIF4AII :	[illegible]	407

eIF4A Cofactors: eIF4B and eIF4H

The helicase activity of eIF4A is appreciably stimulated by two factors, eIF4B and eIF4H (Lawson et al., 1989; Richter-Cook et al., 1998). eIF4B is a dimer and is an RNA binding protein (Methot et al., 1994; Methot et al., 1996a; Methot et al., 1996b). It is required for formation of the 48S pre-initiation complex in an *in vitro* ribosome binding assay (Dmitriev et al., 2003). eIF4B increases the affinity of eIF4A for its binding partners, RNA and ATP. On its own it has no catalytic activity (Grifo et al., 1984) although it stimulates the RNA binding, ATPase and helicase activity of eIF4A (Rogers et al., 2001; Rogers et al., 1999). eIF4B is regulated by its phosphorylation, which is directly coupled to increased translation rates (Duncan and Hershey, 1985; Manzella et al., 1991; Raught et al., 2004; Shahbazian et al., 2006).

eIF4H is another cofactor of eIF4A (Richter-Cook et al., 1998). It possesses an RNA binding domain similar to that found in eIF4B (Richter-Cook et al., 1998) and also stimulates the processivity and helicase activity of eIF4A (Richter et al., 1999; Richter-Cook et al., 1998; Rogers et al., 1999). It is proposed that eIF4H buffers the structural remodelling of eIF4A while performing its enzymatic functions in RNA binding, ATP catalysis and duplex unwinding (Richter et al., 1999).

1.2.4 eIF4E Regulation

eIF4E is the rate-limiting factor in translation initiation in some systems. Accordingly, it is a target of extensive regulation (see also section 1.2.3.1.). Deleterious consequences of its deregulation have been extensively documented (Gingras et al., 1999; Mamane et al., 2004). eIF4E is controlled at the level of transcription, which directly correlates to its

expression levels within the cell. It is also regulated post-translationally by phosphorylation, and by a group of proteins which bind to it directly.

1.2.4.1. Transcriptional regulation of eIF4E

eIF4E mRNA levels are regulated at the transcriptional level by myc, which has two binding sites in the eIF4E promoter (Gingras et al., 1999; Jones et al., 1996). In view of that, *myc* levels are correlated with increased eIF4E mRNA levels after growth factor stimulation in fibroblasts, which both peak late in G1 phase (Rosenwald et al., 1993). In line with these data, increased eIF4E mRNA levels are paralleled with increased myc function in transformed cells (Rosenwald, 1996; Rosenwald et al., 1993) and cells expressing the estrogen receptor-myc fusion protein (Rosenwald et al., 1993).

1.2.4.2. eIF4E Phosphorylation

eIF4E is phosphorylated *in vivo* by Mnk1/2 on serine 209 (S209) (Flynn and Proud, 1995; Joshi et al., 1995; Waskiewicz et al., 1997; Waskiewicz et al., 1999). However, treatment of cells with okadaic acid has revealed another phosphorylated residue, threonine 210 (T210) (Bu et al., 1993). Phosphorylation on T210 was also detected when eIF4E is mutated at its major site of phosphorylation S209 (Whalen et al., 1996). Mnk1/2 does not interact with eIF4E directly but mediates eIF4E phosphorylation through eIF4G (Pyronnet et al., 1999).

Phosphorylation of eIF4E is generally linked with increased translation rates (reviewed in (Gingras et al., 1999; Mamane et al., 2004)). For instance, stimulation of cells with growth factors and mitogens increases translation rates, and this is matched by

enhanced eIF4E phosphorylation. For the most part, the two are synonymous; however, in some cases translation rates are inversely correlated with eIF4E phopshorylation, as it occurs during arsenite-induced stress (Wang et al., 1998). Flies expressing a phosphorylation mutant of eIF4E are smaller in size and are slower in their development than their wild type counterparts, therefore accenting the biological relevance of eIF4E phosphorylation (Lachance et al., 2002).

The co-crystal structure of unphosphorylated eIF4E bound to a cap analog, predicted that phosphorylation of eIF4E at residue S209 would form a salt bridge with lysine 159 (K159) of eIF4E, and that this interaction would stabilize eIF4E interaction with the cap (Marcotrigiano et al., 1997). However, there is a discrepancy in the correlation between eIF4E phosphorylation and its affinity for the cap. Whereas previous data demonstrated that phosphorylated eIF4E has greater affinity for the cap over the unphosphorylated form (Joshi et al., 1995), recent reports have indicated the opposite effect (Scheper et al., 2002; Zuberek et al., 2004). New crystal structure studies of eIF4E demonstrate that the distance between S209 and K159 is too far to form a salt bridge to explain increased binding affinity for the cap, and too far to accommodate the opposing view that phosphorylation of eIF4E decreases cap affinity by electrostatic repulsion between the phosphorylated S209 and the phosphate of the adenosine present in the cap structure (m^7Gppp$\underline{A}$). Therefore, the issue remains divisive (reviewed in (Sonenberg and Dever, 2003). Nevertheless, an explanation put forth to connect increased translation rates with reduced cap affinity, is that the rate of dissociation of eIF4E is increased in these cases and ribosomal initiation complexes are free to scan for the initiation codon (Scheper and Proud, 2002).

1.2.4.3. Regulation by 4E Binding Proteins (4E-BPs)

eIF4E is also regulated through interaction with a family of repressor proteins called 4E-BPs (Richter and Sonenberg, 2005). These members include 4E-BP1, 4E-BP2, 4E-BP3 and 4E-Transporter (4E-T). 4E-BPs share a conserved eIF4E binding sequence motif YXXXXLΦ (where X is any amino acid and Φ represents a hydrophobic residue) that is also found in eIF4G, and whose binding is mutually exclusive with eIF4G (Dostie et al., 2000a; Gingras et al., 1999; Mader et al., 1995; Poulin et al., 1998) (Fig. 1.7A). 4E-BPs and eIF4G make contacts with residues on the convex dorsal surface of eIF4E (Marcotrigiano et al., 1997; Marcotrigiano et al., 1999). Most notably is the tryptophan 73 (W73) residue of eIF4E, which is phylogenetically conserved; mutation of this residue to an alanine (W73A) abrogates interaction of eIF4E with eIF4G, 4EBP1, 2, 3 and 4E-T (Dostie et al., 2000a; Marcotrigiano et al., 1999; Pyronnet et al., 1999). Other notable residues on the convex dorsal surface of eIF4E are His37 and V69 which make contact with the the tyrosine and the leucine of the eIF4E-binding motif, and L131 and L135 of eIF4E, which make contact with the hydrophobic residue (Marcotrigiano et al., 1997; Marcotrigiano et al., 1999).

In the accompanying sections, the regulation of eIF4E via its binding partners will be elaborated; particular emphasis will be placed on the regulation of eIF4E by 4E-T.

<u>*4E-BP1, 2, 3*</u>

Human cDNAs encoding for 4E-BP1, 2, and 3 were cloned by Far Western screening using a ^{32}P-labelled eIF4E probe (Pause et al., 1994a). 4E-BP1 is present in most tissues, but is highly expressed in adipose tissue (Tsukiyama-Kohara et al., 2001). Accordingly, 4E-BP1 knockout mice display differences in metabolic rate and adipose tissue content

from the wild type mice (Tsukiyama-Kohara et al., 2001). 4E-BP2 is primarily expressed in the brain, and 4E-BP2 knockout mice have impaired long-term memory formation (Banko et al., 2005; Tsukiyama-Kohara et al., 2001). 4E-BP3 is mainly expressed in the colon, kidney and liver relative to the other 4E-BPs (Poulin et al., 1998; Tsukiyama-Kohara et al., 2001). 4E-BP1-3 are phosphoproteins (Gingras et al., 1999). Studies characterizing the phosphorylation have been mainly performed on 4E-BP1. 4E-BP1 is phosphorylated on residues Thr 37 and Thr 46 by FRAP/mTOR, even under basal conditions (Gingras et al., 2001). Phosphorylation at these sites are a precedent for subsequent phosphorylation at serum-sensitive residues S65, Thr70 and S83 by the PI3K/Akt pathway (Gingras et al., 2001). Phosphorylation of 4E-BP1 regulates its binding to eIF4E. Hyperphosphorylated 4E-BP1 has reduced affinity for eIF4E whereas hypophosphorylated 4E-BP exhibits the opposite effect (Gingras et al., 1999; Pause et al., 1994a). Treatment with the inhibitor of FRAP/mTOR, rapamycin, results in hypophosphorylated 4E-BP1, increased binding to eIF4E, and decreased translation rates (Beretta et al., 1996). Hence, rapamycin is being explored as a chemotherapeutic agent (Petroulakis et al., 2006).

4E-Transporter (4E-T)

4E-Transporter (4E-T) was also discovered by Far-Western screening with a ^{32}P-labelled eIF4E probe (Dostie et al., 2000a). It binds eIF4E through the same conserved sequence motif as that found in 4E-BPs and eIF4G (Dostie et al., 2000a) (Fig. 1.7A). Mutation of the tyrosine residue, within the eIF4E-binding motif of 4E-T obstructed its interaction with eIF4E, as was reported for other eIF4E-binding proteins (Mader et al., 1995; Poulin et al., 1998). 4E-T is a ubiquitously expressed protein with nucleocytoplasmic shuttling

Figure 1.7. 4E-T sequence alignment across species

(A) Amino acid sequence alignment of the eIF4E-binding site of 4E-T with other eIF4E-binding proteins. Adapted from (Dostie et al., 2000a).

(B) MAP BCM Search Launcher sequence alignment of human 4E-T (Accession no. Q9NRA8) and homologs from mouse (BAB11963), rat (XP_223589) and *X. laevis* (AAH77338). Position of the eIF4E binding site (4EBS) is indicated (brown), arginine rich region (blue), nuclear localization signal (NLS, green), serine rich regions (orange), and nuclear export signals (NES, violet).

(C) Comparison of human 4E-T with *Drosophila* Cup (AAB64427) and Cup-like (AAN71228) proteins.

A

	Sequence
Human	
4E-T	[27]P H R Y T K E E L L D I K E L P[42]
eIF4GI	K K R Y D R E F L L G F Q F I F
eIF4GII	K K Q Y D R E F L L D F Q F M P
4E-BP1	R I I Y D R K F L M E C R N S P
4E-BP2	R I I Y D R K F L L D R R N S P
4E-BP3	R I I Y D R K F L L E C K N S P
Drosophila	
deIF4G	K K Q Y D R E Q L L Q L R E V K
Yeast	
p150	K Y T Y G P T F L L Q F K D K L
p130	K Y T Y G P T F L L Q F K D K L
p20	M I K Y T I D E L F Q L K P S L
Wheat	
iso-eIF4G	R V R Y S R D Q L L D L R K I T

B

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                                          4EBS
human      1 MDRRSMGETESGDAGLLDLEKPPASKCPHRYTKEELLIIKELPHSKQRPSCLSEKYDSDGVWDPEKWHASLYPASGRSSPVESLKKELDTD
mouse      1 MEK-SVADTENGDAGLELLEKLPTSKSPHRYTKEELLIIKERPYSKQRPSCLSEKYDSDGVWDPEKWHASLYPASGRSSPVESLKKESESD
rat        1 MEKRSVDETENGDAGLDLLEKLPTSKSPHRYTKEELLIIKERPYSKQRPSCLSEKYDSDGVWDPEKWHASLYPASGRSSPVESLKKEPESD
X.laevis   1 --MDYREETDRSEPPGDIEEKPPRGESHHSYSKEELLIIKELPHSKERPSCLLDKYDSDGVWDPEKWHSSLYPNSGRSSPVEALKKDCDTE
                                                                                   Arginine rich
human     91 RPSLVRRIVDPRERVKEDDLDVVLSPQRRSFGGGCHVTAAVSSRRSGSPLE-KDSDGLRLLGGRRIGSGRIISARTFEKDHRLSDKDLRD
mouse     90 RPSLVRRIADPRERVKEDDLDVVLSPQRRSFGGGCHVTAAVSSRRSGSPLE-KDSDGLRLLGGRRIGSGRIISARAFEKDHRLSDKDLRD
rat       91 RPSLVRRIADPRERVKEDDLDVVLSPQRRSFGGGCHVTAAVSSRRSGSPLE-KDSDGLRLLGGRRIGSGRIISARAFEKDHRLSDKDLRD
X.laevis  89 RNMLKRRIADPRERVKEDDLDVVLSPQRRSFGGGCHVTAAANSRRSGSPLDNKENESMRLLGSRRIGSGRLMTARGFDRDVRGGEKDREP

human    180 LRDRDREEDFKDKRFRREEGDSKRVFGERRRNDSYTEEEPEWFSAGPTSQSETIELTGFDDKILEEDHKGRKRTRRRTASVKEGIVECNG
mouse    179 LRDRDREEDYKDKRFRREEGDSKRVFGERRRNDSYTEEEPEWFSAGPTSQSETIELTGFDDKILEEDHKGRKRTRRRTASVKEGIVECNG
rat      180 LRDRDREEDYKDKRFRREEGDSKRVFGERRRNDSYTEEEPEWFSAGPTSQSETIELTGFDDKILEEDHKGRKRTRRRTASVREGIVECNG
X.laevis 179 REGRDREKEYKDKRFRREYGDGKRIFGERRRNDSCTEEEPEWFSAGPTSQSETIELIGFDDKILEEDQKGRKRTRRRTTSLKEGV-ECNG
                          NLS                                                  Serine rich
human    270 GVAEEDEVEVILAQEPAADQEVPRDAVLPEQSPGDFDFNEFFNLDK-VPCLASMIEDVLGEGSVSASRFSRWFSNPSRSGSRSSSLGSTP
mouse    269 GVAEEDEVEVILAQEPSADQEVPRDVILPEQSPGEFDFNEFFNLDK-VPCLASMIEDVLGEGSVSASRFSRWFSNPSQSGSRSSSLGSTP
rat      270 GVAEEDEVEVILAQEPSADQEVPRDVILPEQSPGEFDFNEFFNLDK-VPCLASMIEDVLGEGSVSASRFSRWFSNPSRSGSRSSSLGSTP
X.laevis 268 GVLEE----VALPPAHAADQEVPEEAVLPEPAPGEFDFNEFFNLDKSVPGLAS-------------------------------------
                                                                                         NES1
human    359 HEELERLAGLEQAILSPGQNSGNYFAPIPLEDHAENKVDILEMLQKAKVDLKPLLSSLSANKEKLKESSHSGVVLSVEEVEAGLKGLKVD
mouse    358 HEELERLAGLEQAVLSPGQNSGNYFAPIPSEDHAENKVDILEMLQKAKVDLKPLLSSLSANKEKLKESSHSGVVLSVEEVEAGLKGLKVD
rat      359 HEELERLAGLEQAVLSPGQNSGNYFAPIPSEDHAENKVDILEMLQKAKVDLKPLLSSLSANKEKLKESSHSGVVLSVEEVEAGLKGLKVD
X.laevis 317 ------------------------------------------------------------------------------------------

human    449 QQVKNSTPFMAEHLEETLSAVTNNRQLKKDGDMTAFNKLVSTMKASGTLPSQPKVSRNLESHLMSPAEIPGQPVPKNILQELLGQPVQRP
mouse    448 QQMKNSTPFMAEHLEETLSAASSNRQLKKDGDMTAFNKLVNTMKASGTLPTQPKVSRNVESHLLAPAEIPGQPVSKNILQELLGQPVQRP
rat      449 QQIKNSTPFMAEHLEETLSAVTSNRQLKKDGDMTAFNKLVNTMKASGTLPTQPKVSRNLESHLLAPAEIPGQPVPKNILQELLGQPVQRP
X.laevis 317 ----------------------------------------------------------RKQESAVLSS-DVATTPLQRNILQELLGQPV-SD
                                                                                       NES 2
human    539 ASSNLLSGLMGSLEPTTSLLGQRAPSPPLSQVFQTRAASADYLRPRIPSPIGFTPGPQQLLGDPFQG-MRKPMSPITAQMSQLELQQAAI
mouse    538 ASSNLLSGLMGSLEATASLLSQRAPSPPMSQVFRTQAASADYLHPRIPSPIGFPSGPQQLLGDPFQG-MRKPMSPVSAQMSQLELQQAAI
rat      539 ASSNLLSGLMGSLEPTASLLSQRAPSPPMSQVFRTQAASADYLHPRIPSPIGFPSGPQQLLGDPFQG-MRKPMSPVTAQMSQLELQQAAI
X.laevis 349 SSSHVLSNLMGSLDS--SLLQQRSPSPQIPQVFPTRASSADYLRNHIPSPIGFASGPSKFLGEQFPGGIPREDGPMIAQVSPLELQQAAI
              NES 2
human    628 EGLALPHDLAVQAANFYQPGFGKPQVDRTRDGFRNRQQ-RVTKSPAPVHRGNSSSPAPAASITSMLSPSFTPTSVIRKMYESKEKSKEEP
mouse    627 EGLALPHDLAVQTAPFYQPGFSKPQVDRTRDGLRNRQQ-RMSKSPAPMHGGNSSSPAPAASITSMLSPSFTPTSVIRKMYESREKTKEEM
rat      628 EGLTLPHDLAVQTTPFYQPGFSKPQVDRTRDGLRNRQQ-RMTKSPAPMHRGNSSSPAPAASITSMLSPSFTPTSVIRKMYESREKSKEEI
X.laevis 437 DGF-IPHEIADQAANYYHPGYGKVQMDKGRENFRSRQQPRVNKSPVANMRGVSLSPGPGASVTSMLSPSFTPTSVIRKMYESREKIKDEP
                      Serine rich
human    717 ASGKAALEDSKEDTQKASEENLLSSSSVPSADRDSSPTTNSKLSALQKSSCSTPLSQANRYTKEQDYRPKATGRKTPTLASPVPTTPFLR
mouse    716 APGMVVPEDGKEDTQKTSEENLLSSNPIPNTDQDSS-TTNPKLSTLQKSSCSTPLSQTNRYTKEQDYRPKTAGRKTPTLASPVPGTPFLR
rat      717 APGTVVPEDGKEDAQKASEENLLSSNSISNSDQDSSPTANPKLSTLQKSSCSTPLSQTNRYTKEQDYRPKAAGRKTPTLASPVPGTPFLR
X.laevis 526 TEKQNVSE--KEGGQGTLEDGSLPNSIEQDSPQDTSLVNGNKVSVSRHTSCTPPLPHQSRYTKDQDYRPKSTGRRTPTVSSPVPGASFLQ

human    807 PVHQVPLVPHVPMVRPAHQLHPGLVQRMLAQGVHPQHLPSLLQTGVLPPGMDLSHLQGISGPILGQPFYPL-PAASHPLLNPRPGTPLHL
mouse    805 PTHQVPLVPHVPIVRPAHQLHPGLVQRLLAQGVHPQHLPSLLQAGVLPPGIDMAPLQGLSGPLLGQPLYPLVSAASHPLLNPRPGTPLHL
rat      807 PTHQVPLVPHVPIVRPAHQLHPGLVQRLLAQGVHPQHLPGLLQAGVLPPGIDIAPLQGLSGPLLGQPLYPLVSAASHPLLNPRPGTPLHL
X.laevis 614 PLHQLPLVP---VVRPSHQLHPGLVQRMLAQGVPPQRLS-LLQAGLLPPGIDLPPLHALSPSVLGQPFYPL-SPAGHPLLNQRPANPLQL

human    896 AMVQQQLQRSVLHPPGSGSHAAAVSVQTTPQNVPSRSGLPHMHSQLEHRPSQRSSSPVGLAKWFGSDVLQQPLPSMPAKVISVDELEYRQ
mouse    895 AVMQQQLQRSVLHPPGSSSQAAAISVQT-PQNVPSRSGMPHMHSQLEHRTSQRSSSPVGLAKWFGSDVLQQPLPSMPTKVISVDELEYRQ
rat      897 AVMQQQLQRSVLHPPGSSSQAAAINVQT-PPNVPSRSGLPHMHSQLEHRTSQRSSSPVGLAKWFGSDVLQQPLPSMPTKVISVDELEYRQ
X.laevis 699 AMMQQSMQRS------------GSGLHAPPLGMTNR---PNMY--MEQRASQRSSSPVGLAKWFGSDVLQQPLPSMPAKVISVDELEYRQ
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C

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human     150 -------------------LGGRRIGSGRI------------------------------------------------------IS
cup       434 SIIQSQPPQPQTEFQDPAIVNQRRIGSGRL--NHSKWGYNDEDYHSYHNGKSQHMEEVNSKNSKNMTVLQFFDNGEISSQPQRRPNTPVM
cup-like  214 ---------------------ERRIGSGRLLPRSDNWDYKNEKTV---EASIENEKETSPNGSGSTSSLN------QHNQSQHRSRT-FS

human     163 ARTFEKDHRLSDKDLRDLRDRDRERDFKDKEFRREFGDSKRVFG---ERRRNDSYTE--EEPEWFSAGPTSQSETIELTGFDDKILEEDE
cup       522 GMSINRSENDTLHSNESSEDLSRANENYVKRVMSGFLVVSKPKSRDVEDRHHRRYRNQNEEPEWFSCGPTSRLDTIELCGFDE---DEEK
cup-like  273 GRLVERVPEVTDRRFQYDSKKSFDRQGINNRRISG----KEPFSTQSRSKRGNSYLIH-EEPEWFSAGPKSQLETIDLHGFEDLEKNEER

human     248 K---------------------------------GRKRTRRRTASVKEGIVECNGGVAEEDEVEVILAQEPAADQEVPRDAVLPEQSPG
cup       609 MLKEGNKNHGLGETERETSKQKMDHKYKWTHAEPMGRSKYMPKHDTNNNHNVENMNNVMATEHQQQKEEKRPGSGRSFQFDKF--NQSQQ
cup-like  358 SVTEDKNNQIQQLDKNLDAQASKDEASMRNSNDSLNFREVIPSDEKKHT------DENVVTSIQNSTDLGHPNKNKPIQMQPS--QNPES

human     304 DFDFNEFFNLDKVPCLASMIEDVLGEGSVSASRFSRWFSNPSRSGS--------------------------------------------
cup       697 NYESSSYVNHQQPPQTQPQQMQQQSNTNTNNSKFMSFFANEGNSSSSSLNEFFKQAINQGHGNNPEQPKSLGHIGQMPSVDQLEAKWRRN
cup-like  440 EFNFDAFLNMHPLDNSVLSNDETGKSDSKGTSRFSRWFRQKEAANNNEFPGFRESHAQEKRG-------------IPSVKDLEAQMIKV
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activity (Dostie et al., 2000a). It contains one classical bipartite nuclear localization signal (NLS) and two nuclear export signals (NES), which fit the consensus of leucine-rich nuclear protein export signals. At steady state, 4E-T is cytoplasmic and concentrated in foci (Dostie et al., 2000a). Upon treatment with Leptomycin B (LMB), an inhibitor of the CRM-1 protein export pathway, 4E-T accumulates in the nucleus (Dostie et al., 2000a). 4E-T imports eIF4E into the nucleus by the importin α/β pathway, hence the name 4E-Transporter (Dostie et al., 2000a). Importin α is an adaptor molecule of importin β, which is the actual receptor molecule. Importin α interacts with basic rich NLSs, such as that present in 4E-T. The role of 4E-T as the transporter of eIF4E was deciphered using a GST-pull down assay with purified components, in which eIF4E was pulled down by GST-tagged importin β receptor (in the company of importin α) only in the presence of 4E-T (Dostie et al., 2000a). Furthermore, the requirement of the interaction of eIF4E with 4E-T for its nuclear import was demonstrated *in vivo,* using overexpressed eIF4E and 4E-T in HeLa cells, and examining their cellular localization in the presence of LMB. Whereas wild type 4E-T caused nuclear accumulation of eIF4E upon LMB treatment, a mutant of 4E-T which abrogates binding to eIF4E, caused nuclear accumulation of 4E-T but not of eIF4E in LMB-treated cells (Dostie et al., 2000a). 4E-T is a phosphoprotein (Pyronnet et al., 2001). The phosphorylated form of 4E-T has a reduced affinity for eIF4E in nocodazole-treated cells (i.e. when cells are arrested in mitosis) (Pyronnet et al., 2001). More recently, the hypophosphorylated form of 4E-T was shown to correlate with decreased translation rates during prolonged hypoxia (Koritzinsky et al., 2006).

A database search identified 4E-T homologs in mouse, rat, and *Xenopus*, which possess considerable homology to human 4E-T and contain an eIF4E binding site (4EBS), NLS, and NES1/2 which are conserved (Fig. 1.7B) (Dostie et al., 2000a). Furthermore, the mouse homolog, Clast4, is also a phosphoprotein (Ballif et al., 2004; Villaescusa et al., 2006). The *Drosophila* Cup protein and the Cup-like protein (gene product CG32016) share limited homology with human 4E-T (h4E-T) in the region encompassing amino acids 150-349 (Dostie et al., 2000a) (Fig. 1.7C). The *Drosophila* Cup protein is required for repression of *oskar* mRNA translation and localization of *oskar* to the posterior pole (Wilhelm and Smibert, 2005). The interaction of Cup with eIF4E is necessary for repressing *oskar* mRNA translation (Wilhelm and Smibert, 2005). The *C. elegans* clone F26F3 (GenBank accession No. S43585) also shows limited homology with h4E-T in this region (Dostie et al., 2000a). Even though a yeast homolog of 4E-T was not identified by database search, the eIF4E-binding protein with an inhibitory effect on cap-dependent translation in *S. cerevisiae*, p20, shares homology with the eIF4E-binding motif of h4E-T (Altmann et al., 1997; de la Cruz et al., 1997).

Approximately 12-33% of eIF4E was localized to the nucleus by immunofluorescence analysis and cellular fractionation studies (Dostie et al., 2000b; Lejbkowicz et al., 1992). In the nucleus, it colocalizes with splicing factors in speckles, an event that is sensitive to treatment with a cap analog (Dostie et al., 2000b). Coversely, inhibition of transcription with 5,6-dichloro-1-beta-D-ribobenzimidazole (DRB) leads to a more pronounced speckled staining (Dostie et al., 2000b). The localization of eIF4E in speckles is regulated by the Clk/Sty kinase. Overexpression of the wild type Clk/Sty kinase causes a release of eIF4E from splicing factor speckles, whereas a kinase-

impaired mutant causes rounding up speckles (Dostie et al., 2000b). Because eIF4E does not contain an arginine/serine rich phosphorylation domain (RS domain), characteristic of SR (serine/arginine rich) proteins which are substrates of the Clk/Sty kinase, it may be regulated through another protein which is a direct substrate of this kinase (Dostie et al., 2000b). Since eIF4E is localized with splicing factors in speckle domains, it may be involved in splicing-related events, such as mRNA export and NMD (Dostie et al., 2000b).

There is substantial evidence which indicates that the nuclear CBC is essential for mRNA splicing (Izaurralde et al., 1994; Lewis et al., 1996a; Lewis et al., 1996b), and there is some evidence in favour of its role in mRNA export (Ishigaki et al., 2001; Izaurralde et al., 1992; Lejeune et al., 2002; Shen et al., 2000; Visa et al., 1996)(section 1.2.3.1). However, eIF4E may take part in the processing of a subset of mRNA. Stable overexpression of eIF4E in NIH3T3 caused a shift in the nucleocytoplasmic distribution of certain mRNAs, namely growth stimulatory proteins such as cyclin D1 (Rousseau et al., 1996). It was demonstrated that increased cyclin D1 protein levels was the result of improved export of cyclin D1 mRNA and not due to increased association with ribosomes (Rousseau et al., 1996). This is the first evidence which suggested that eIF4E is involved in the export of a subset of mRNAs. It has since been shown that the cap-binding activity of eIF4E is necessary for the export of cyclin D1 mRNA (Cohen et al., 2001).

Evidently, the regulation of eIF4E activity by 4E-T has important consequences on translation homeostasis; 4E-T sequesters the limiting factor of translation into the nucleus. Once in the nucleus eIF4E associates with splicing factors and regulates the

expression of certain mRNAs. The order of events and further biochemical characterization of eIF4E activity and regulation in the nucleus has yet to be elucidated.

eIF4G Regulation: impact on eIF4E activity

eIF4E activity and availability is also regulated by eIF4G binding. eIF4G is a phosphoprotein (Raught et al., 2000). eIF4G is phosphorylated at its C-terminal region through the PI3K/Akt and FRAP/mTOR pathways (Raught et al., 2000). Phosphorylation at the C-terminus site seems to be regulated by the N-terminal region (Raught et al., 2000). It is not clear how phosphorylation of eIF4G affects its function in translation (Gingras et al., 1999; Raught et al., 2000). Since PABP appears to govern eIF4F crosslinking ability through eIF4G, one can imagine that the availability of PABP will impact on eIF4G/eIF4E affinity for the cap (Kahvejian et al., 2005).

1.3 Eukaryotic mRNA Decay

The lifetime of an mRNA is limited so that gene expression is normalized and kept under control. mRNA turnover is crucial for three main reasons (Coller and Parker, 2004; Moore, 2005; Parker and Song, 2004): The most obvious reason is that it regulates protein levels and therefore maintains basal levels of gene expression. An example of problems which arise as a consequence of impaired mRNA destruction include an increase in *c-fos* mRNA levels within the cell, which have been correlated with the onset of cancer (Wilusz et al., 2001b). Secondly, mRNA decay is necessary when it comes to eliminating messages which are faulty, for instance messages which contain a premature termination codon or messages which lack a termination codon. The best illustrated

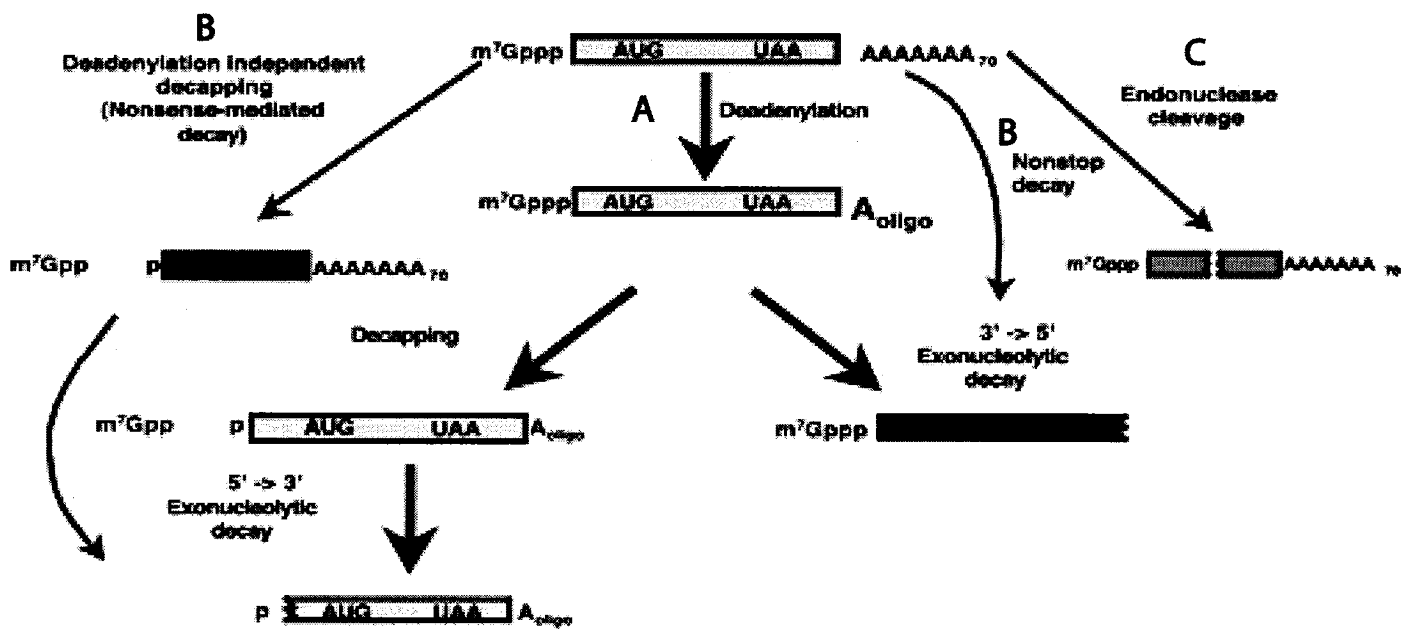
example of the harmful consequences of this process going astray, is the case of β-Thalassemia. β-Thalassemia arises when β-globin mRNA containing a PTC within the 3'part of the gene is not eliminated, and leads to a truncated protein product with a dominant negative function (Holbrook et al., 2004). Thirdly, mRNA turnover is a defence mechanism in response to viral infection. This was first demonstrated in plants but host cell defence against viral infection by RNA interference (RNAi) has been more recently reported in mammals (Schutz and Sarnow, 2006).

Eukaryotic mRNA decay pathways can be grouped into three categories (Fig. 1.8): 1) general mRNA decay which implies the most frequent pathway of mRNA turnover and consists of removal of the polyA tail (deadenylation) followed by either 5'-3'digestion of the mRNA body or 3'-5'digestion (Coller and Parker, 2004; Parker and Song, 2004; Tourriere et al., 2002; Wilusz et al., 2001b); 2) specialized mRNA decay pathways, which eliminate aberrant messages such as NMD, a process involved in eliminating mRNAs containing a PTC (Amrani et al., 2006; Behm-Ansmant and Izaurralde, 2006; Conti and Izaurralde, 2005; Maquat, 2004), and non-stop decay (NSD) which destroys mRNAs which are devoid of a stop codon (Maquat, 2002); 3) RNA interference (RNAi) which involves the destruction of mRNAs by short (21-23 nucleotides) double stranded RNA with perfect complementarity to its target, and induce endonucleolytic cleavage of the target mRNA. Degradation of the mRNA may be elicited by exogenous short interfering RNA (siRNA) or endogenous microRNA (miRNA) (Valencia-Sanchez et al., 2006). mRNA decay relies on signals inherent in *cis*-acting elements within the 5'UTR and 3'UTR of the message and depends on *trans*-acting factors which bind to these regions. mRNA decay is also influenced by translation

Figure 1.8. Pathways of eukaryotic mRNA decay

Adapted from (Coller and Parker, 2004).

Eukaryotic mRNA decay pathways can be grouped into three categories: (A) general mRNA decay which is prompted by removal of the polyA tail (deadenylation) followed by either 5'-3'digestion or 3'-5'digestion of the mRNA body; (B) specialized mRNA decay pathways, which eliminate aberrant messages such as the nonsense-mediated decay (NMD) and non-stop decay (NSD) pathways; (C) RNA interference (RNAi) which involves the destruction of mRNAs by short (21-23 nucleotides) double stranded RNA and induce endonucleolytic cleavage of the target mRNA.


B
Deadenylation independent decapping (Nonsense-mediated decay)
m7Gppp
AUG
UAA
AAAAAAA 70
C
Endonuclease cleavage
A
Deadenylation
B
Nonstop decay
A oligo
m7Gpp
p
Decapping
3' -> 5' Exonucleolytic decay
5' -> 3' Exonucleolytic decay

and is contingent upon the translation machinery (Dreyfuss et al., 2002; Moore, 2005).

The following sections will focus on deadenylation-dependent general mRNA decay with particular emphasis on the 5'-3'exonucleolytic decay pathway, followed by a description of the NMD pathway which makes part of the specialized pathways of decay mentioned above. Reference to RNAi will be made briefly, and an explanation of the location of mRNA decay in cytoplasmic P-bodies will conclude this chapter.

1.3.1 Deadenylation-Dependent Decay

The 5'-3'and 3'-5'exonucleolytic pathways both rely on removal of the polyA tail as the primary event in the digestion process (Muhlrad and Parker, 1992; Shyu et al., 1991). This is achieved by a group of enzymes called deadenylases. Three different enzyme complexes exist as deadenylases in eukaryotes. They include Ccr4/Pop2/Not1 complex, Pan2/Pan3 complex, and poly(A) ribonuclease (PARN) (reviewed in (Parker and Song, 2004)).

1.3.1.1 Deadenylase Complexes

Ccr4/Pop2/Not1 complex is the enzyme complex with major deadenylase activity in yeast. This complex is conserved in higher eukaryotes. In yeast, Ccr4p and Pop2p both have deadenylase activity while Not1-NotSp and Caf4, Caf16, Caf40 and Caf130p are auxiliary proteins which aid its function (Denis and Chen, 2003; Parker and Song, 2004; Tucker et al., 2001). Studies performed in yeast demonstrated that Ccr4p constitutes the major catalytic component of this complex (Chen et al., 2002; Tucker et al., 2002). Pop2p exhibits nuclease activity *in vitro*, and is needed for mRNA degradation *in vivo*

(Daugeron et al., 2001; Thore et al., 2003). Therefore, the Ccr4/Pop2/Not1 complex may have two nucleases, Ccr4 and Pop2 that are regulated under different conditions and have different substrate specificities. Alternatively, Pop2 may just improve the nuclease activity of Ccr4 by stabilizing its conformation and interactions with the mRNA (Parker and Song, 2004).

Pan2p/Pan3p nuclease activity was discovered in yeast Ccr4p-deleted strains (Tucker et al., 2001). This complex is conserved from yeast to mammals. Pan2/Pan3 complex is involved in trimming the polyA tail of long messages to ~55-75 nucleotides in yeast (Brown and Sachs, 1998) and less than 110 nucleotides in mammals (Yamashita et al., 2005). It was recently demonstrated by *in vivo* studies that cytoplasmic mRNA degradation in mammalians occurs by the dual action of Pan2/Pan3 and Ccr4/Caf1 complexes. Pan2/Pan3 clips the poly(A) tail to less than 110 nucleotides, and in the second phase of deadenylation, Pan2/Pan3 surrenders to the Ccr4/Caf1 complex for complete nuclease digestion of the poly(A) tail (Yamashita et al., 2005). The reasons for this collaborative effort will become apparent below. Pan2 is the catalytic subunit while Pan3 is an accessory protein in this complex.

The enzyme complex with major deadenylase activity in mammalian cells was thought to be PARN, otherwise known as DAN (deadenylating nuclease), based on *in vitro* decay assays (Gao et al., 2000; Korner and Wahle, 1997). PARN was also shown to be responsible for poly(A) removal during *Xenopus* oocyte maturation (Dehlin et al., 2000). While present in most eukaryotes, a homolog of PARN in yeast *S. cerevisiae* and *Drosophila* does not exist (Parker and Song, 2004). PARN mediates the rapid degradation of various ARE-containing messages (Gherzi et al., 2004; Lai et al., 2003;

Tran et al., 2004). Its action is stimulated by several message specific AU-binding proteins such as TTP (tristetraprolin), KSRP (K homology Splicing Regulatory Protein) and RHAU (RNA helicase associated with AU-rich element), which recruit it to the message and enable rapid deadenylation of the mRNA. Furthermore, PARN is implicated in rapid deadenylation during NMD in mammalian cells (Lejeune et al., 2003).

What governs the specificity of the deadenylase enzyme complexes for their substrates? The Ccr4/Pop2/Not1 complex is inhibited by PABP and thus, it cannot access its substrate if it is covered by PABP (Tucker et al., 2002; Viswanathan et al., 2003). Similarly to Ccr4p, PARN is inhibited by PABP, but unlike Ccr4p, its activity is dependent on cap recognition (Dehlin et al., 2000; Gao et al., 2000; Korner and Wahle, 1997; Martinez et al., 2001). Contrary to Ccr4 and PARN, PAN (Pan2/Pan3) is stimulated by PABP, consistent with its physiological role as the poly(A) tail trimmer (Sachs and Deardorff, 1992). It seems logical that the reported "biphasic" deadenylation in mammalian cells occurs through PAN first and then Ccr4. Because PAN is stimulated by PABP, it will bind the 3'end of the mRNA and digest it down to less than 100 nucleotides; the resulting loss of PABP molecules will alleviate the inhibition of Ccr4 activity and promote eIF4F disassembly by loss of eIF4G-PABP interaction, and therefore stimulate Dcp2 activity (Yamashita et al., 2005). Alternatively, the loss of PABP molecules bound to the poly(A) after PAN digestion could promote PARN activity, which is stimulated by the cap structure.

1.3.2 3'-5'Decay and the Enzymes Involved

It is proposed that the major decay pathway in mammalian cells occurs via 3'-5'digestion by the exosome following deadenylation, based on *in vitro* studies performed in mammalian cell extracts (Chen et al., 2001; Mukherjee et al., 2002; Wang and Kiledjian, 2001). Most of the data comes from studies done on AU-rich mRNAs (Chen et al., 2001; Mukherjee et al., 2002). It has been demonstrated by several groups that the exosome is recruited to these messages via interaction with AU binding proteins (Chen et al., 2001; Gherzi et al., 2004; Lykke-Andersen and Wagner, 2005; Tran et al., 2004). Alternatively, the exosome may be targeted directly to an RNA sequence with specific affinity for any of the exosomal components; for instance, PmSc175, an exosomal subunit, can recruit the exosome to ARE-containing mRNAs (Mukherjee et al., 2002). However, it should be noted that ARE-mediated decay does not exclusively necessitate 3'-5'digestion but may also undergo 5'-3'decay given that AU-binding proteins, TTP and BRF-1, can interact with enzymes of this pathway as well (Fenger-Gron et al., 2005; Lykke-Andersen and Wagner, 2005). In addition, more recently, *in vivo* experiments demonstrated that ARE-mRNAs undergo predominantly 5'-3'decay when enzyme components of either pathway are depleted by RNAi (Stoecklin et al., 2006).

1.3.2.1. The Exosome (3'-5'exonuclease)

In yeast, the majority of mRNAs are degraded by deadenylation-dependent 5'-3'decay (Decker and Parker, 1993; Muhlrad et al., 1994; Muhlrad et al., 1995; Schwartz and Parker, 1999; Tourriere et al., 2002). Mutations which impaired 5'-3'degradation in yeast revealed a separate pathway which effects 3'-5'exonucleolytic digestion by a complex called the exosome (Anderson and Parker, 1998; Mitchell et al., 1997; Muhlrad

et al., 1995). The exosome collectively refers to a complex with an estimated 10 proteins in yeast, which have exonuclease activity (Parker and Song, 2004; Raijmakers et al., 2004). Originally, the exosome was characterized on the basis of its nuclear function in RNA processing and degradation of ribosomal RNA (rRNA), small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA), as well as degradation of nuclear pre-mRNA and mRNA (Parker and Song, 2004; Raijmakers et al., 2004). However, the exosome is not confined to the nucleolus and nucleoplasm, it is also localized to the cytoplasm and is required for cytoplasmic 3'-5' decay (Allmang et al., 1999; Lejeune et al., 2003; Raijmakers et al., 2004). The nuclear specificity of the exosome is conferred by the Rrp6p subunit in yeast, which is absent in the cytoplasmic exosome (Allmang et al., 1999; Raijmakers et al., 2004).

The exosome is proposed to be the equivalent of the proteasome for RNA (van Hoof and Parker, 1999). The exosome contains enzymes which bear similarity to RNase PH exonucleases which are phosphorylases that digest RNAs at their 3'end by employing phosphate as a nucleophile (Parker and Song, 2004; Symmons et al., 2002; van Hoof and Parker, 1999). Based on structural studies performed on polynucleotide phosphorylase (PNPase) from bacteria, it was proposed that the exosome forms a ring-like structure with active sites centred toward the cavity of the exosome (Aloy et al., 2002; Parker and Song, 2004; Raijmakers et al., 2004; Symmons et al., 2002; van Hoof and Parker, 1999). The reasons for sequestering the active sites to the center may be to avoid non-specific random cleavage of substrates. Furthermore, separating the recognition of the substrate from the degradation reaction, would allow for an additional level of regulation of this multiprotein complex (van Hoof and Parker, 1999). The rationale for multiple

exonucleases within the complex is that the various enzymes may be differentially regulated under certain growth conditions, may have different substrate specificities, or may perform different functions on a single substrate (van Hoof and Parker, 1999).

The exosome is conserved in eukaryotes. Human homologs of exosomal subunits with exonuclease activity have been identified (Allmang et al., 1999; Mitchell and Tollervey, 2000; Raijmakers et al., 2004). Components of the human exosome consist of core proteins Rrp4, Csl4, Rrp40, Rrp41, Rrp46, Mtr3, Rrp42, OIP2, Pm/Sc-175, as well as Pm/Scl-100 (hRrp6p) and Dis3 (Raijmakers et al., 2004). As mentioned above, the human exosome is recruited to its substrate by AU binding proteins or through direct interaction with RNA.

1.3.2.2. The Scavenger Decapping Enzyme

The scavenger decapping enzyme, DcpS, is a pyrophosphatase belonging to the histidine triad (HIT) family (Liu et al., 2002). The HIT motif consists of His-X-His-X-His-X, where X is a hydrophobic residue, and is necessary for hydrolyzing the cap. The substrate of DcpS is free cap (m^7GpppX), 7-methyl GDP (m^7Gpp) or short RNA with less than 10 nucleotides linked to the cap (Liu et al., 2002) (Fig. 1.9 and Table 1.1). In contrast, this enzyme is not able to decap long RNA substrates in mammals (Liu et al., 2002). The cleavage product of DcpS is m^7GMP. DcpS catalyses hydrolysis of the capped dinucleotide generated by 3'-5'exosome-mediated decay (Wang and Kiledjian, 2001). In accordance with this, DcpS was found to coimmunoprecipitate with exosomal subunits in mammalian cells (Wang and Kiledjian, 2001). Furthermore, DcpS hydrolyzes the m^7GDP byproduct of Dcp1/2 (see below), to m^7GMP (van Dijk et al., 2003). The

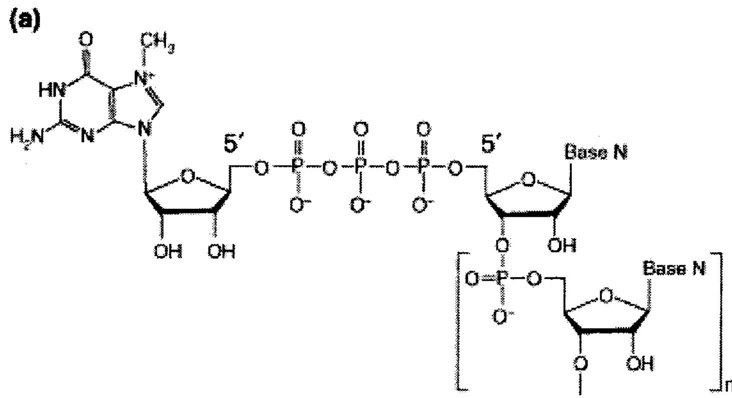
Figure 1.9. Cap structure and metabolism

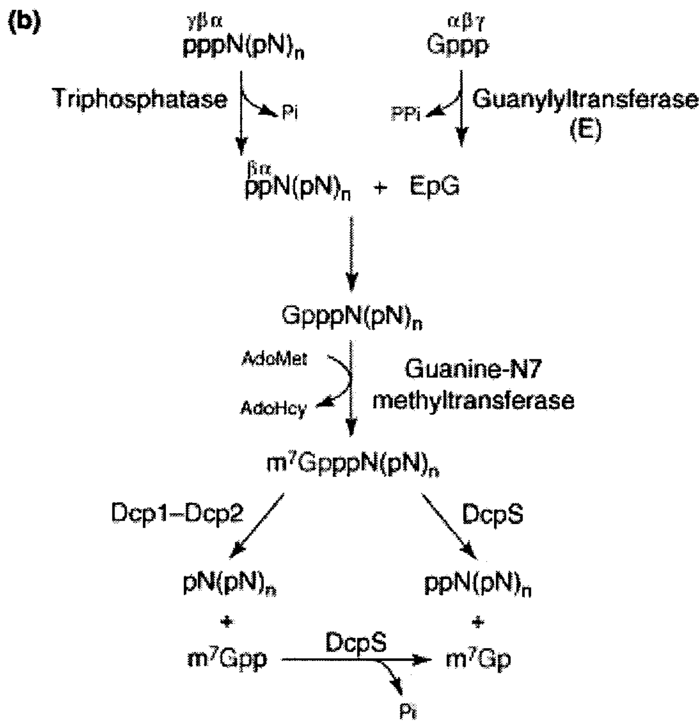
(A) Chemical structure of the mRNA cap.

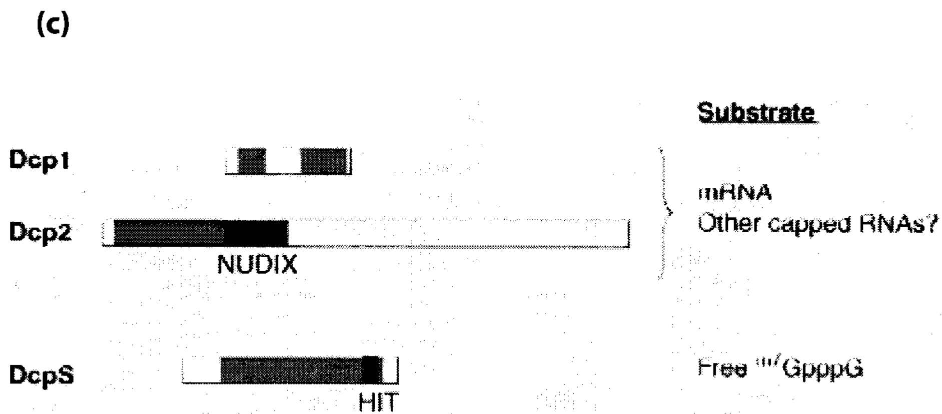
(B) Enzymatic synthesis and degradation of the mRNA cap.

(A) and (B) adapted from (Gu and Lima, 2005).

(C) Schematic of eukaryotic decapping proteins. Dcp2 contains the NUDIX domain that is essential for enzymatic activity. The active site of DcpS contains a HIT (histidine triad) domain. Blue shaded regions show areas of evolutionary conservation, and black shaded regions show known functional motifs. Known substrates for the decapping enzymes are indicated on the right. Adapted from (Fillman and Lykke-Andersen, 2005) with modification.


(a)
CH3
HN
H2N
N
N+
5′
5′
O
OH OH
Base N
OH
O=P
Base N
OH
n


(b)
pppN(pN)n
Gppp
Triphosphatase
Pi
PPi
Guanylyltransferase
(E)
ppN(pN)n + EpG
GpppN(pN)n
AdoMet
AdoHcy
Guanine-N7
methyltransferase
m7GpppN(pN)n
Dcp1–Dcp2
DcpS
pN(pN)n
ppN(pN)n
+
+
m7Gpp
DcpS
m7Gp
Pi
Current Opinion in Structural Biology


(c)
Substrate
Dcp1
Dcp2
NUDIX
mRNA
Other capped RNAs?
DcpS
HIT
Free m7GpppG

scavenger decapping enzyme is a critical effector in both 5'-3' and 3'-5' decay because it prevents the unwanted sequestering of residual unhydrolyzed cap by eIF4E and from interfering with mRNAs to be translated, since DcpS can compete with eIF4E for binding to a free cap but not cap attached to an RNA moiety (Liu et al., 2004).

There are two homologs of DcpS in *S. cerevisiae,* Dcs1p and Dcs2p (Liu et al., 2002). Dcs1p and Dcs2p bear equal level of identity to DcpS and contain the characteristic HIT motif. However, Dcs1p has decapping activity similar to DcpS, whereas Dcs2p does not have catalytic activity (Liu et al., 2002). Dcs2p is known to bind to Dcs1p and, therefore, it is proposed that it may regulate its activity.

1.3.3 5'-3'Decay Pathway and the Enzymes Involved

1.3.3.1. Decapping Enzymes Dcp1 and Dcp2

In eukaryotes, deadenylation-dependent 5'-3' decay is proceeded by decapping of the mRNA by a holoenzyme complex consisting of Dcp1 and Dcp2 (Beelman et al., 1996; Dunckley and Parker, 1999; Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002b). The Dcp1: Dcp2 complex catalyzes hydrolysis of the cap bound to an RNA moiety consisting of at least 25 nucleotides (Piccirillo et al., 2003; van Dijk et al., 2002). The product of hydrolysis is a 5'monophosphate mRNA and m^7GDP, which is then a substrate for the scavenger decapping enzyme (Fig. 1.9 and Table 1.1). 5'-3'mRNA decay is the most common pathway of degradation utilized by yeast (Decker and Parker, 1993; Muhlrad et al., 1994; Muhlrad et al., 1995; Schwartz and Parker, 1999).

Dcp1 and Dcp2 were originally identified in *S. cerevisiae.* Dcp1p was the first

Table 1.1 Enzymes involved in mRNA decay

Adapted from (Coller and Parker, 2004) with modification.

TABLE 1.1 Enzymes involved in mRNA decay *		
Protein	Function	Features
Deadenylation		
Ccr4p	Catalytic subunit of the deadenylase	Homology to Mg2+ dependent endonucleases
Pop2p	Regulator of deadenylation, may also have deadenylase activity	Homology to RNaseD
Pan2p/Pan3p	Minor deadenylases, required for poly(A) length control	Pan2p has homology to RNaseD
PARN	Mammalian deadenylase	Homology to RNaseD. Can bind to 5' cap, which stimulates deadenylase activity
Decapping		
Dcp1p	Major component of decapping holoenzyme	EVH1/WH1 domain
Dcp2p	Catalytic subunit of decapping holoenzyme	NUDIX motif, conserved Box A and Box B motif
Exonucleases		
Xrn1p	Major cytoplasmic 5'-3' exonuclease	
Rat1p	Nuclear 5'-3' exonuclease	
Rrp4, Rrp40p, Rrp41p,Rrp42p, Rrp43p,Rrp44p, Rrp45p,Rrp46p, Mtr3p, Cs14p	A complex of 3'-5' exonucleases termed the exosome	Domain organization similar to that of bacterial PNPase

* Note that mammalian orthologs have been identified for all these enzymes; PARN is not present in *S. cerevisiae.*

subunit identified with decapping activity (LaGrandeur and Parker, 1998) and Dcp2p was later identified in a yeast strain containing mutated Dcp1p (Dunckley and Parker, 1999). Homologs of Dcp1p and Dcp2p were later identified in humans (Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002b). There are two homologs of Dcp1 in humans, Dcp1a and Dcp1b (Lykke-Andersen, 2002) (Fig. 1.9 and Table 1.1). They are both distantly related to the yeast ortholog and bear similarity to Dcp1p primarily in their N-terminal region, which incidentally contains residues important for decapping activity in yeast Dcp1p. The two human Dcp1 homologs bear very little similarity over their entire length but contain 68% identity in their N-terminal region. Human Dcp2 contains 36% identity to the yeast homolog and possesses a mutT or Nudix motif which is conserved from yeast to humans (Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002b). The Nudix motif is present among members of a large family of pyrophosphatases called NUDIX (nucleoside diphosphate linked to an X moiety) hydrolases. The Nudix motif is composed of a 23 amino acid consensus sequence which contains several conserved glutamate residues that coordinate the action of a divalent metal ion (Mildvan et al., 2005; Parker and Song, 2004) and is critical for the decapping activity of Dcp2 (Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002b). Mutations within this region lead to a loss of decapping activity in both yeast and mammals (Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002b). In addition to cap recognition and hydrolysis by the Nudix motif, a conserved region flanking the Nudix motif, called Box B, is important for the RNA binding ability of Dcp2, which is also necessary for its decapping activity (Piccirillo et al., 2003). The RNA binding ability of Dcp2 likely ensures that it functions only on capped RNA on which initiation complexes have been disrupted

(Fillman and Lykke-Andersen, 2005).

Dcp2 is the subunit with intrinsic decapping activity, whereas Dcp1 has no catalytic activity on its own, but binds to Dcp2 *in vitro* and *in vivo* (Beelman et al., 1996; Dunckley and Parker, 1999; Lykke-Andersen, 2002; Steiger et al., 2003) and modulates its activity (Lykke-Andersen, 2002; She et al., 2004). The crystal structure of Dcp1 from *S. cerevisiae* has revealed two structurally conserved regions important for decapping (She et al., 2004). The N-terminal domain of Dcp1 contains a proline-rich site (PRS) through which it may bind proteins that regulate the Dcp1:Dcp2 complex. The other site is present on the concave surface of Dcp1, opposite of the PRS site, and is essential for decapping activity. This site does not seem to be necessary for binding to Dcp2 and therefore may be vital to coordinating the metal ion or inducing conformational changes in Dcp2 that are pertinent to its enzymatic activity. Another region critical for decapping, is a conserved hydrophobic patch situated in the vicinity of the former described region (She et al., 2004).

The crystal structure of Dcp2 from *S. pombe* has been recently described (She et al., 2006). A C-terminally truncated Dcp2 was used in these studies. The Nudix motif, which is vital for decapping activity *in vitro* and *in vivo* is found in the C-terminal portion. A conserved region on the surface of the N-terminal domain mediates the interaction with Dcp1. The flexibility of this region compared to the C-terminal domain affirms the fact that interaction with Dcp1 enhances the intrinsic catalytic activity of the C-terminus (She et al., 2006).

1.3.3.2 Regulation of Decapping by Dcp1:Dcp2

<u>Inhibitors of decapping</u>

Decapping is regulated by a slew of factors which either positively or negatively influence the activity of the decapping enzymes (Coller and Parker, 2004; Fillman and Lykke-Andersen, 2005; Parker and Song, 2004) (Table 1.2). Decapping is negatively affected by translation initiation factors (Schwartz and Parker, 1999). The cap binding protein, eIF4E, is in direct competition with the decapping enzyme and its dissociation from the cap is necessary for Dcp1/2 to gain access to this structure (Schwartz and Parker, 2000). Therefore, translation and general mRNA decay are antagonistic. Yeast strains containing mutations in all the translation initiation factors tested, decreased the half life of the reporter mRNA in *S. cerevisiae* (Schwartz and Parker, 1999); an increase in the rate of deadenylation and decapping was observed. The poly(A) tail is an entity in itself which regulates decapping (Coller and Parker, 2004). It is well known that deadenylation occurs prior to decapping: the time it takes for decapping to commence coincides with the time it takes for deadenylation to complete (Decker and Parker, 1993). Furthermore, decapping intermediates for the most part contain short (A) lengths (Decker and Parker, 1993; Muhlrad et al., 1994; Muhlrad et al., 1995). Moreover, mRNAs containing poly(A) tails are resistant to decapping in *in vitro* extracts (Wilusz et al., 2001a). PABP is the major protein associated with the poly(A) tail and is a key player in regulating decapping (Coller and Parker, 2004; Wilusz et al., 2001a). PABP also physically associates with eIF4G at the 5'end resulting in circularization of the mRNA (Kahvejian et al., 2001). Therefore, PABP is inhibitory to decapping, presumably by preventing the release of the eIF4F complex from the 5'end, since it is believed to have a stimulatory effect on eIF4F binding to the cap (Kahvejian et al., 2005), and is therefore an impediment for the decapping enzyme to access the cap. Alternative explanations to

Table 1.2. Regulators of mRNA decapping in yeast and mammals

Adapted from (Coller and Parker, 2004) with modification.

TABLE 1.2 Regulators of yeast/mammalian mRNA decapping			
Protein	Properties	Function	Significant interactions
Pab1p*	Contains four N-terminal RRM domains and a proline-rich C terminus	Major protein associated with poly(A) tail. Blocks mRNA decapping and stimulates translation. Primary coupler of deadenylation and decapping.	eIF-4G, eRF3, Pan2/3p
eIF-4E*	Cap-binding protein	Component of the eukaryotic translational initiation complex, eIF-4F. Blocks mRNA decapping by competing with Dcp1/2p for access to the cap.	Lsm7p, eIF-4G, eIF- 4A, eIF-4B, Pab1p
Lsm1–7p*	Sm-like proteins	Required for the efficiency of decapping *in vivo*. Forms a heteroheptameric ring complex and interacts with the mRNA after deadenylation. May facilitate the assembly of the decapping complex.	Dcp1p, Dcp2p, Dhh1p, Pat1p, Xrn1p, Upf1p
Pat1p†	88kDa protein with no recognizable sequence motifs	Interacts with both polyadenylated and deadenylated transcripts. Required for efficiency of both decapping and formation of P bodies in vivo. May "seed" the decapping complex on the mRNA.	Dcp1p, Dcp2p, Lsm1–7p, Dhh1p, Xrn1p, Crm1p
Dhh1p*	Member of the ATP-dependent DExD/H box helicase family	Required for the efficiency of decapping *in vivo*. Homologs across species are required for translational repression during mRNA storage events.	Dcp1p, Dcp2p, Lsm1–7p, Ccr4p, Pop2p, Caf17p, Pbp1p, Edc3p
Edc1p, Edc2p	Small, basic proteins with weak homology to each other	Required for efficient decapping in vitro. Directly binds to the mRNA substrate.	Dcp1p, Dcp2p
Edc3p*	Contains five conserved domains	A general and mRNA-specific regulator of decapping. Regulates the decapping of the RPS28a mRNA.	Dcp1p, Dcp2p, Dhh1p, Crm1p, Rps28ap, Nup157p, Lsm8p
Puf3p†	Pumillo-like protein, contains eight PUF repeats	Messages specific activator of mRNA deadenylation and decapping. Homologs facilitate translational repression. Regulates the decapping of the COX17 mRNA.	
Upf1p, Upf2p, Upf3p*	Upf1p is an ATP-dependent RNA helicase	Required for non-sense- mediated decapping.	eRF1, eRF3, Dcp2p, Upf2p, Lsm1p
Hedls or Ge-1‡	Contains a WD40 repeat region	Enhances decapping activity of Dcp2 and Promotes the interaction between Dcp1 and Dcp2	Dcp1, Dcp2

* mammalian ortholog present

† no mammalian ortholog

‡ no yeast homolog

the inhibitory role of PABP in the decapping process, independent of the poly(A) tail, have been put forth since: a) tethered Pabp1 (yeast PABP) can inhibit decapping independent of the poly(A) tail (Coller et al., 1998), b) deletions of the eIF4G binding site of Pabp1 can still inhibit decapping (Coller et al., 1998; Schwartz and Parker, 1999), c) when translation initiation factors are mutated in yeast they do not bypass deadenylation which suggests that PABP may function independently of its interaction with the initiation complex (Schwartz and Parker, 1999). Thus, it has been suggested that PABP can either interact directly with a protein which inhibits the decapping enzyme or it may bind the cap directly (Coller and Parker, 2004; Khanna and Kiledjian, 2004). Alternatively, it may function in inhibiting the localization of translating pools of mRNAs away from degradation enzyme factories (see section 1.4) (Coller and Parker, 2004).

Positive regulators of decapping

Decapping is also regulated by a host of factors which enhance its activity (see Table 1.2 for summary). These factors have been mainly characterized in *S. cerevisiae* and include Dhh1p, Lsm1p-7p, Pat1p and the enhancers of decapping (Edcp) proteins; they are collectively referred to as decapping factors (Coller and Parker, 2004; Parker and Song, 2004).

Dhh1p is a member of the DEAD box family of RNA helicases. It interacts with the decapping enzymes in RNAseA-treated extracts (Coller et al., 2001). The interaction of Pat1p and Lsm1p-7p with the Dcp1p:2p complex, however, is RNA-dependent (Bouveret et al., 2000; Tharun and Parker, 2001). When each of these factors is deleted in *S. cerevisiae,* there is a rise in deadenylated mRNAs which are capped, which suggests

that they are important for decapping (Bouveret et al., 2000; Coller et al., 2001; Tharun et al., 2000). Dhh1p is a putative RNA helicase which stimulates the decapping activity of Dcp1 *in vitro,* and interacts with all the decapping factors and with the deadenylase complex; its RNA unwinding activity is thought to be important for conformational mRNP changes at the 5'end and for making the cap accessible to decapping enzymes (Coller et al., 2001; Fischer and Weis, 2002). The Lsm1-7 complex is a group of proteins which have RNA-binding activity, and possess an Sm-like motif (from which they derived their name) which mediates protein-protein interactions and binding to RNA (He and Parker, 2000). The related complex, Lsm 2-8, is a nuclear RNA-binding heptameric complex which is involved in pre-mRNA splicing (He and Parker, 2000). In contrast, Lsm1p-7p is involved in cytoplasmic mRNA degradation and stimulates decapping (Bouveret et al., 2000; Tharun et al., 2000). Lsm1p-7p proteins assemble on deadenylated mRNAs which no longer have translation factors PABP, eIF4E and eIF4G bound on the mRNA (Tharun and Parker, 2001). Therefore, the Lsm1-7 complex is a critical marker of mRNAs bound for decay, and its binding to the mRNA may promote mRNP rearrangements which accelerate removal of translation factors and association of decapping factors. Lsm1p-7p interacts directly with Pat1p (Tharun and Parker, 2001). Pat1p, unlike Lsm1p-7p, is bound to mRNAs on which translation factors have not been displaced and remains bound after they have been removed (Tharun and Parker, 2001). Therefore, Pat1p is a bridging molecule between a translationally active state of the mRNP and one receptive for decay. Pat1p may function to recruit proteins which assign the mRNA for decapping and decay. The Edcp proteins are a family 3 proteins identified in *S. cerevisiae* which interact with the Dcp1p:Dcp2p complex and enhance decapping.

They bind mRNA and stimulate decapping directly through the Dcp1p:Dcp2p complex *in vitro* (Schwartz et al., 2003). Edc1p and Edc2p are not essential for decapping; yeast strains in which each of these factors is deleted alone had no significant effect on mRNA decay rates. However, deletion of Edc1p or Edc2p in yeast strains partially compromised for decapping, significantly decreased mRNA decay rates. Accordingly, overexpression of either these proteins increased mRNA decay rates in strains defective in mRNA decapping (Schwartz et al., 2003). Edc3p is not homologous to Edc1p and Edc2p. It interacts with the Dcp1p: Dcp2p complex itself and enhances decapping (Kshirsagar and Parker, 2004).

Homologs of Lsm1p-7p, Dhh1p, Pat1p, and Edc3p are also present in higher eukaryotes (Fillman and Lykke-Andersen, 2005). Human homologs of Lsm1p-7p (Lsm 1-7) (Achsel et al., 1999), Dhh1p (referred to as rck/p54) (Smillie and Sommerville, 2002) and hEdc3 (Fenger-Gron et al., 2005) have been identified and linked to mRNA decay mainly by colocalization of these factors in P-bodies (Cougot et al., 2004; Fenger-Gron et al., 2005; Ingelfinger et al., 2002; Sheth and Parker, 2003), discrete cytoplasmic foci where mRNA decay takes place (see section 1.4), and by interaction studies with decapping enzymes (Fenger-Gron et al., 2005; Fillman and Lykke-Andersen, 2005; Lykke-Andersen and Wagner, 2005). Two additional factors that have been identified in humans and lack an observable homolog in yeast are GW182 and Hedls (human enhancer of decapping large subunit, otherwise known as Ge-1) (Eystathioy et al., 2002; Eystathioy et al., 2003; Fenger-Gron et al., 2005; Yu et al., 2005). Hedls interacts with Dcp2 in RNAse treated extracts and mediates the interaction of Dcp2 with Dcp1 (Fenger-Gron et al., 2005). Furthermore, Hedls promotes hydrolysis of the cap by Dcp2 in an *in*

vitro decapping assay (Fenger-Gron et al., 2005). Therefore, Hedls is an enhancer of decapping in human cells. hEdc3 appears to be necessary for the interaction of rck/p54 with Dcp1a (Fenger-Gron et al., 2005). GW182 is a large protein which contains multiple glycine/trytophan repeats (GW repeats) (Eystathioy et al., 2002). This protein was associated to mRNA decay by its colocalization with the decapping enzyme in discrete cytoplasmic foci using serum containing antibodies against the autoantigen GW182, from patients with motor and sensory neuropathy (see section 1.4) (Eystathioy et al., 2003). GW182 contains an RNA recognition motif (RRM) and is bound to a subset of mRNAs in HeLa (Eystathioy et al., 2002). GW182 plays a critical role in RNAi (see section 1.3.5) (Jakymiw et al., 2005; Liu et al., 2005a; Rehwinkel et al., 2005).

1.3.3.3. Xrn exonucleases

Once decapping is complete, the 5'monophosphate mRNA becomes a substrate of the 5'-3'exonuclease, Xrn1. Xrn1 is present in yeast and is conserved in other eukaryotes (Bashkirov et al., 1997; Ingelfinger et al., 2002; Johnson and Kolodner, 1991; Kolodner et al., 1987). Deletion of Xrn1 in yeast leads to an accumulation of deadenylated, decapped mRNA intermediates (Hsu and Stevens, 1993; Schwer et al., 1998). Rat1 (Xrn2) exists in yeast and humans and is localized in the nucleus where it may function in 5'-3'digestion of nuclear RNAs (Amberg et al., 1992; Johnson, 1997).

1.3.4 Nonsense Mediated Decay (NMD)

Nonsense mediated decay (NMD) is considered a specialized pathway of mRNA decay that rids the cell of messages containing a premature termination codon (PTC), which

originates from mutated genes as a result of nonsense or frameshift mutations, or that arises from transcriptional or splicing errors. It is also implicated in the regulation of normal transcripts in about ~10% of the yeast, human and *Drosophila* transcriptome (Behm-Ansmant and Izaurralde, 2006; Conti and Izaurralde, 2005; Maquat, 2004).

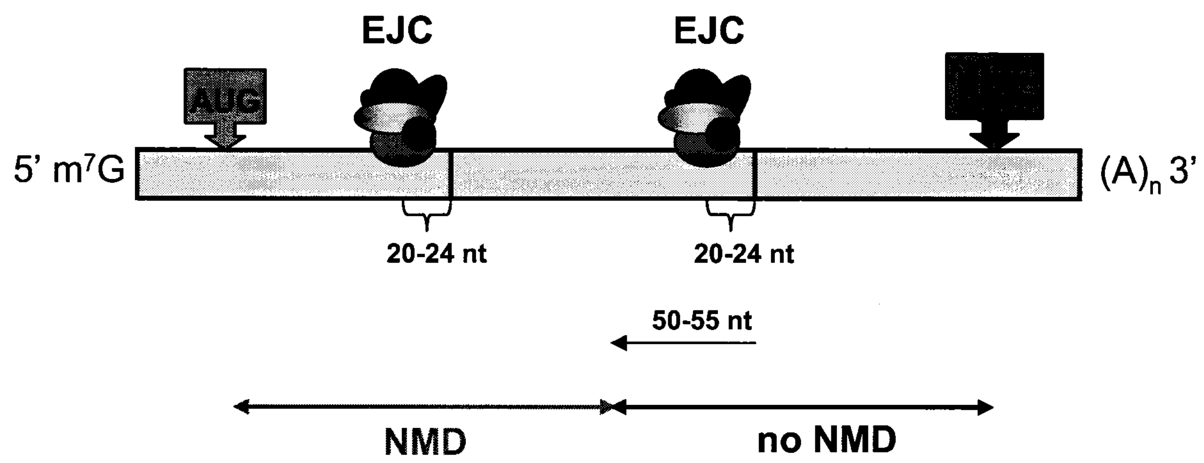
NMD is an RNA surveillance pathway which is conserved in yeast, *Drosophila, C. elegans* and mammals (Conti and Izaurralde, 2005). In all eukaryotes, a surveillance complex is formed upon recognition of a PTC, however, how a stop codon is considered premature and the mechanism involved in the decay of the message differs across organisms. In this section, only the mechanism involved in the mammalian NMD pathway will be discussed. The importance of this pathway is underscored by the fact that approximately 30% of human genetic disorders are the result of a PTC in the mRNA (Holbrook et al., 2004). To put this into perspective, a drug that promotes readthrough of a PTC, which is a result from an inherited mutation in the gene linked to cystic fibrosis, is currently in clinical trials and shows much promise (Ainsworth, 2005).

1.3.4.1. Splicing dependency of mammalian NMD

NMD is a splicing dependent event in mammals. During splicing, a group of proteins are deposited 20-24 nucleotides upstream of the exon-exon junction, which is termed the exon-junction complex (EJC) (Kataoka et al., 2000; Le Hir et al., 2000a; Le Hir et al., 2000b) (Fig. 1.10). The EJC forms a stable, position-dependent and sequence-independent contact with the mRNA and communicates the position of the PTC; it is essentially a mark for destruction of the mRNA if found downstream of a termination codon (Le Hir et al., 2001b; Le Hir et al., 2000a; Le Hir et al., 2000b). A stop codon is

Figure 1.10. Splicing dependency of NMD

Position-dependent effects of the EJC on NMD. Figure modelled against (Lejeune and Maquat, 2005). When a termination codon is located greater than 50-55 nt upstream of the last exon-exon junction, the mRNA will be subjected to NMD, as indicated by the red underlying arrow.


EJC
EJC
AUG
5' m7G
(A)n 3'
20-24 nt
20-24 nt
50-55 nt
NMD
no NMD

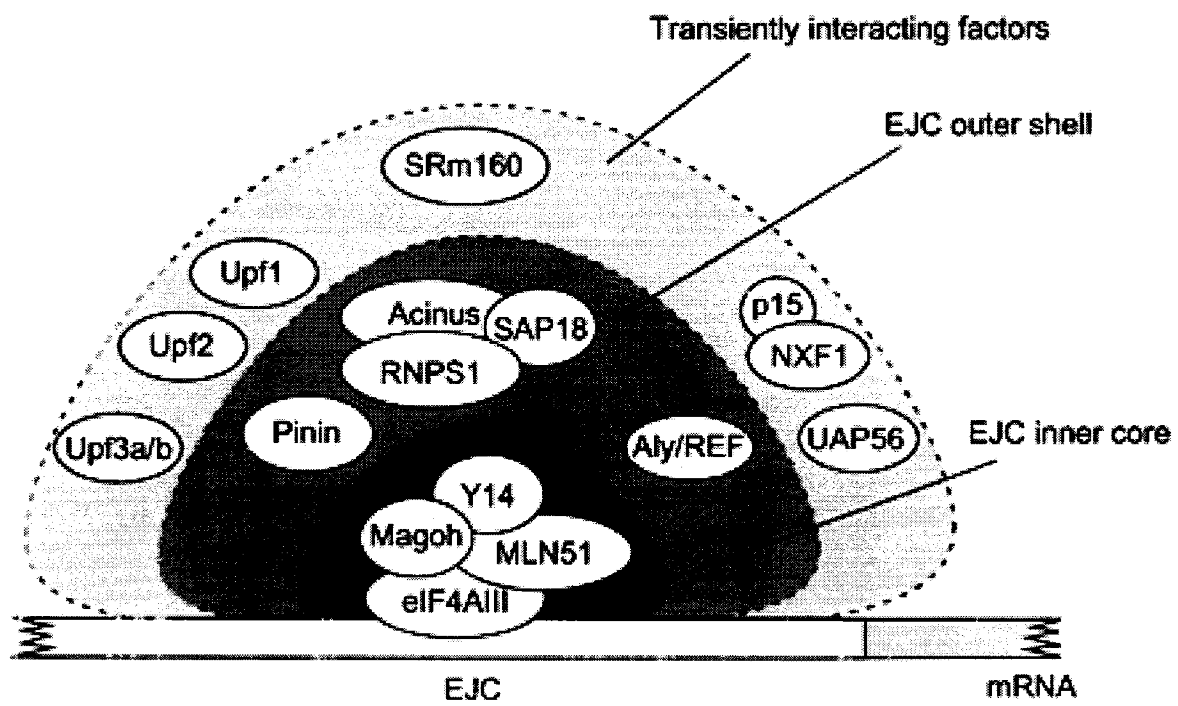
considered premature if it is found at least 50-55 nucleotides upstream of a spliced intron (Fig.1.10) (Carter et al., 1996; Nagy and Maquat, 1998; Zhang et al., 1998a; Zhang et al., 1998b). In fact, siRNA against components of the EJC inhibits NMD; on the other hand, tethering of the EJC factors to the 3' end of a normal transcript results in decay of that message (Gehring et al., 2003; Lykke-Andersen et al., 2000; Lykke-Andersen et al., 2001; Mendell et al., 2002).

1.3.4.2 Components of the EJC

As mentioned above, during splicing the splicesome deposits a complex of proteins at the exon-exon junction. Some of the proteins at this position remain bound to the mRNA once it exits the nucleus and enters the cytoplasm, while other proteins are displaced from the EJC along its journey (Dreyfuss et al., 2002; Moore, 2005) (Fig. 1.11). The latter set of proteins are considered accessory factors and aid in either splicing (for instance, SRm160) or in export of the mRNA through the nucleopore complex (for instance, UAP56, REF/Aly and TAP) (Le Hir et al., 2001b). EJC factors which remain bound to spliced mRNA, at the position of the EJC, even upon entry of the mRNA into the cytoplasm include Y14, Magoh, Barentsz, and RNPS1 (Degot et al., 2004; Kataoka et al., 2001; Kataoka et al., 2000; Kim et al., 2001b; Le Hir et al., 2001a; Le Hir et al., 2001b; Lykke-Andersen et al., 2001) (Fig. 1.11). In addition to these proteins, a component of the trimeric surveillance complex (see section 1.3.4.4), Upf3, is often considered a component of the EJC because it has a nuclear distribution at steady-state, it binds specifically to spliced mRNAs at the postion of the EJC, and it shuttles between the nucleus and the cytoplasm which suggests that it exits with spliced mRNAs from the

Figure 1.11. The EJC core and auxiliary factors

Three spheres of EJC factors. The minimal EJC core likely consists of a tetrameric complex containing eIF4AIII *(note: our studies and others have identified eIF4AIII as a novel component of the EJC, see Chapter 2)*, MLN51, Magoh, and Y14, with eIF4AIII providing direct contact to the mRNA. All factors in this core are shuttling proteins and most likely follow the mRNA to the cytoplasm. Proteins in the outer shell were all found by mass spectrometry of the in vitro-derived EJC. RNPS1, Acinus, and SAP18 can stably associate and may bind the EJC core as a trimeric complex. However, RNPS1 may also bind alone, e.g., via interactions with Pinin. SAP18, RNPS1, and Aly/REF are shuttling proteins, whereas Acinus and Pinin are nuclear restricted. Transiently interacting factors are proteins not identified in the *in vitro*-derived EJC, but which likely interact dynamically with either the EJC core or outer sphere proteins. Adapted from (Tange et al., 2005).



Transiently interacting factors
EJC outer shell
EJC inner core
SRm160
Upf1
Upf2
Upf3a/b
Acinus
SAP18
RNPS1
Pinin
Aly/REF
p15
NXF1
UAP56
Y14
Magoh
MLN51
eIF4AIII
EJC
mRNA

nucleus (Kim et al., 2001a; Lykke-Andersen et al., 2000). Binding assays have substantiated the formation of such a complex (EJC) and have also delineated the specificity and the relevance of these interactions with respect to NMD. Y14 and Magoh directly interact and form a heterodimer *in vitro* and *in vivo* (Kataoka et al., 2001; Le Hir et al., 2001a). The association of Y14 and Magoh is necessary for cell viability in *Drosophila* S2 cells (Le Hir et al., 2001a). siRNA against Y14 inhibits NMD while tethering of Y14 to a reporter induces NMD (Gehring et al., 2003; Lykke-Andersen et al., 2001). A connection between Y14 and NMD is further supported by the fact that Y14 also interacts with Upf3 in RNAse treated extracts. The interaction of Y14 with Upf3 is necessary for NMD mediated by tethered Upf3 (Gehring et al., 2003). RNPS1 can interact with the Upf complex in RNAse treated extracts and is therefore proposed to be a key mediator between the EJC and the surveillance complex (Lykke-Andersen et al., 2001). Tethering of RNPS1 to a reporter also induces NMD (Lykke-Andersen et al., 2001). Barentsz has RNA-binding activity and associates with EJC components. Furthermore, Barentsz colocalizes with Magoh in nuclear speckels, which are sites where splicing factors are stored (Degot et al., 2004; Le Hir et al., 2001a). It is not surprising that EJC proteins which are deposited by the splicesome onto the mRNA (i.e. Y14, Magoh, RNPS1 and Barentsz), are also found in this location (Degot et al., 2004; Kataoka et al., 2001; Kataoka et al., 2000; Le Hir et al., 2001a; Lykke-Andersen et al., 2001). The localization of Barentsz in nuclear speckles and its binding to Magoh and RNA, occurs through the same region called SELOR (speckle localizer and RNA binding module) (Degot et al., 2004).

The EJC is thought to be removed during the first round of translation (Dostie and

Dreyfuss, 2002) (see below). Y14 cosediments with mRNAs at the top of the sucrose gradient and with 80S monosome fractions in a ribosome profiling assay of HeLa cell extracts. Furthermore, Y14 is retained on mRNAs in which translation is inhibited by a hairpin structure in its 5' UTR, *in vitro* and *in vivo,* which indicates that translation is required to remove Y14 from mRNAs (Dostie and Dreyfuss, 2002). The dependency of NMD on translation is discussed below.

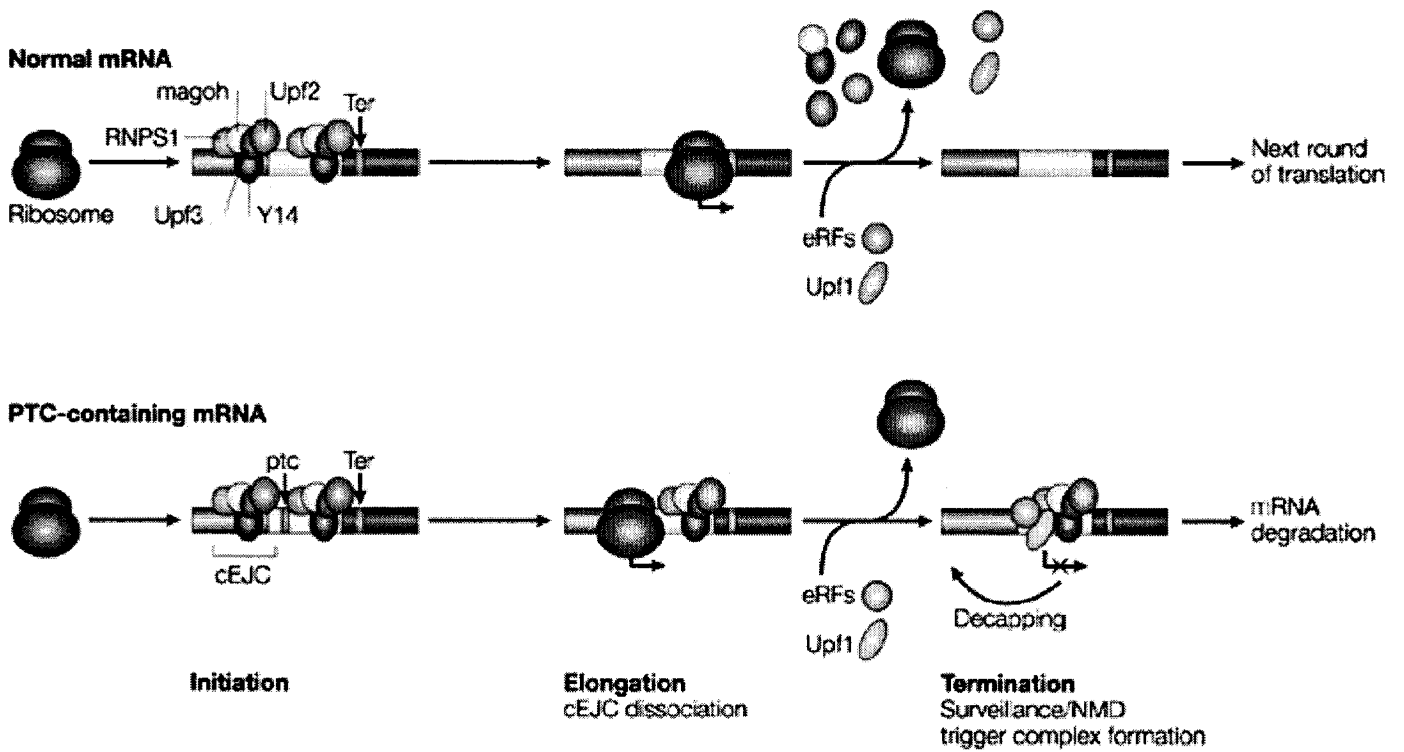
1.3.4.3. Translation Dependency of NMD

NMD is dependent on ongoing translation (Fig. 1.12). The reliance of NMD on translation is supported by several lines of evidence: 1) mutations within the initiator AUG, 2) suppressor tRNAs and 3) hairpin structures within the 5'UTR of the mRNA, which all inhibit translation, also inhibit NMD. This is manifested by an accumulation of PTC-containing mRNAs (Belgrader et al., 1993; Le Hir et al., 2001a; Wang et al., 2002a); 4) protein synthesis inhibitors with different modes of action inhibit NMD (Carter et al., 1995) and 5) infection with poliovirus, which results in eIF4G cleavage at its N-terminus and host cell shutoff of protein synthesis, also inhibits NMD (Carter et al., 1995). Altogether, these data point to the involvement of a surveillance mechanism which relies on scanning of the ORF by the ribosome for a termination codon in the wrong context.

It is proposed that NMD occurs during a pioneer round of translation, which is defined as the first time the reading frame is scanned by the ribosome, and is believed to occur in association with a different set of translation factors which function uniquely in NMD (Chiu et al., 2004; Ishigaki et al., 2001; Lejeune et al., 2002; Lejeune et al., 2004).

Figure 1.12 Translation dependency of NMD

Model of the dependency of NMD on the position of a PTC relative to the EJC and the translating ribosome. Adapted from (Dreyfuss et al., 2002) with modification.


Normal mRNA
magoh
Upf2
Ter
RNPS1
Ribosome
Upf3
Y14
Next round
of translation
eRFs
Upf1
PTC-containing mRNA
ptc
Ter
cEJC
mRNA
degradation
Decapping
Initiation
Elongation
cEJC dissociation
Termination
Surveillance/NMD
trigger complex formation
Nature Reviews | Molecular Cell Biology

It has been reported that messages subject to NMD are bound by CBP20/80 at the cap instead of eIF4E and that the poly(A) tail is bound by PABP2 not in lieu of PABP1 but simultaneously with PABP1 on the mRNA (Ishigaki et al., 2001; Lejeune et al., 2002). More recently, it was shown that eIF4G is necessary for NMD and that it directly interacts with CBP80 (Lejeune et al., 2004; McKendrick et al., 2001). In accordance with the role of CBP20/80 in NMD, 4E-BP1 inhibited translation of a reporter luciferase but did not inhibit NMD (Chiu et al., 2004). In contrast, PAIP2 (Poly(A) Binding Protein Interacting Protein 2), an inhibitor of PABP1 function (Khaleghpour et al., 2001a; Khaleghpour et al., 2001b), inhibited both translation of a reporter luciferase mRNA and NMD (Chiu et al., 2004).

It is debatable where the pioneer round of translation takes place, i.e. the nucleus or the cytoplasm (Iborra et al., 2004; Nathanson et al., 2003; Wilkinson and Shyu, 2002). Most of the PTC-containing mRNAs analyzed to date are degraded in the nuclear fraction (Wilkinson and Shyu, 2002). Some have thus postulated that the first round of translation takes place in the nucleus by a ribosome-like particle, and is based mainly on evidence that some translation factors are localized to the nucleus and that amino acid incorporation occurred in permeabilized cells or purified nuclei (Iborra et al., 2001). However, the latter has met considerable debate and it is presumably more commonly accepted that the mRNA is likely to be read by cytoplasmic ribosomes as it egresses from the nucleus, but that its degradation *per se* co-fractionates with the nucleus (Bohnsack et al., 2002; Nathanson et al., 2003). Since mRNA decay is associated mainly with the nuclear fraction and since some translation factors are localized in the nucleus, including those reported to function in NMD [i.e. CBP80 (Izaurralde et al., 1994), PABP2 (Calado

and Carmo-Fonseca, 2000), and eIF4G (McKendrick et al., 2001)], it is conceivable that nuclear translation initiation factors are assembled on the mRNA in the nucleus and are exported to the cytoplasm where they are then engaged by the ribosome.

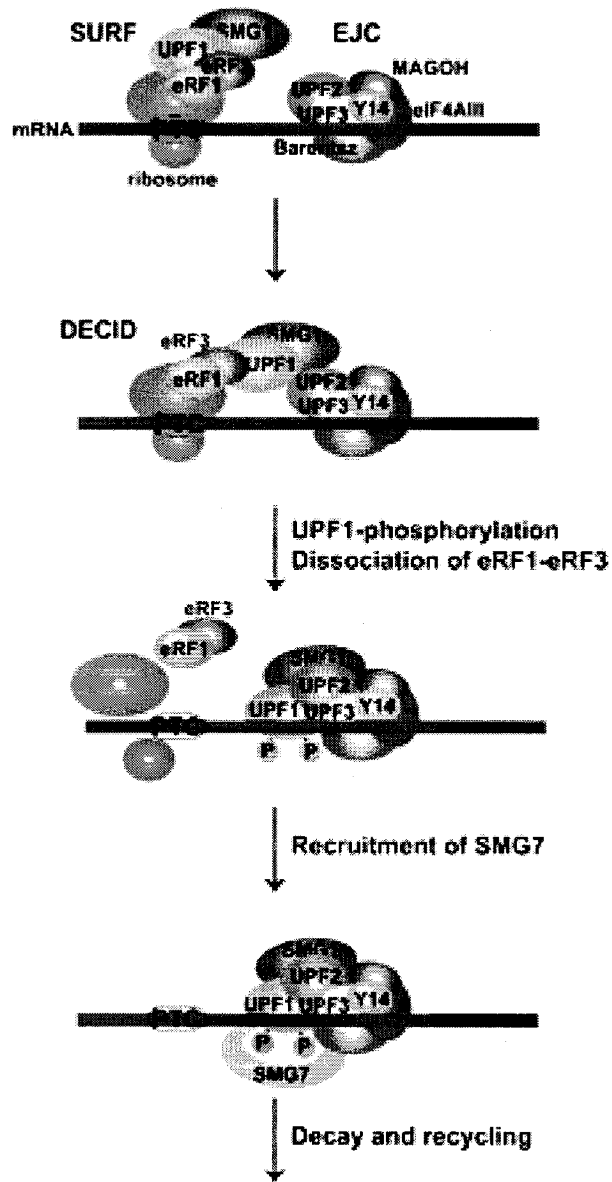
1.3.4.4. The Trimeric Surveillance Complex: Upf1:Upf2: Upf3

The "effector" arm of NMD refers to the mechanism involved in the decay of the PTC-containing mRNA. The signal for decay comes from the surveillance complex which is composed of 3 core proteins: Upf1, Upf2, Upf3. These proteins are conserved in all eukaryotes and are essential for NMD in all organisms (Behm-Ansmant and Izaurralde, 2006; Conti and Izaurralde, 2005). As mentioned above, Upf3 is a nuclear protein which is associated with the EJC and remains associated at the EJC after export from the nucleus (Kim et al., 2001a; Lykke-Andersen et al., 2000). Upf2 is localized at the perinuclear interface; it is proposed that Upf2 joins Upf3 upon exit from the nucleus (Lykke-Andersen et al., 2000). Upf1 is a cytoplasmic protein which contains ATP-dependent RNA helicase activity (Lykke-Andersen et al., 2000). Its helicase activity is required for NMD (Sun et al., 1998; Weng et al., 1996). Upf1 is also involved in translation termination and interacts with translation release factors eRF1 and eRF3 (Czaplinski et al., 1998). If the ribosome encounters a stop codon upstream of an EJC during translation, a premature translation termination event bridges Upf1, associated with release factors eRF1 and eRF3 at the stop site, with downstream bound Upf2 and Upf3. The recruitment of Upf1 results in the formation of the surveillance complex: Upf1:Upf2:Upf3. Formation of the Upf1:Upf2:Upf3 trimeric complex is supported by interaction data, genetic studies and even structural data (Behm-Ansmant and Izaurralde,

2006). Upf2 interacts directly with Upf3 and Upf1 (Behm-Ansmant and Izaurralde, 2006; Kadlec et al., 2004). Upf2 contains three MIF4G domains. The last of the three MIF4G is involved in the interaction with Upf3 (Kadlec et al., 2004). The binding site of Upf2 is mapped to the RNA-binding domain of Upf3 (Kadlec et al., 2004). The binding sites on both Upf2 and Upf3 are conserved among eukaryotes. Upf2 makes contact with Upf1 through both its N and C-terminal regions (Behm-Ansmant and Izaurralde, 2006).

Formation of the surveillance complex elicits Upf1 phosphorylation, which in turn prompts decay of the mRNA (reviewed in (Behm-Ansmant and Izaurralde, 2006). The phosphorylation of Upf1 is required for NMD to occur and is mediated by SMG-1, a phosphoidyl-inositol-3 (PI3)-like kinase. Based on recent data from Kashmina *et al*, a model for surveillance complex formation and Upf1 phosphorylation has emerged (Behm-Ansmant and Izaurralde, 2006; Kashima et al., 2006). When premature translation termination occurs, a complex of proteins consisting of at least eRF1, eRF3, Upf1, and SMG-1 is assembled, and is referred to as the SURF complex (Fig. 1.13). Interaction of Upf1 with downstream bound Upf/ EJC factors instigates Upf1 phosphorylation by SMG-1 and dissociation of eRF1 and eRF3. So Upf1 phosphorylation requires SMG-1, Upf2 and Upf3. The bridging of Upf1 between release factors and EJC components results in the formation of an intermediate complex termed DECID (decay-inducing complex). After Upf1 phosphorylation and release factor dissociation, SMG7 is recruited to the EJC and promotes dephosphorylation of Upf1. A 14-3-3-like domain has been mapped as the binding site of Upf1 on SMG7. SMG7 recruits other proteins, SMG5 and SMG6, which presumably aid in the dephosphorylation of Upf1. SMG7 has been shown to coimmunoprecipitate with

Figure 1.13. Trimeric complex assembly and recruitment of SMG7 triggers decay
See text for details. (Red and blue boxes) Exons; (PTC) premature translation termination codon. Adapted from (Behm-Ansmant and Izaurralde, 2006).


SURF
SMG1
EJC
UPF1
eRF1
MAGOH
UPF2
UPF3
Y14
eIF4AIII
mRNA
ribosome
DECID
eRF3
SMG1
UPF1
eRF1
UPF2
UPF3
Y14
UPF1-phosphorylation
Dissociation of eRF1-eRF3
eRF3
eRF1
UPF2
UPF1
UPF3
Y14
P
P
Recruitment of SMG7
UPF2
UPF1
UPF3
Y14
P
P
SMG7
Decay and recycling

phosphorylated Upf1, SMG5 and protein phosphatase PP2A. SMG6, in turn, has been shown to interact with PP2A and Upf1; however, it is ambiguous whether it is in the same complexes containing SMG5 and SMG7. Therefore, it has been suggested that a minimal complex of SMG5 and SMG7 instigates dephosphorylation of Upf1 by PP2A, so that Upf1 can be recycled into another round of NMD.

1.3.4.5. mRNA Decay Enzymes in NMD

Binding of SMG7 to Upf1 is thought to coincide with the recruitment of decay enzymes (Unterholzner and Izaurralde, 2004). Tethering SMG7 to a reporter transcript, regardless of whether it contains a PTC, promotes its degradation (Unterholzner and Izaurralde, 2004). The increased turnover of SMG7-bound transcripts is mediated by its C-terminal domain. Coincidentally, the C-terminal domain of SMG7 is needed for localization in foci where mRNA decay occurs (see section 1.4); the ability of tethered SMG7 to induce decay of bound messages relies on Dcp2 and Xrn1 (Unterholzner and Izaurralde, 2004). siRNA against Dcp2 or Xrn1 inhibits the mRNA decay function of full length SMG7 and of a deletion mutant of SMG7 containing only the C-terminal domain (Unterholzner and Izaurralde, 2004). Overexpression of SMG7 results in the recruitment of coexpressed SMG5 and coexpressed Upf1 in mRNA decay foci (Unterholzner and Izaurralde, 2004). SMG7 interacts with phosphorylated Upf1 and SMG5 via its N-terminal 14-3-3-like domain (Unterholzner and Izaurralde, 2004). Upf1 in turn has been shown to interact with Dcp2 in a yeast two hybrid assay (Dunckley and Parker, 1999). Therefore, SMG7 appears to be the link between the changes in the phosphorylation state of Upf1 with mRNA decay, i.e. SMG7 induces decay of mRNAs bound by phosphorylated Upf1 by

recruiting decay enzymes and targeting their decay in discrete foci in the cytoplasm (Unterholzner and Izaurralde, 2004).

Degradation of PTC-containing mRNAs involves the same enzymes utilized in general mRNA decay. In *S. cerevisiae,* PTC-containing mRNAs, in general, bypass deadenylation of the mRNA and undergo rapid decapping by Dcp1/2 and 5'-3'decay by Xrn1 (Muhlrad and Parker, 1994). Some PTC-containing mRNAs are also degraded by the 3'-5'exonucleolytic pathway, whereby they undergo rapid deadenylation, digestion of the mRNA by the exosome and the Ski complex (Cao and Parker, 2003; Mitchell and Tollervey, 2003). In mammalian cells, both pathways have been reported to elicit decay of PTC-containing mRNAs (Chen and Shyu, 2003; Lejeune et al., 2003).

1.3.4.6. Alternative NMD Pathways in Mammalian Cells

Two alternative complexes with differential requirements for Upf2 have recently been reported to elicit NMD (Behm-Ansmant and Izaurralde, 2006; Gehring et al., 2005). Both complexes require Upf1, however, they have different sensitivities to depletion of the various EJC components, most notably there seems to be a discrepancy in the requirement of one of the core proteins of the surveillance complex, Upf2. In one NMD pathway, reporter transcripts that are triggered for NMD by tethering known EJC components is not inhibited by Upf2 depletion (Gehring et al., 2005). In another pathway, Upf2 depletion inhibits NMD triggered by tethering some but not all EJC factors discussed above (Gehring et al., 2005). Endogenous targets of the alternative NMD pathways have been identified by gene expression profiles in cells treated with siRNA agaiinst Upf1, Upf2, RNPS1 or Barentsz (Gehring et al., 2005). These alternative

NMD pathways may be minor pathways in mammalian NMD (Behm-Ansmant and Izaurralde, 2006). Another possibility is that low levels of Upf2 remaining after siRNA mediated knockdown are sufficient to sustain NMD in these assays. Notably, the tethering assays may not reflect NMD that is studied by PTC-containing reporter mRNAs and may not mirror true NMD (Behm-Ansmant and Izaurralde, 2006) (see General Discussion, section 4.2).

Besides its role in mRNA stability, the EJC stimulates other functions such as mRNA export (Le Hir et al., 2001b) and translation (Nott et al., 2004; Wiegand et al., 2003) (see General Discussion for further detail).

1.3.5 RNA Interference (RNAi)

The third group which constitutes one of the mRNA degradation pathways utilized by eukaryotes, involves the destruction of target transcripts by short RNAs which are complementary to the mRNA (for review and references therein refer to (Murchison and Hannon, 2004; Valencia-Sanchez et al., 2006)). This mode of RNA silencing is carried out by two types of small RNAs, microRNAs (miRNAs) and short interfering RNAs (siRNAs). miRNA/siRNA silence their targets by inducing endonuclease cleavage of the mRNA but it is also likely that they, alternatively, induce deadenylation and decapping of the message (see below). miRNAs also silence specific targets not by inciting cleavage of the mRNA but by repressing its translation. Interestingly, miRNAs regulate approximately 30% of human genes. RNAi by siRNAs and miRNAs will be discussed briefly here.

siRNAs and miRNAs are ~21-25 nucleotides in length. They are processed from

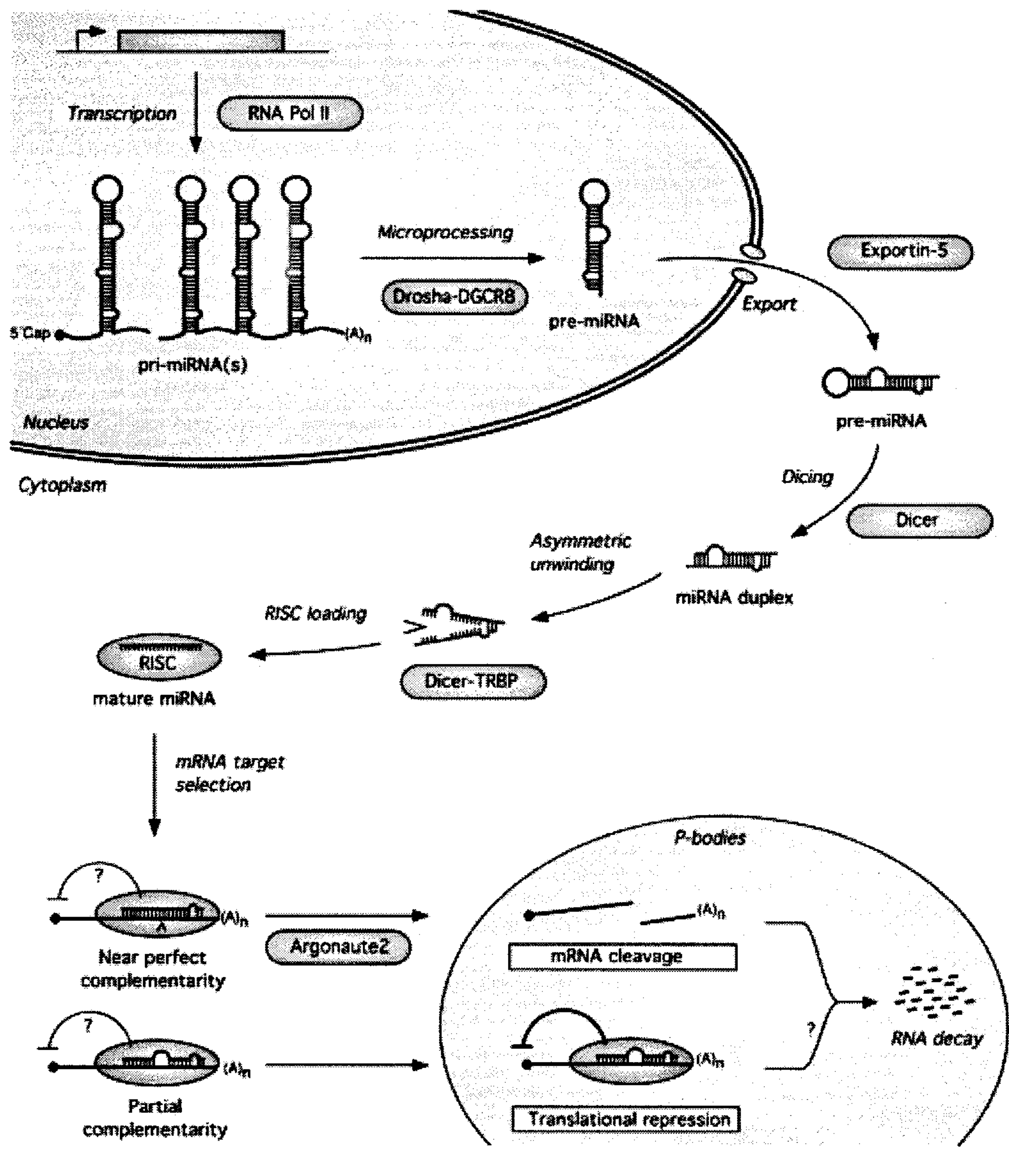
different precursors. miRNAs are processed from noncoding regions of mRNAs produced by RNA Polymerase II, which form a stem loop structure (Fig. 1.14) . These structures are metabolized in the nucleus by Drosha, an RNAse III enzyme, and its interacting partner, DGCR8. After cleavage, the stem loop RNA is reduced to a 65-75 nucleotide precursor miRNA (pre-miRNA), which is exported from the nucleus by exportin 5. In the cytoplasm, the pre-miRNA is processed further by another RNAse III endonuclease, DICER, which yields the ~21-25 nucleotide double stranded RNA (dsRNA). The dsRNA is incorporated into a RNA-induced silencing complex (RISC) which is composed of several proteins, including a group called Argonaute (Ago) proteins. It is this complex which effects RNA silencing of the target mRNA (reviewed in (Valencia-Sanchez et al., 2006)). siRNA is produced by processing long dsRNA which can either be provided exogenously (for example, by viruses or by transfection of cells in culture) or from endogenous precursors (Sontheimer and Carthew, 2005). They are also metabolized by DICER and incorporated into a RISC complex. One of the key players in the RISC complex is Argonaute 2. This protein has been identified as the "slicer"protein, i.e. the protein which catalyzes the endonuclease cleavage of the mRNA target (Meister et al., 2004).

The specificity of the short RNA to the target governs whether the target will be degraded or whether it will be translationally repressed. In general, perfect base pairing of the siRNA/miRNA favours degradation of the mRNA, whereas imperfect base pairing by a miRNA results in translational repression (Humphreys et al., 2005; Jing et al., 2005; Olsen and Ambros, 1999; Petersen et al., 2006; Pillai et al., 2005; Valencia-Sanchez et al., 2006). However, some mismatching by miRNA can allow for mRNA cleavage in

Figure 1.14. RNA silencing

Adapted from (Wienholds and Plasterk, 2005).

Although the miRNA processing pathway is illustrated here, siRNAs are processed from long dsRNA (which have been provided exogenously or which are derived endogenously) by DICER in the cytoplasm and incorporated into a RISC complex in a similar fashion as miRNA, but the protein components which constitute the siRISC may differ from miRISC. The mRNA targeted by the siRNA is then degraded in P-bodies like the miRNA illustrated in the diagram, which induces cleavage of its target.


Transcription
RNA Pol II
Microprocessing
Drosha-DGCR8
pre-miRNA
5'Cap
(A)n
pri-miRNA(s)
Nucleus
Cytoplasm
Exportin-5
Export
pre-miRNA
Dicing
Dicer
Asymmetric
unwinding
miRNA duplex
RISC loading
Dicer-TRBP
RISC
mature miRNA
mRNA target
selection
P-bodies
?
(A)n
Near perfect
complementarity
Argonaute2
mRNA cleavage
RNA decay
Partial
complementarity
Translational repression

some cases (Valencia-Sanchez et al., 2006; Wu et al., 2006). Furthermore, while slicer activity requires binding to one site of the target mRNA, efficient translation repression by miRNA requires binding to many sites on the target. The repression of translation by miRNA reportedly occurs by interruption of translation initiation (Humphreys et al., 2005; Pillai et al., 2005) or a step in translation after initiation (Olsen and Ambros, 1999; Petersen et al., 2006). It has been proposed that the two mechanisms of repression are not mutually exclusive but that miRNA-mediated repression may influence a combination of translation events (Olsen and Ambros, 1999; Valencia-Sanchez et al., 2006). Furthermore, a relationship of miRNA/siRNA to mRNA decapping and deadenylation-dependent decay and/or translation repression is suggested by the localization of target mRNAs and RISC components in P-bodies with decapping factors (see section 1.4) (Jakymiw et al., 2005; Liu et al., 2005a; Liu et al., 2005b; Sen and Blau, 2005). Therefore, localization of mRNAs in P-bodies may represent an additional level of miRNA-mediated translational control (Valencia-Sanchez et al., 2006).

1.4 Processing Bodies (P-bodies)

1.4.1 Composition of P-bodies, sites of mRNA decay enzymes

Processing bodies (P-bodies) are cytoplasmic foci, visible by light microscopy, where mRNA decay takes place (Coller and Parker, 2004; Fillman and Lykke-Andersen, 2005; Parker and Song, 2004). These bodies were originally identified as mRNA graveyards in yeast, *S. cerevisiae,* by immunostaining against proteins involved in decapping and 5'-3'exonuclease digestion (Sheth and Parker, 2003). The Dcp1/Dcp2 decapping complex,

Xrn1, and decapping activators Dhh1p, Pat1p, and Lsm1-7 as well as mRNA have all been reported to localize in these foci (Sheth and Parker, 2003). Mammalian counterparts of the yeast P-body also exist. The mammalian homologs of Dcp1/2, Xrn1, Lsm1-7 have all been localized to cytoplasmic foci (Cougot et al., 2004; Ingelfinger et al., 2002; Liu et al., 2004; van Dijk et al., 2002) which are referred to as mammalian processing bodies (P-bodies) but are also known as Dcp bodies (Cougot et al., 2004) or GW bodies (Eystathioy et al., 2003), because of the localization of the human GW182 autoantigen in these foci. The human homolog of dhh1p, p54/Rck, is also concentrated in P-bodies (Cougot et al., 2004). In addition, human Edc3 and Hedls, are localized in P-bodies (Fenger-Gron et al., 2005). Contrary to yeast, the Ccr4 deadenylase is partially localized in mammalian P-bodies (Cougot et al., 2004). Components of the exosome, however, are excluded from P-bodies, at least in yeast (Coller and Parker, 2005).

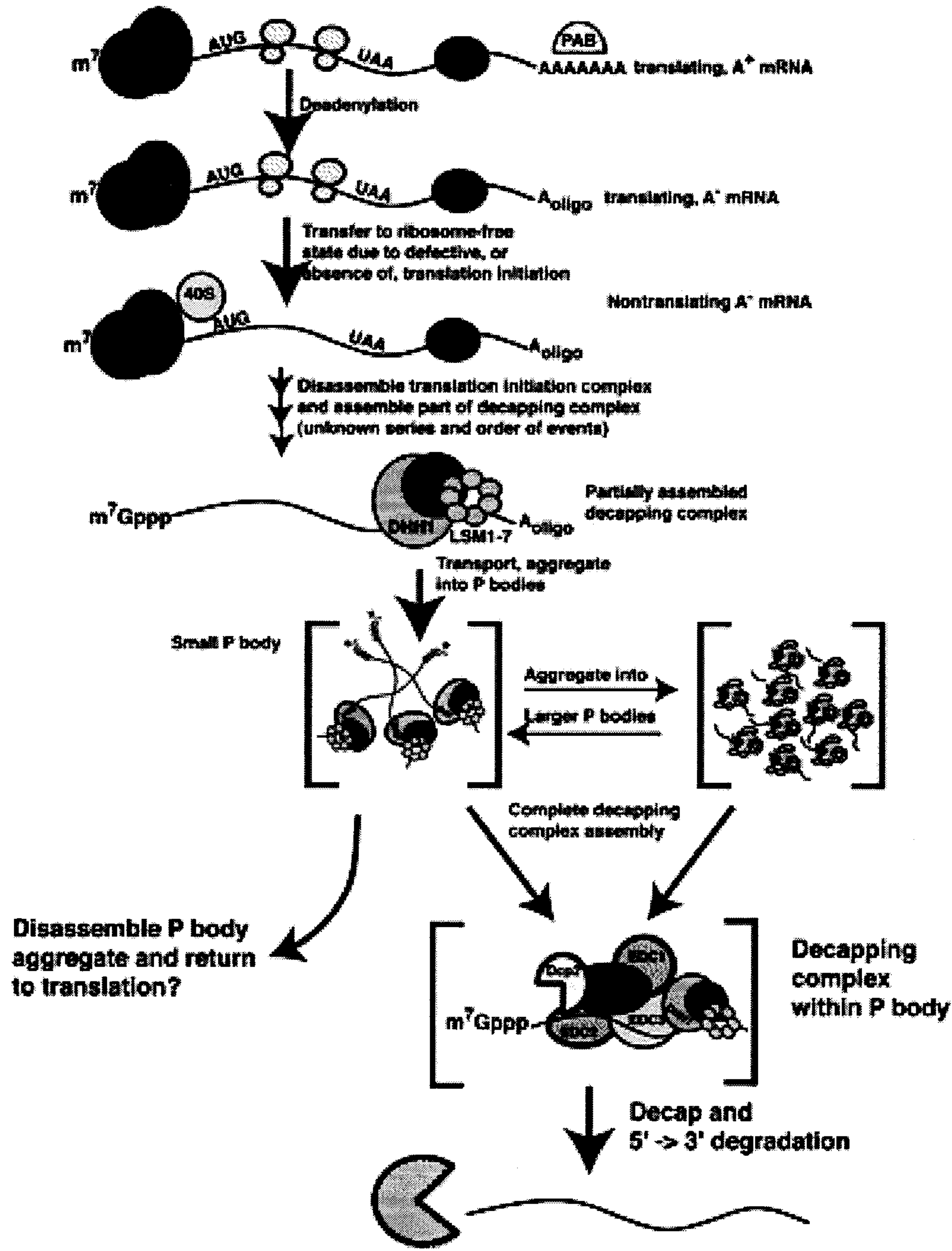
The size and number of P-bodies varies depending on the flow of mRNA going through the decapping step (Coller and Parker, 2004). In yeast cells depleted of Dcp1, Dcp2, Lsm1 or Xrn1 there is an increase in the size and number of P-bodies (Sheth and Parker, 2003). It is reasoned that mRNAs are trapped at the decapping step and cannot be degraded. Accordingly, depletion of Xrn1 from HeLa cells resulted in an accumulation of poly(A) RNA in P-bodies (Cougot et al., 2004). In contrast, deletion of decapping factors Pat1p or Dhh1p results in a loss of yeast P-bodies which suggests that these proteins mark the mRNP for decay in P-bodies (Coller and Parker, 2005; Sheth and Parker, 2003). Incidentally, these proteins, in addition to being activators of decapping, are inhibitors of translation (Coller and Parker, 2005). Treatment with the translation inhibitor cycloheximide results in the disappearance of P-bodies in yeast and mammalian cells

(Cougot et al., 2004; Sheth and Parker, 2003). Cycloheximide traps polysomes on the mRNA and reflects the state of an mRNA which is not accessible to decapping enzymes and exonucleases, hence the decrease in mRNAs going through decapping and a decrease in P-bodies. Thus, the mRNA is needed for localization of decapping factors in P-bodies. In accordance with the lack of ribosomes in P-bodies, translation factors are absent from the foci (Coller and Parker, 2005; Teixeira et al., 2005). Immunoprecipitation of Lsm complex components, revealed that Lsm1p-7p coimmunopurifies with mRNA which is not bound by eIF4E, eIF4G and PABP in *S. cerevisiae* (Tharun and Parker, 2001). This led to the hypothesis that there is a rearrangement in the composition of factors bound to the mRNA which coincides with loss of PABP and with deadenylation (Coller and Parker, 2004) (Fig. 1.15). A rearrangement in the mRNP from a translationally active state to an mRNP destined for decay is believed to be necessary for its localization in P-bodies, where it is either degraded or remains in a translationally repressed state, sequestered from the translating pool of mRNA (Brengues et al., 2005; Coller and Parker, 2005; Teixeira et al., 2005).

1.4.2 P-bodies: sites of translationally repressed mRNA

Bodies with similar morphology exist in *Xenopus, Drosophila* and *C. elegans* (Coller and Parker, 2004). In these organisms, the bodies represent sites where translationally repressed mRNPs, consisting of maternal mRNA, are kept for later activation needed in development. There is evidence in yeast which also favours that P-bodies are sites containing repressed mRNPs which can be released and reactivated. The evidence in support of this includes: 1) cycloheximide treatment reduces P-bodies even in strains in

Figure 1.15. Hypothesis of mRNA decapping and translational repression in yeast
Adapted from (Coller and Parker, 2004).



m7
AUG
UAA
PAB
AAAAAAA translating, A+ mRNA
Deadenylation
m7
AUG
UAA
Aoligo
translating, A- mRNA
Transfer to ribosome-free state due to defective, or absence of, translation initiation
40S
m7
AUG
UAA
Aoligo
Nontranslating A- mRNA
Disassemble translation initiation complex and assemble part of decapping complex (unknown series and order of events)
m7Gppp
DHH1
LSM1-7
Aoligo
Partially assembled decapping complex
Transport, aggregate into P bodies
Small P body
Aggregate into
Larger P bodies
Complete decapping complex assembly
Disassemble P body aggregate and return to translation?
m7Gppp
Decapping complex within P body
Decap and 5' -> 3' degradation

which decapping is absent (Δdcp1p), 5'-3'degradation is compromised (ΔXrn1), or 3'-5' degradation is compromised (ΔSki2) (Brengues et al., 2005); furthermore, the number of P-bodies is also reduced after cycloheximide treatment in a ΔSki2 strain, which is also impaired in decapping by a temperature sensitive mutation (*dcp1-2 ski2Δ*) (Brengues et al., 2005). This collectively suggests that mRNPs can exit P-bodies in the absence of mRNA degradation. 2) Glucose deprivation induces an increase in the size and number of P-bodies; however, GFP-labelled mRNA is restored into the translating pool and away from P-bodies when glucose is resupplemented into the media. Moreover, glucose resupplementation is accompanied by a disassembly of P-bodies to normal levels (i.e. such as seen before glucose deprivation) even in *dcp1-2* strains (Brengues et al., 2005). 3) Yeast strains containing a temperature sensitive mutant of eIF3 (*prt1-63*) display an increased number of P-bodies during glucose deprivation similar to wild type strains, but the disassembly of P-bodies does not occur in the *prt1-63* after glucose resupplementation like in the wild type strains; therefore, the disassembly of P-bodies requires translation to be functional (Brengues et al., 2005). 4) Finally, another striking example which favours P-bodies as sites where translationally silenced mRNAs are stored, comes from the co-localization of RISC components (Ago1 and Ago2) with Dcp1/2 and GW182 in mammalian P-bodies (Jakymiw et al., 2005; Liu et al., 2005a; Liu et al., 2005b; Rehwinkel et al., 2005; Sen and Blau, 2005). Furthermore, interaction of Ago2 with Dcp1/2 and GW182 has been verified (Jakymiw et al., 2005; Liu et al., 2005a; Liu et al., 2005b). Moreover, mRNAs which are the target of miRNAs and shown to be silenced in a slicer-independent manner (i.e. not cleaved by Ago2 but inhibited at the level of translation) also localize to P-bodies (Liu et al., 2005b). Depletion of GW182 by

siRNA not only disrupts P-bodies but impairs RNA silencing of both a miRNA and siRNA - targeted reporter (Jakymiw et al., 2005; Liu et al., 2005a; Rehwinkel et al., 2005). On the other hand, transfection of siRNA into HeLa cells results in an accumulation of GW182 to P-bodies (Jakymiw et al., 2005). Whether or not repression of translation by miRNA is the effect or the cause of localization of mRNP into P-bodies remains to be determined (see General Discussion, Chapter 4). Furthermore, interaction of Ago1 and Ago2 with Dcp1/2 and its colocalization with these factors in P-bodies suggests that silencing by miRNA can result in deadenylation and/or decapping of certain messages and their degradation; indeed some miRNAs are able to elicit mRNA degradation (see section 1.3.5).

1.4.3 P-bodies and NMD

Given that P-bodies are sites of mRNA decay, it is conceivable that factors involved in the "effector arm" of NMD may also localize in cytoplasmic foci. Overexpressed SMG5 and SMG7 (see section 1.3.4.5) localize in P-bodies with endogenous P-body factors (Unterholzner and Izaurralde, 2004). The localization of SMG5 to P-bodies is dependent on its interaction with SMG7. The C-terminal fragment of SMG7 is necessary for its localization in P-bodies and for eliciting decay when tethered to an mRNA. Incidentally, overexpression of SMG7 also recruits Upf1 in P-bodies (Unterholzner and Izaurralde, 2004). The C-terminal domain of SMG7, however, is not sufficient to accumulate Upf1 in P-bodies and bypasses the need for Upf1 in a tethering assay. Based on these data, a model for the role of SMG7 in the NMD pathway has been proposed (Behm-Ansmant and Izaurralde, 2006; Conti and Izaurralde, 2005). The N-terminal fragment of SMG7

interacts with SMG5 and phosphorylatd Upfl whereas the C-terminal domain is required for recruiting mRNA decay enzymes and for concomitant localization in P-bodies where the mRNA will likely be degraded. In this regard, SMG7 is an important link between PTC recognition and mRNA decay. It has yet to be determined whether NMD mRNA targets are present in mammalian P-bodies (see General Discussion, Chapter 4).

1.5 Rationale for the studies performed

The studies performed here focused on the potential role in mRNA decay of two factors first characterized in this laboratory, termed eIF4AIII and 4E-T (Dostie et al., 2000a; Li et al., 1999). Both studies were prompted by the unique localization of these factors within the cell by immunostaining. eIF4AIII, unlike its other family members eIF4AI and eIF4AII, localized predominantly to the nucleus (J. Dostie unpublished data; (Bohnsack et al., 2002; Holzmann et al., 2000; Lejbkowicz et al., 1992)). Interestingly, eIF4AIII was present in nuclear speckles reminiscent of splicing factor storage sites (Holzmann et al., 2000). Furthermore, eIF4AIII peptides were recovered from mass spectrometry analysis of the human splicesomal C complex (Jurica et al., 2002; Rappsilber et al., 2002; Zhou et al., 2002). Because eIF4AIII localized to the nucleus and is recruited to the splicesomal complex, we suspected a function of eIF4AIII in NMD, an mRNA decay process that is dependent on splicing and which cofractionates with the nucleus.

We reported that 4E-T is the nuclear import factor of eIF4E (Dostie et al., 2000a). In these studies, the localization of 4E-T at steady state revealed a predominantly cytoplasmic localization with a prominent punctate staining. At the time, the nature of

these foci eluded us. Within recent years, studies performed in mammalian cells and *S. cerevisiae* revealed the localization of mRNA decay enzymes in discrete cytoplasmic foci (Bashkirov et al., 1997; Ingelfinger et al., 2002; Sheth and Parker, 2003; van Dijk et al., 2002). mRNA decay intermediates were localized to these foci in yeast, which were coined P-bodies, providing the first direct evidence that mRNA decay can occur in specific sites of the cytoplasm. Thereafter, the function of 4E-T in mRNA decay was addressed. Indeed, we report that eIF4AIII is a novel component of the EJC and is required for NMD in Chapter 2, and that 4E-T is a *bonafide* component of P-bodies in mammalian cells which functions in mRNA decay in Chapter 3.

CHAPTER 2

A nuclear translation-like factor eIF4AIII is recruited to the mRNA during splicing and functions in nonsense-mediated decay

2.1 ABSTRACT

In eukaryotes, a surveillance mechanism known as nonsense-mediated decay (NMD) degrades the mRNA when a premature-termination codon (PTC) is present. NMD requires translation to read the frame of the mRNA and detect the PTC. During pre-mRNA splicing, the exon-exon junction complex (EJC) is recruited to a region 20-24 nucleotides upstream of the exon junction on the mature mRNA. The presence of a PTC upstream from the EJC elicits NMD. eIF4AIII is a nuclear protein that interacts physically or functionally with translation initiation factors eIF4G and eIF4B, respectively, and shares strikingly high identity with the initiation factors eIF4AI/II. Here we show that siRNA against eIF4AIII, but not against eIF4AI/II, inhibits NMD. Moreover, eIF4AIII, but not eIF4AI, is specifically recruited to the EJC during splicing. The observations that eIF4AIII is loaded onto the mRNA during splicing in the nucleus, has properties related to a translation initiation factor, and functions in NMD raises the possibility that eIF4AIII substitutes for eIF4AI/II during NMD.

2.2 Introduction

Ribosome recruitment to the mRNA in eukaryotes requires the RNA helicase, eukaryotic initiation factor 4A (eIF4A), which is thought to unwind 5' secondary structure in the mRNA and facilitate binding of the small ribosomal subunit (1). eIF4AI and eIF4AII are two similar gene products that are functionally redundant for translation initiation (2); eIF4AI accounts for the majority (about 75%) of total eIF4A in a rabbit reticulocyte lysate (3). eIF4AIII, which is ~70% identical to eIF4AI/II (4), cannot substitute for these proteins in an *in vitro* mRNA ribosome binding assay (4). Nevertheless, a relationship of eIF4AIII to a translation-like mechanism is suggested by the observation that eIF4AIII requires the initiation factor eIF4B for efficient RNA unwinding activity (4). In addition, eIF4AIII interacts with another essential initiation factor, eIF4G (4). eIF4AIII can inhibit translation in a rabbit reticulocyte lysate when added in large amounts (4), whereas it may have a stimulatory effect on selective mRNA translation in *Xenopus* oocytes (5). Importantly, in contrast to eIF4AI/II, which are cytoplasmic, eIF4AIII is nuclear where it co-localizes with splicing factors in speckle domains (6).

Nonsense-mediated decay (NMD) is an RNA surveillance mechanism that rids the cells of defective mRNAs bearing a PTC (7-9). About 30% of inherited genetic disorders are the consequence of mutations that create a PTC (10). NMD is both a splicing and translation-dependent event (11). An exon-exon junction complex (EJC) is assembled 20-24 nucleotides upstream of the exon-exon junction on the mRNA as a result of splicing (12, 13). The EJC is composed of mRNA export factors (such as Aly/REF) and factors that are required for NMD (such as RNPS1, Y14, mago and Upf3) (11, 14, 15). Many of these splicing-dependent mRNA binding proteins are localized in

the nucleus but shuttle between the nucleus and the cytoplasm (11, 14). The EJCs are positional markers of introns; when a termination codon is present at least 50-55 nucleotides upstream of a spliced intron, i.e. upstream of an EJC, it is recognized as premature (16-19). In mammalian cells, intronless, PTC-bearing mRNAs are not subject to NMD, demonstrating the importance of splicing for NMD (20-22). In addition to being splicing-dependent, NMD also requires translation. Protein synthesis inhibitors (23), mutation of the initiation codon (24), hairpin structures in the 5'UTR (25, 26) and suppressor tRNAs all inhibit NMD (24).

NMD is thought to occur during the so-called pioneer round of translation, which is defined as the first time the reading frame of the mRNA is read (27). According to a current view, the pioneer round of translation occurs when the cap and polyA tail are bound by the nuclear proteins CBC and PABP2, whereas bulk translation occurs when the cytoplasmic proteins eIF4E and PABP are bound (27). Because eIF4AIII is localized to the nucleus, is a homologue of a translation factor necessary for bulk translation, and interacts with translation factors (eIF4B and eIF4G), it is conceivable that it could function in the pioneer round of translation and thus in NMD (27).

Here we report that knockdown of eIF4AIII using RNA interference (RNAi) results in inhibition of NMD, whereas knockdown of eIF4AI and II fails to inhibit NMD. We also demonstrate that eIF4AIII, but not eIF4AI, associates with mRNA during splicing at a region containing the EJC. Finally, we show that eIF4AIII is a nucleocytoplasmic shuttling protein. Together, these data indicate that eIF4AIII is loaded onto the mRNA during splicing in the nucleus and then functions during NMD.

2.3 Results

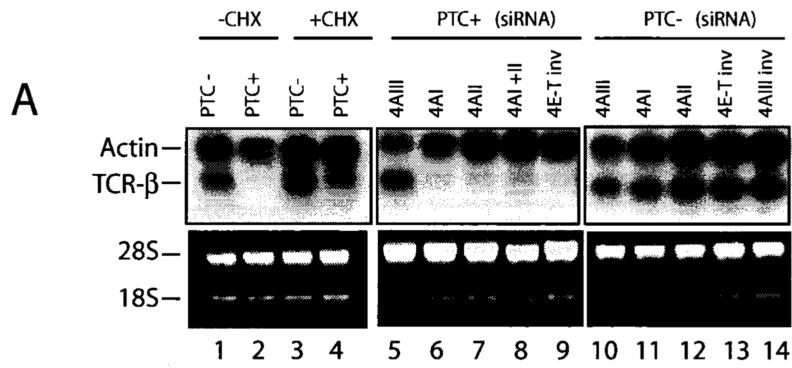
RNA interference (RNAi) was carried out in HeLa cells to determine whether eIF4AIII plays a role in NMD. To assay for NMD, we analyzed the levels of TCR-β mRNA, a transcript that undergoes NMD in a nuclear-associated manner (24). Wild–type and PTC containing TCR-β transcripts were analyzed (23, 24). Treatment of cells with small interfering RNA (siRNA) against eIF4AIII reduced its level by 80% (Fig.2.1B, lane 4). In HeLa cells expressing a PTC-containing TCR-ß gene, the levels of the mutant mRNA were very low relative to the levels of the wild type mRNA, indicating that NMD has occurred (Fig. 2.1A, compare lane 1 to 2). As expected, NMD was strongly inhibited upon the addition of the translation inhibitor, cycloheximide (Fig. 2.1A, lane 4)(23). NMD was significantly inhibited in cells treated with siRNA against eIF4AIII (Fig. 2.1A, lane 5; siRNA against eIF4AIII is accompanied by a decrease in actin, which is presumably a consequence of increased cell death), but not in cells treated with a negative control siRNA (4E-T inverted; 4E-T inv(28); Fig. 2.1A, lane 9). Furthermore, siRNA against eIF4AIII inhibits decay of PTC+ TCR-β mRNA to a similar extent as siRNA against Upfl (29, 30). We conclude that eIF4AIII functions in NMD.

To determine whether siRNA against eIF4AI and eIF4AII affects NMD, we treated HeLa cells with siRNAs against these factors (see Table 2.1 for a list of sequences). The siRNA treatments resulted in a specific reduction of protein levels of 75% for eIF4AI and 95% for eIF4AII (Fig. 2.1B). Strikingly, and in sharp contrast to the results obtained with RNAi to eIF4AIII, knockdown of eIF4AI and II (Fig. 2.1B, lanes 2 and 7, respectively) failed to inhibit NMD (Fig. 2.1A, lanes 6 and 7); the PTC-containing mRNA was present at levels similar to those seen in untransfected cells or cells treated

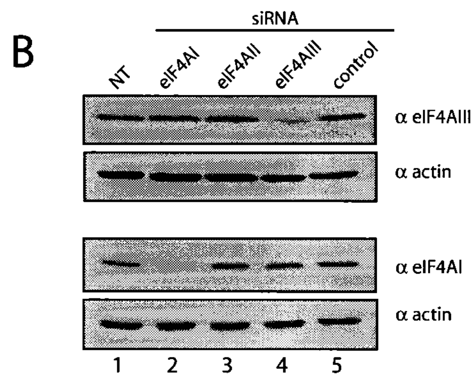
Figure 2.1. RNAi against eIF4AIII impairs NMD.

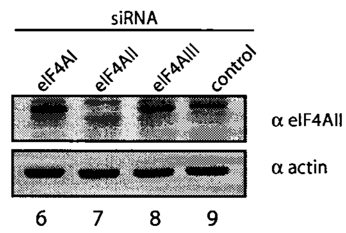
(A) Northern blot analysis of polyadenylated [(poly(A)] RNA hybridized with Vβ8.1 probe(23). Lanes 1 and 2 represent stable cell lines expressing wild type TCR-β mini-gene and PTC-containing TCR-β mini-gene, respectively. Cycloheximide treatment in both cell lines was performed for 2h at 37°C (100ug/ml final) (lanes 3 and 4). siRNAs were transfected into stable cell lines expressing a PTC-containing TCR-β mini-gene (PTC+) (lanes 5-9) and wild type TCR-β mini-gene (PTC-) (lanes 10-14). β-actin served as a loading marker. The 28S and 18S ribosomal RNA serve to demonstrate equal loading for eIF4AIII siRNA treated samples because siRNA against eIF4AIII is accompanied by decreased levels of actin due to cell death (see Results). CHX, cycloheximide. (B and C) Western analysis of the levels of translation initiation factors after knockdown. Thirty micrograms of protein extract was resolved by SDS/PAGE. Proteins were quantitated against β-actin (Sigma) as a loading control and the level of protein in negative control (4E-T inv) transfected cells was normalized to 100. NT, nontransfected .

A



B





C

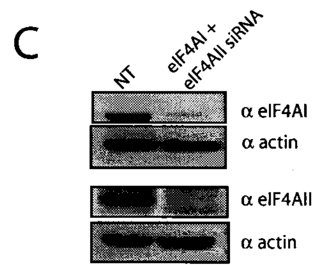


Table 2.1 Summary of the siRNA used for gene expression knockdown

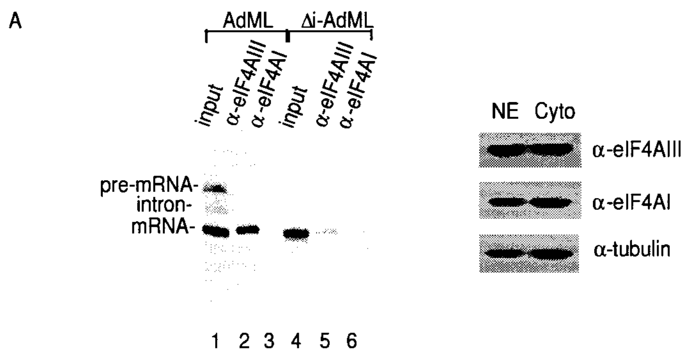
Table 1. Summary of the small interfering RNA (siRNA) used for gene expression knockdown

Name	Position in the open reading frame	siRNA Structure
Synthetic siRNA		
eIF4AI	226-244	5'- GCCCAAUCUGGGACUGGGA dTdT -3' 3'- dTdT CGGGUUAGACCCUGACCCU -5'
eIF4AII	122-140	5'- AGGAGUCUCUCCUUCGUGG dTdT -3' 3'- dTdT UCCUCAGAGAGGAAGCACC -5'
eIF4AIII	75-93	5'- AGUGGAAUUCGAGACCAGC dTdT -3' 3'- dTdT UCACCUUAAGCUCUGGUCG -5'
4E-T Inverted	935-953	5'- CGUACCGUGGAAUAGUUCC dTdT -3' 3'- dTdT GCAUGGCACCUUAUCAAGG -5'

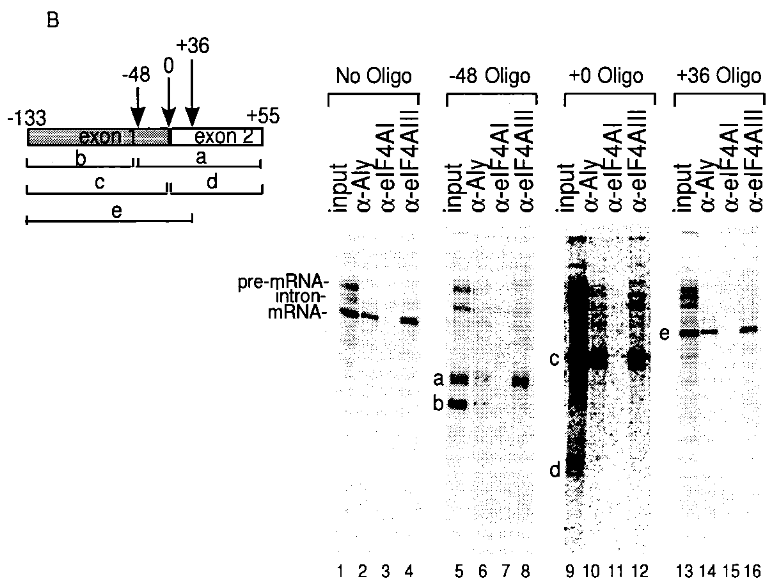
with the negative control siRNA (28) (lane 9). To determine whether eIF4AII compensates for the loss of eIF4AI function or vice versa, a double knockdown was performed (Fig. 2.1C). No effect on NMD was observed (Fig. 2.1A, compare lane 8 to 9). Thus, a large reduction in the levels of the cytoplasmic eIF4A translation initiation factors failed to affect NMD, whereas a similar reduction in eIF4AIII exhibited a major inhibitory effect on NMD. These data indicate that eIF4AIII, but not eIF4AI/II, functions in NMD, although we cannot rule out the possibility that the small amounts of remaining eIF4AI/II are sufficient for NMD (see Discussion). Notably, the effect of siRNA against eIF4AIII is not caused by an increase in transcription of TCR-β as is evident in the wild type cell line (PTC-) (Fig. 2.1A, compare lane 10 to 13 and 14). Furthermore, the observation that eIF4AI and eIF4AII siRNA fail to inhibit NMD, is not the result of inhibition of transcription by these siRNA (compare lanes 11 and 12 to lanes 13 and 14).

To obtain additional evidence that eIF4AI and eIF4AIII have distinct roles in NMD, we next asked whether either of these proteins associates with spliced mRNA. After splicing, the mature mRNA is packaged into a specific spliced mRNP complex for transport to the cytoplasm (31). To determine whether eIF4AIII and/or eIF4AI are present in the spliced mRNP we performed *in vitro* splicing of AdML pre-mRNA in HeLa nuclear extract followed by immunoprecipitations (Fig. 2.2A, *Left*). Significantly, the spliced mRNA is immunoprecipitated by the eIF4AIII antibody but not by the eIF4AI antibody (Fig. 2.2A, compare lanes 2 and 3). As shown in Fig. 2.2A (R*ight*), eIF4AI and eIF4AIII are present in both nuclear and cytoplasmic extracts. As eIF4AI is a cytoplasmic protein (32-34), this protein, like tubulin (Fig.2.2A, *Right*), is likely to be a contaminant in the nuclear extract. Thus, the failure to immunoprecipitate the mRNA

Figure 2.2. eIF4AIII, but not eIF4AI, associates with the EJC on spliced mRNA. (A) eIF4AIII, but not eIF4AI, is associated with spliced mRNA but not with intronless mRNA *(Left)*. Western analysis showing the presence of eIF4AIII, eIF4AI, and α-tubulin in the nuclear (NE) and cytoplasmic (Cyto) extracts *(Right)*. (B) eIF4AIII associates with the region of the mRNA that contains the EJC. *(Left)* Schematic of AdML mRNA showing the locations of oligonucleotides. The numbers indicate the middle of each 12mer oligonucleotide. (The RNA fragments containing the EJC are designated a, c, and e.) *(Right)* The total reaction after splicing and oligonucleotide-directed RNase H cleavage was used for the immunoprecipitations and is shown in lanes designated input. Immunoprecipitations were carried out with the indicated antibodies after cleavage with each oligonucleotide. The RNA fragments containing the EJC that were immunoprecipitated are indicated (a, c, and e). The RNA fragments that do not contain the EJC are also indicated (b and d). A darker exposure of lanes 9-12 is included in order to detect fragment d (55nt) generated with the +0 oligonucleotide (lane 9). The 13 nt RNA fragment generated with the +36 oligonucleotide is not detected.



A
AdML
Δi-AdML
input
α-eIF4AIII
α-eIF4AI
input
α-eIF4AIII
α-eIF4AI
pre-mRNA-
intron-
mRNA-
1 2 3 4 5 6
NE Cyto
α-eIF4AIII
α-eIF4AI
α-tubulin



B
+36
-48
0
-133
+55
exon 1
exon 2
b
a
c
d
e
No Oligo
-48 Oligo
+0 Oligo
+36 Oligo
input
α-Aly
α-eIF4AI
α-eIF4AIII
pre-mRNA-
intron-
mRNA-
a
b
c
d
e
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

with the eIF4AI antibody is not due to the absence of this protein from the nuclear extract. The eIF4AI antibody is functional for immunoprecipitation assays as determined by immunoprecipitation/Western blot (data not shown). Together, these data indicate that eIF4AIII, but not eIF4AI, is specifically associated with the mRNA.

To determine whether the association of eIF4AIII with the mRNA is splicing dependent, we transcribed a construct that is identical to that encoding AdML pre-mRNA except that it is lacking the intron. This intronless mRNA (Δi-AdML) was then incubated in the nuclear extract and used for immunoprecipitations. Significantly, the Δi-AdML mRNA is immunoprecipitated very poorly by the eIF4AIII or eIF4AI antibody (Fig. 2.2A, lanes 4-6). We conclude that eIF4AIII is specifically associated with mRNA in a splicing-dependent manner. This result is consistent with previous studies showing that eIF4AIII is specifically associated with highly purified spliceosomal complexes (35-38).

Proteins that function in NMD as well as proteins that function in mRNA export are localized on the spliced mRNA to a distinct region located 20-24 nts upstream of the exon-exon junction (12, 13, 15). The proteins that are bound to this region are referred to as the exon-exon junction complex (EJC)(11-14). The EJC proteins were originally localized on the mRNA using a combination of immunoprecipitation and oligonucleotide-directed RNase H cleavage of the mRNA (12). Thus, we next used this approach to determine where eIF4AIII is bound on the mRNA. After *in vitro* splicing, three different 12-mer oligos were used for RNase H digestion (see schematic, Fig. 2.2B). Immunoprecipitations were carried out with antibodies to eIF4AI and eIF4AIII. In addition, we used an antibody to Aly, a known EJC protein (12), as a positive control. In lanes 1-4, immunoprecipitations carried out without RNase H digestion demonstrate that

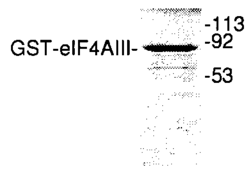
eIF4AIII and Aly antibodies, but not eIF4AI antibodies, immunprecipitate spliced mRNA but not pre-mRNA *in vitro*. The mRNA fragments containing the exon-exon junction, bands labelled as a, c, and e, were generated by digestion with a -48 oligonucleotide (Fig. 2.2B, lanes 5-8), +0 oligonucleotide (Fig. 2.2B, lanes 9-12) and +36 oligonucleotide (Fig. 2.2B, lanes 13-16), respectively. Fragments containing the exon-exon junction region are immunoprecipitated by Aly (Fig. 2.2B, lanes 6, 10, and 14) and eIF4AIII antibodies (Fig. 2.2B, lanes 8, 12 and 16), but not by eIF4AI antibodies (Fig. 2.2B, lanes 7, 11 and 15). The products of the digestion lacking the exon-exon junction (labelled b and d) are immunoprecipitated by neither eIF4AIII nor Aly antibodies. We note that the 3' RNase H digestion products, which lack the 5'cap (e.g fragments a and d), are more readily degraded than the 5' RNase H digestion products, which contain the cap (e.g. fragments b, c and e). The 13nt fragment generated with the +36 oligo is not detected at all, most likely because it is rapidly degraded as it is very small and uncapped. A long exposure of the gel shows that fragment d, which does not contain the EJC, is not immunoprecipitated by any of the antibodies (Fig. 2.2B, lanes 9-12). Together, these results indicate that eIF4AIII, like Aly, is present on the region of the mRNA that contains the EJC.

To determine whether eIF4AIII associates with spliced mRNA *in vivo*, GST-eIF4AIII or buffer alone (Fig. 2.3A) was pre-injected into *Xenopus* oocyte cytoplasm. After incubation of the oocytes, the eIF4AIII was imported into the nucleus (Fig. 2.3B). UAP56, which serves as a marker for the nucleus, is detected in the nuclei of pre-injected oocytes (Fig.2.3B). A mixture of ^{32}P-pre-mRNA, U1 and U6 snRNAs (Fig. 2.3C, lane 1) was then injected into nuclei of the pre-injected oocytes and incubated to allow splicing.

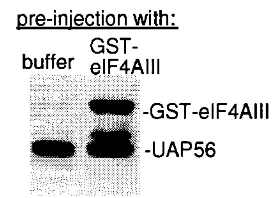
Figure 2.3. eIF4AIII is associated with spliced mRNA *in vivo.*

(A) Coomassie-stained gel showing GST-eIF4AIII used for microinjection into oocyte cytoplasm. (B) Western analysis of *Xenopus* oocyte nuclei after pre-injection showing the presence of UAP56, a known nuclear protein, and recombinant GST-eIF4AIII. (C) Inj indicates the total RNA before injection and I indicates the RNA species present in the nuclear lysates prior to the addition of glutathione beads. Total RNA unbound (U) and bound (B) to the beads is shown.

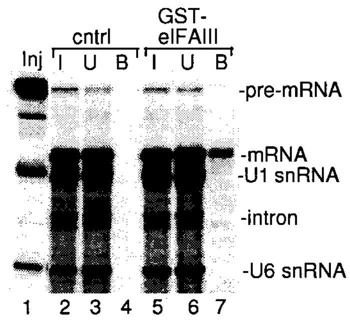
A



B



C

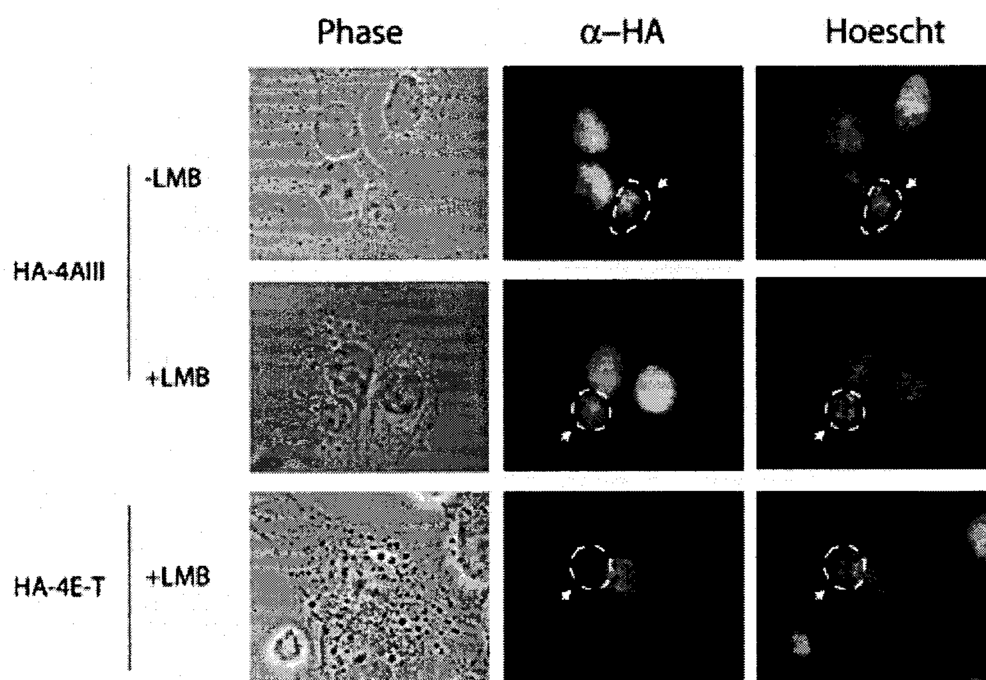


The nuclei were then isolated and the nuclear lysates incubated with glutathione beads. The spliced mRNA, but not the pre-mRNA or snRNAs, is bound to the beads when the oocytes contain GST-eIF4AIII (Fig. 2.3C, lane 7). In contrast, no RNAs bind to the beads in the buffer control (Fig. 2.3C, lane 4) or when other nuclear GST-fusion proteins (GST-Sir2 and GST-ΔC Aly (39)) are pre-injected (data not shown). We conclude that eIF4AIII is specifically associated with spliced mRNA *in vivo*. The observation that eIF4AIII is present in the EJC and is associated with mRNA is consistent with the possibility that eIF4AIII plays a direct role in NMD. Moreover, eIF4AIII is recruited to the mRNA during splicing, while the mRNA is still in the nucleus.

To determine whether eIF4AIII might accompany the mRNA to the cytoplasm together with other EJC components (Y14, magoh, Aly/REF), we used a heterokaryon assay (40), that measures the ability of a protein to shuttle between the nucleus and the cytoplasm. A hemagglutinin-tagged eIF4AIII expression plasmid was transfected into HeLa cells, followed by fusion to murine NIH 3T3 cells. Three hours after fusion, eIF4AIII was detected in most (>95%) of the fused murine nuclei (Fig. 2.4). Incubation with leptomycin B (LMB), which inhibits CRM1-dependent protein export, had no effect on eIF4AIII shuttling (Fig. 2.4) even after overnight incubation (data not shown). In contrast, Leptomycin B abolished export of 4E-T, a protein known to be a substrate of the CRM1 receptor (28). We conclude that eIF4AIII exits the nucleus in a CRM1-independent fashion and thus may accompany the mRNA via the TAP-mediated export pathway (41-43). The observation that eIF4AIII shuttles, suggests that it can conceivably participate in NMD in the cytoplasm and/or nucleus (33, 44, 45).

Figure 2.4. eIF4AIII is a shuttling protein.

eIF4AIII and 4E-T localization was detected by staining with anti-HA antibody (1/1000 dilution, Covance/Babco,) and Texas red-conjugated anti–mouse IgG (Molecular Probes). Human and mouse nuclei are differentiated by staining with Hoechst dye 33258 (Sigma). The mouse nucleus is indicated by an arrow. Leptomycin B (LMB, Sigma) treatments were performed at 5ng/ml final for 5h. Images were processed with a *Nikon Eclipse E800* microscope at 60X magnification.



Phase
α–HA
Hoescht
-LMB
HA-4AIII
+LMB
HA-4E-T
+LMB

2.4 Discussion

Here we have shown that RNAi of eIF4AIII, a protein with striking similarity to the well-known cytoplasmic initiation factors eIF4AI/II, inhibits NMD. In contrast, RNAi of eIF4AI/II had no effect on NMD, suggesting that these proteins do not have a function in NMD. Although we cannot rule out the possibility that small amounts of eIF4AI/II are sufficient for NMD, we also show that eIF4AIII, but not eIF4AI, is associated with the EJC, a complex known to function in NMD. Thus, both the RNAi data and the biochemical studies support a role for eIF4AIII and not its close homolog eIF4AI, in NMD. We note that eIF4AI knockdown effectively inhibits translation (>80%) of poliovirus, which is confined to the cytoplasm and is more dependent on eIF4AI for translation than other viral and cellular mRNAs (46, 47) (see Appendix Fig. A1). Thus, eIF4AI knockdown affects its function in cytoplasmic translation.

The early recruitment of eIF4AIII to the spliced mRNA raises the possibility that the protein functions in an initial step of NMD. It was previously proposed that NMD occurs during the pioneer round of translation (27). Considering (*i*) the structural and functional similarities between eIF4AIII and eIF4AI/II, i.e. its interaction with the middle domain of eIF4G1 and its helicase activity stimulated by eIF4B, (*ii*) the essential role of eIF4AIII in NMD, (*iii*) the presence of eIF4AIII at the EJC (our results; (30, 34, 48)) and the recruitment of eIF4AIII to the mRNA while it is still in the nucleus, it is plausible that eIF4AIII is required for NMD because it substitutes for eIF4AI/II in the pioneer round of translation. Another, but not mutually exclusive possibility, is that eIF4AIII functions as a structural component of the EJC and that its helicase activity is required for remodeling the components of the EJC. This may be necessary for its positioning, likely through

Upf2 and Upf3, in proximity to the ribosome and its associated factors to communicate the site of the PTC. In a previous study, Upf2, was shown to interact with eIF4AI (49). It is possible that this interaction occurs during a later step of NMD or functions in cytoplasmic NMD, as this interaction was shown in yeast, where NMD appears to be a purely cytoplasmic process.

It is still a matter of debate whether the first round of translation takes place in the nucleus or the cytoplasm. Given that eIF4AIII is a nucleocytoplasmic shuttling factor, it can conceivably function in either compartment. It was reported earlier that the nuclear cap binding complex, nCBC, substitutes for the cytoplasmic cap-binding protein, eIF4E, in the pioneer round of translation (27). Other potential translation initiation factors that could function uniquely in NMD include PABP2 and an eIF4G-like protein (35, 36). Similar to eIF4AIII, these proteins are present in the nucleus and are specifically recruited to spliceosomes (35, 36). eIF4G-like contains a region which is similar to the domain in eIF4G that interacts with eIF4AIII (4). By analogy to the cytoplasmic initiation complex, eIF4G-like may interact with both eIF4AIII and nCBC. The cytoplasmic translation initiation factor PABP1 interacts with eIF4G and the poly(A) tail, and PABP2 is a candidate for this protein in the nuclear initiation complex. Together, these observations raise the possibility that NMD involves a set of previously uncharacterized translation initiation factors that are loaded onto the mRNA early in its biogenesis in the nucleus.

2.5 Materials and Methods

2.5.1 siRNA Transfections

siRNA transfections were performed in HeLa cells using Lipofectamine Plus Reagent (Invitrogen). One day prior to siRNA transfection cells were trypsinized to achieve 50-60% confluency. For a 6-well plate, 15µl of siRNA duplex (20µM annealed duplex from Dharmacon) was mixed with 100ul OPTI-MEM medium and 10µl Plus Reagent and incubated for 15 minutes at room temperature. In a separate tube, 20µl Lipofectamine (Invitrogen) was added to 100µl OPTI-MEM. Lipofectamine mix was added to pre-complexed RNA mix and incubated for an additional 15 min. RNA and protein were harvested 48 hours post-transfection for use in Northern and Western blots, respectively. For double knockdown of eIF4AI and eIF4AII, eIF4AII siRNA was transfected using conditions described above, 12 hours later eIF4AI siRNA was transfected in the same manner and cells were collected 60 hours post-transfection.

2.5.2 Western Blot

Cells were washed twice and scraped in ice-cold PBS and pelleted at maximum speed for 10 minutes in a microcentrifuge. Cell pellets were frozen on dry ice and thawed in RIPA buffer (150mM NaCl/50mM Tris-HCl pH 7.5/1% NP-40/0.5% sodium deoxycholate/0.1% SDS and a cocktail of protease inhibitors [Roche Diagnostics]), incubated on ice for 10 min and centrifuged for 5 min at maximum speed to recover the supernatant. The amount of protein loaded is indicated in the figure legends. Proteins were separated on a SDS/10% PAGE , transferred to a nitrocellulose membrane

(0.22uM), blocked overnight in 5% non-fat milk, and incubated with antibodies (anti-eIF4AIII (4), anti-eIF4AI (4), and anti-eIF4AII (50)) prepared in 1%BSA/PBST (0.5% Tween) at a dilution of 1/1000 (except for 4AII which was used at 1/500 dilution) for 1h at room temperature, washed four times for 15 minutes with PBST (0.5% Tween), and incubated with secondary antibody (1/5000) for 1h at room temperature.

2.5.3 NMD Assay

Cells stably expressing TCR-β wt (3C1) and TCR-β containing a PTC (7C3) were a generous gift from Miles Wilkinson and Melissa Moore(51). Total RNA was isolated using Qiagen (Valencia, CA) RNAeasy kit according to the manufacturer's instructions. For Northern blots, 3ug of total RNA was separated on a 1.3% agarose/formaldehyde gel, transferred to a Hybond-N membrane (Amersham Pharmacia) and probed with ^{32}P-labeled, random-primed DNA probes: TCR-β V_{β}8.1 and human actin probes were prepared from plasmids β117 and β290, respectively(23, 52). Cycloheximide treatment was performed at a final concentration of 100ug/ml for the indicated amount of time.

2.5.4 Immunoprecipitation of Spliced mRNA and Oocyte Injections

Oligonucleotide-directed RNase H cleavage and immunoprecipiation was performed essentially as described(12). Briefly, endogenous RNase H digestion was accomplished by adding each cDNA oligo to a final concentration of 0.5μM to *in vitro* splicing reactions and incubating the reaction for 10 min at 30°C. For no oligo controls, equivalent volumes of TE buffer was added to the splicing reactions. Immunoprecipitated RNAs were recovered by phenol/chloroform extraction and ethanol

precipitation. RNA species were analyzed on 15% denaturing polyacrylamide gels and visualized by Molecular Dynamics PhosphorImager. Oocyte microinjection was carried out according to Luo. et al(31). The plasmid encoding GST-eIF4AIII was constructed by cloning the cDNA encoding human eIF4AIII protein into protein expression vector pGEX5 at *Bam*HI and *Xho*I sites. GST pull-down of the in vivo complexes was done as described (53).

2.5.6 Heterokaryon Assay

HeLa and NIH 3T3 fusions were performed as previously described(54). HA-tagged eIF4AIII and 4E-T were previously reported(4, 28) and were transfected into HeLa cells by the calcium phosphate method. Cells were treated with 5ng/ml LMB (Sigma) 3h before cell fusion. Cells were subsequently washed with PBS 1X and cell fusions were formed by incubation for 2 min in 50% polyethylene glycol (PEG) 8000 in PBS. Cells were washed extensively with PBS and returned to fresh media containing 100ug/ml cycloheximide and 5ng/ml leptomycin B. After 2h, cells were fixed and processed for immunofluorescence as described previously(28).

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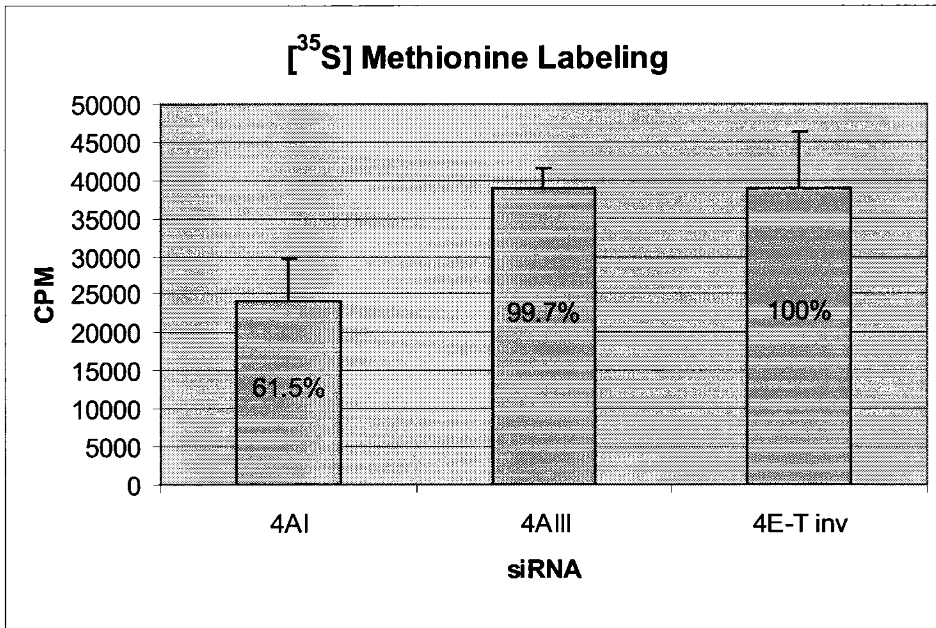
APPENDIX (TO CHAPTER 2)

Figure A1. Effect of eIF4A RNAi on cellular translation measured by ^{35}S-methionine incorporation into TCA precipitable material.

(A) siRNA treatment was performed as described in Materials and Methods (Chapter 2). Forty-eight hours later cells were labeled with ^{35}S-methionine for 60 minutes and assayed for incorporation of ^{35}S-methionine into acid-insoluble material in 5ul-aliquots of translation samples (Schoenberg, D.R., mRNA Processing and Metabolism, *Methods and Protocols*, Svitkin and Sonenberg, Chapter 13, Section 3.8). Radioactivity was determined by liquid scintillation counting and normalized to actin levels by NIH *Image* software. The result is an average of three independent experiments. 4E-T inv = negative control. (B) Knockdown efficiency by Western of eIF4AI and eIF4AIII in two independent experiments.

A



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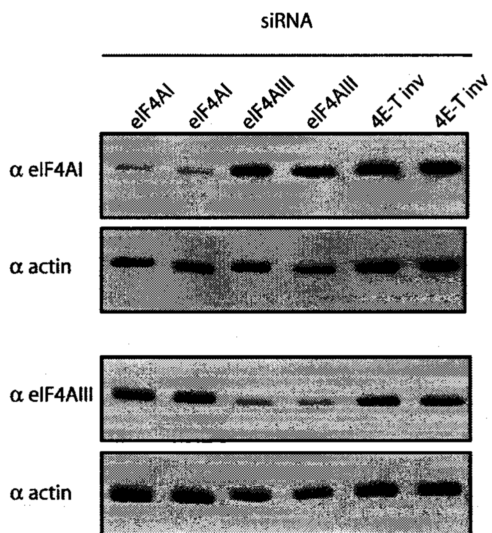
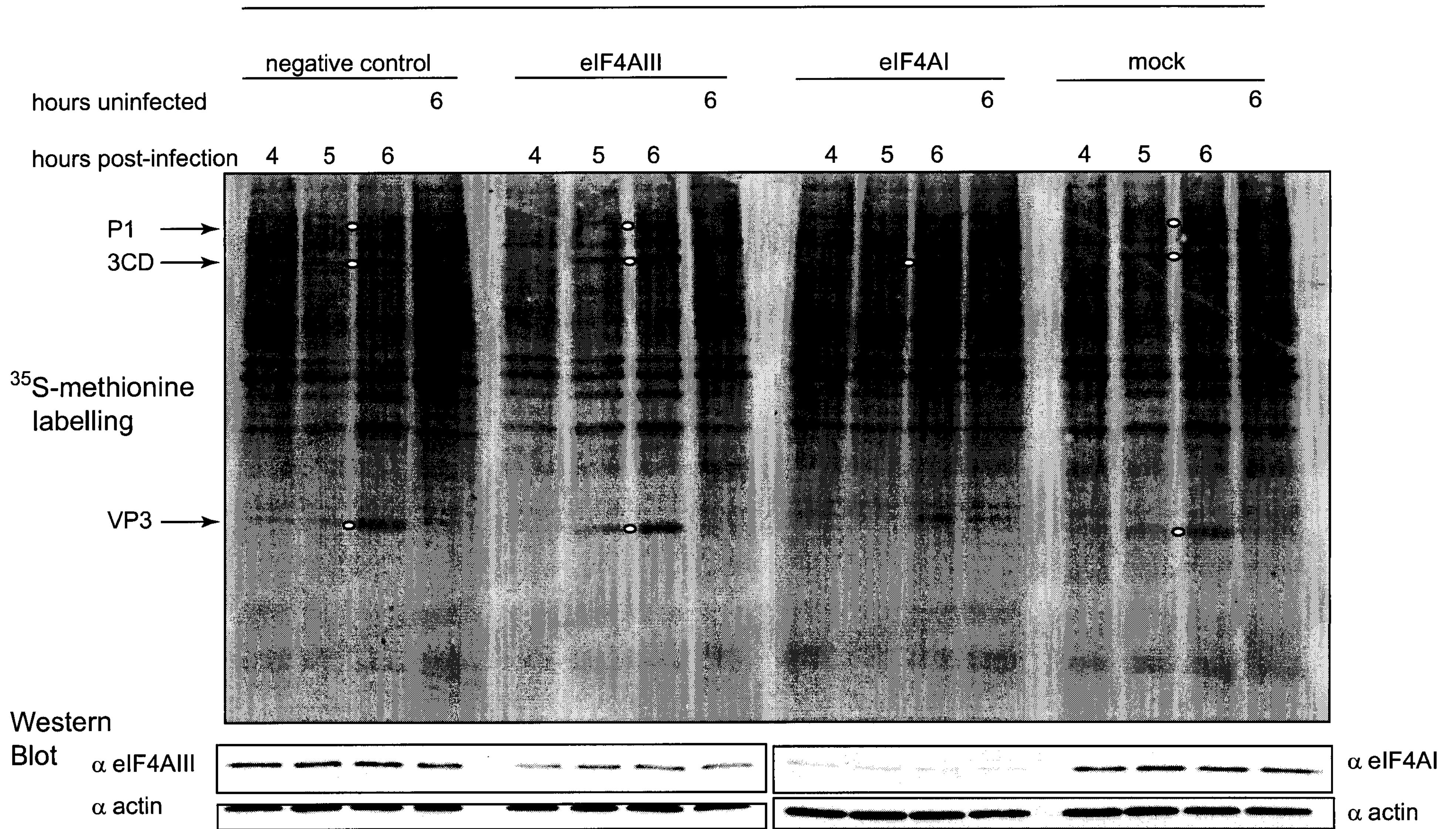


Figure A2. Effect of eIF4A RNAi on poliovirus translation.

HeLa CCL2 were transfected with siRNA in a 24-well plate with lipofectamine plus reagent (see Chapter 2, Materials and Methods). 48h later, cells were infected with the Mahoney strain of poliovirus type 1 at a multiplicity of infection (M.O.I) of 5 plaque-forming units (pfu) per cell. After virus adsorption at room temperature for 30 min, cells were further incubated at 37°C. At the indicated time points after infection, the medium was replaced with media containing 10μCi/mL [^{35}S]-methionine. After further incubation for 30 minutes at 37°C, the cells were washed once in PBS and lysed in 1X laemmli-sample buffer. Uninfected cells were radiolabelled and collected at 6 hours. Twenty microliters of cell lysate was resolved by 15% SDS-PAGE, transferred to a nitrocellulose membrane and subjected to autoradiography. Viral proteins are indicated by ellipsoids. The membrane was subsequently subjected to Western analysis with anti-eIF4AI and eIF-4AIII antibodies. β-Actin served as a loading control.


siRNA
negative control
eIF4AIII
eIF4AI
mock
hours uninfected
6
6
6
6
hours post-infection
4 5 6
4 5 6
4 5 6
4 5 6
P1
3CD
35S-methionine labelling
VP3
Western Blot
α eIF4AIII
α actin
α eIF4AI
α actin

CONNECTING TEXT

Studies performed in yeast *S. cervisiae* unequivocally demonstrated that mRNA can be degraded in distinct sites within the cytoplasm. mRNA decay enzymes and decapping factors involved in the 5' to 3' decay pathway, as well as mRNA decay intermediates, were stained in specific sites of the cytoplasm in yeast, which were called processing bodies (P-bodies). We previously reported that 4E-T is a nucleocytoplasmic shuttling protein which imports eIF4E to the nucleus. At steady state, 4E-T is predominantly localized in the cytoplasm with a prominent staining in discrete cytoplasmic foci. The nature of these foci, at the time, was unbeknownst to us. The resemblance of 4E-T staining to that seen with decapping factors in yeast P-bodies, and the resemblance of P-bodies to particles in other organisms where repressed mRNAs gather, prompted our study of the role of 4E-T in translation repression and mRNA decay. In Chapter 3, we show that 4E-T is a component of mammalian P-bodies, as well as is its binding partner, eIF4E. We demonstrate that a 4E-T/eIF4E interaction results in the inhibition of cap-dependent translation, that 4E-T is essential to the formation of P-bodies and is required for mRNA turnover. In summary, we describe a distinct function of 4E-T from its role in the nuclear import of eIF4E, which may contribute to translational homeostasis.

CHAPTER 3

A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay

3.1 Abstract

4E-Transporter (4E-T) is one of several proteins which bind the mRNA 5'cap-binding protein, eukaryotic initiation factor 4E (eIF4E), through a conserved binding motif. We previously showed that 4E-T is a nucleocytoplasmic shuttling protein, which mediates the import of eIF4E into the nucleus. At steady state, 4E-T is predominantly cytoplasmic and is concentrated in bodies that conspicuously resemble the recently described Processing bodies (P-bodies), which are believed to be sites of mRNA decay. In this report, we demonstrate that 4E-T colocalizes with mRNA decapping factors in *bona fide* P-bodies. Moreover, 4E-T controls mRNA half life, because its depletion from cells using short interfering RNA (siRNA) increases mRNA stability. The 4E-T binding partner, eIF4E, also is localized in P- bodies. 4E-T interaction with eIF4E represses translation, which is believed to be a prerequisite for targeting of mRNAs to P-bodies. Collectively, these data suggest that 4E-T interaction with eIF4E is a priming event in inducing mRNP rearrangement and transition from translation to decay.

3.2 Introduction

The regulation of mRNA biogenesis and decay plays an important role in the control of gene expression. mRNA decay regulates protein levels, eliminates aberrant mRNAs, and is an essential host defense mechanism during viral infection (Coller and Parker, 2004). There are 3 major mRNA decay pathways (Coller and Parker, 2004; Parker and Song, 2004). The general pathway involves deadenylation followed by decapping of the mRNA. In yeast, transcripts are primarily degraded in a 5' to 3'direction by the Xrn1 exonuclease (Coller and Parker, 2004; Muhlrad et al., 1995; Parker and Song, 2004; Schwartz and Parker, 1999), whereas in higher eukaryotes, mRNAs mostly are eliminated in a 3' to 5' fashion by the exosome after deadenylation (Chen et al., 2001; Hilleren et al., 2001; Mukherjee et al., 2002; Tourriere et al., 2002; Wang and Kiledjian, 2001; Wilusz et al., 2001). mRNA degradation also can occur through a second pathway which involves endonucleolytic cleavage of mRNAs (Schoenberg and Chernokalskaya, 1997), and in addition, through specialized mechanisms, including nonsense-mediated decay and nonstop decay (For reviews see (Coller and Parker, 2004; Parker and Song, 2004; Tourriere et al., 2002; Wilusz et al., 2001).

mRNA decapping is a crucial step in general and specialized mRNA decay (Parker and Song, 2004). Decapping is mediated by a heterodimeric complex composed of Dcp1 and Dcp2 (Beelman et al., 1996; Dunckley and Parker, 1999; Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002). Numerous factors that regulate decapping activity have been identified in yeast and mammals. The Lsm 1-7 complex, Pat1p, Dhh1p, Edc1p, Edc2 and Edc3 are positive regulators of decapping, characterized in yeast (Coller and Parker, 2004; Parker and Song, 2004).

Most mammalian mRNAs studied are the target of degradation by the 3'-5'exosome (Chen et al., 2001; Mukherjee et al., 2002; Wang and Kiledjian, 2001). Nonetheless, decapping activity similar in function to the yeast Dcp1p has been detected in HeLa cells (Gao et al., 2001) and mammalian homologues of Dcp1/2 (Lykke-Andersen, 2002; Wang et al., 2002), the Lsm proteins (Achsel et al., 1999), and Dhh1p (Smillie and Sommerville, 2002) also exist. Furthermore, Xrn1 homologs have been identified in mammals (Bashkirov et al., 1997; Ingelfinger et al., 2002). However, it has not been demonstrated unequivocally whether deadenylated-decapped mRNAs are subject to 5'or 3'exonucleolytic decay in mammalian cells *in vivo* (discussed in (Mukherjee et al., 2002; Wang and Kiledjian, 2001; Wilusz and Wilusz, 2004).

The mRNA 5' cap structure and the 3' polyA tail play important roles in the decapping process (Coller and Parker, 2004; Wilusz et al., 2001). The polyA inhibits decapping, likely through the polyA binding protein (PABP) interaction with eIF4G, a component of the eIF4F cap-binding complex (Coller and Parker, 2004). eIF4F also contains the cap binding protein, eukaryotic initiation factor 4E (eIF4E), and the RNA helicase, eIF4A (Gingras et al., 1999). The interaction of PABP with eIF4G enhances the binding of eIF4F to the mRNA (Kahvejian et al., 2005), and thus, hinders the access of the decapping complex to the cap. Consequently, translation and decapping are antagonistic. Consistent with this idea, mutations in eIF4E and eIF4G can lead to an increase in decapping in yeast (Schwartz and Parker, 1999). Importantly, decapping requires the removal of eIF4F from the 5'cap structure, which, in turn, necessitates a transition from a translationally active mRNP to one destined for decay. This transition entails the replacement of initiation factors on the mRNA with decapping factors (Coller

and Parker, 2004; Parker and Song, 2004; Teixeira et al., 2005; Tharun and Parker, 2001). The switch from translation to decay occurs concomitantly with aggregation of mRNPs into distinct cytoplasmic foci, termed processing bodies (P-bodies) in yeast (Sheth and Parker, 2003), or GW bodies (Eystathioy et al., 2003) or Dcp1a bodies in mammals (Cougot et al., 2004); these are referred to as P-bodies throughout this article. The decapping enzyme, Dcp1/2, and decapping associated factors, Lsm1-7, Dhh1p/p54 and Xrn1, are concentrated in these foci (Cougot et al., 2004; Ingelfinger et al., 2002; Liu et al., 2004; Sheth and Parker, 2003; van Dijk et al., 2002). However, little is known about the recruitment mechanism of mRNAs to P-bodies, or how initiation factors are exchanged with decapping enzymes.

We reported earlier the cloning of 4E-Transporter (4E-T), which interacts with eIF4E through a conserved eIF4E recognition motif (YXXXXLΦ, where Φ is a hydrophobic amino acid) that is found also in eIF4G and 4E-BP. 4E-T is a nucleocytoplasmic shuttling protein that mediates the nuclear import of eIF4E through the importin α/β pathway. At steady state, 4E-T is predominantly cytoplasmic and is concentrated in bodies that conspicuously resemble the recently described P-bodies (Dostie et al., 2000a; Sheth and Parker, 2003). Here, we report that 4E-T colocalizes with eIF4E and decapping factors in *bona fide* P-bodies and controls mRNA turnover. Significantly, depletion of 4E-T from cells enhances mRNA stability.

3.3 Results

3.3.1 4E-T colocalizes with decapping factors

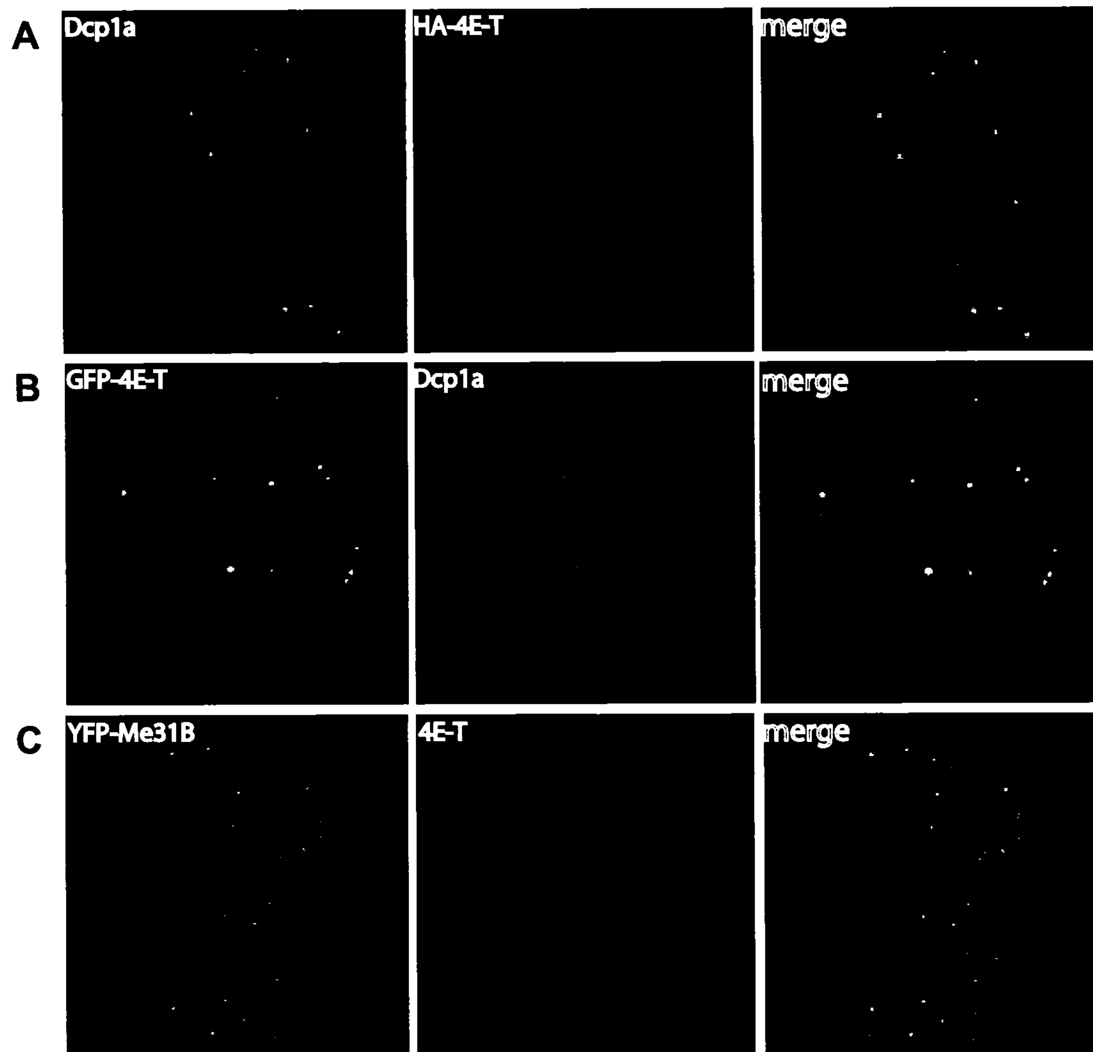
To determine whether 4E-T associates with P-bodies, the localization of 4E-T and P-body components was analyzed by immunofluorescence in HeLa cells. Cells transfected with HA-4E-T were analyzed by double-label immunofluorescence microscopy using anti-HA antibody and an affinity-purified anti-Dcp1a antibody that recognizes the endogenous protein (see Materials and Methods). Transfected HA-4E-T and endogenous Dcp1a colocalized in the cytoplasm in discrete foci that strongly resemble P-bodies (Fig. 3.1 A). Similar results were obtained with a GFP- tagged 4E-T (Fig. 3.1 B). To show that endogenous 4E-T also localizes to P-bodies, its colocalization with Me31B was studied. [Me31B is the *Drosophila* homolog of the human decapping factor Dhh1p/p54, and similar to Dcp1a, is an established marker of P-bodies (Sheth and Parker, 2003; Tseng-Rogenski et al., 2003). Me31B shares 71% identity with Dhh1p/p54 and was shown to form cytoplasmic granules in germline cells (Nakamura et al., 2001)]. HeLa cells transfected with myc-EYFP-Me31B were stained with an affinity purified anti-4E-T antibody, and analyzed by microscopy. Endogenous 4E-T colocalizes with myc-EYFP-Me31B in discrete cytoplasmic foci (Fig. 3.1 C). Thus, endogenous and transfected 4E-T colocalize with *bona fide* decapping factors in cytoplasmic foci, which fulfil the criteria of P-bodies.

3.3.2 4E-T downregulates cap-dependent translation

In the yeast *Saccharomyces cerevisiae*, targeting of mRNAs to P-bodies is believed to occur as a consequence of cessation of translation (Schwartz and Parker, 1999; Teixeira

Figure 3.1. 4E-T colocalizes with decapping factors in P-bodies.

(A and B) Transfected 4E-T colocalizes with endogenous Dcp1a. HeLa cells transfected with HA-4E-T (A) or GFP-4E-T (B) were stained with anti-Dcp1a to visualize endogenous Dcp1a. The localization of HA-4E-T was determined by indirect immunofluorescence with anti-HA (Covance) and Alexa Fluor 594 anti-mouse IgG. Dcp1a was revealed with anti-Dcp1a and either Alexa Fluor 488 anti-rabbit IgG (A) or Alexa Fluor 594 anti-mouse IgG (B). The colocalization of HA-4E-T or GFP-4E-T with Dcp1a appears yellow (right). (C) Endogenous 4E-T colocalizes with transfected myc-EYFP-Me31B. HeLa cells were transfected with myc-YFP-Me31B and colocalization with endogenous 4E-T was performed with anti-4E-T and Texas-Red conjugated anti-rabbit IgG. Colocalization appears yellow (right).

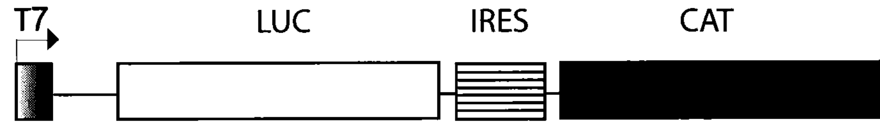

A
Dcp1a
HA-4E-T
merge
B
GFP-4E-T
Dcp1a
merge
C
YFP-Me31B
4E-T
merge

et al., 2005; Tharun and Parker, 2001). Thus, it is conceivable that changes in mRNP composition that lead to translational arrest result in targeting the mRNA to P-bodies in mammals. 4E-T interacts with eIF4E through a conserved binding site, which is also found in eIF4G (Mader et al., 1995), and thus might promote targeting of mRNAs to P-bodies by inhibiting translation initiation. To address this possibility, the inhibitory effect of 4E-T on translation was examined *in vivo*. HeLa cells infected with recombinant vaccinia virus vTF7-3 (to synthesize the T7 RNA polymerase in the cytoplasm) were co-transfected transiently with plasmid DNA containing a 4E-T cDNA and a bicistronic reporter construct expressed from a T7 promoter. The bicistronic reporter construct consists of the luciferase (LUC) and chloramphenicol acetyl transferase (CAT) cistrons, separated by the poliovirus internal ribosome entry site (IRES) (Pause et al., 1994) (Fig. 3.2 A). Translation of the LUC cistron proceeds in a cap-dependent manner, whereas translation of the CAT cistron is cap-independent and serves as a surrogate control for mRNA levels. [Note that quantification of the RNA by Northern blotting could not be performed because the vaccinia virus T7 system produces heterogenous RNA transcripts, generating smeared bands (Fuerst and Moss, 1989). This is attributed to the lack of an authentic T7 RNA polymerase termination sequence. However, this has no deleterious effect on mRNA turnover or translation (Fuerst and Moss, 1989)]. Only a fraction (5-10%) of T7 transcribed RNAs are capped by the vaccinia capping enzyme (Fuerst and Moss, 1989). However, uncapped RNA is translated inefficiently both *in vivo* and *in vitro* (Both et al., 1975; Fuerst and Moss, 1989; Peng and Schoenberg, 2005; Shatkin, 1976; Sonenberg et al., 1980), and unpublished data). Thus, even if only a small fraction of the T7 transcribed RNAs are capped in the T7 vaccinia virus system, the uncapped

Figure 3.2. 4E-T inhibits cap-dependent translation *in vivo.*

(A) Schematic presentation of the bicistronic reporter plasmid (pGEM-LUC-POLIRES-CAT). HeLa cells were infected with the vaccinia virus vTF7-3, and transiently transfected with the reporter plasmid and pcDNA3-4E-T constructs. (B, bottom panel) Western blot analysis of the transfected cells was performed with anti-4E-T antibody. *Lane 1*, pcDNA3; *lane* 2, pcDNA3-4E-T; *lane* 3, pcDNA3-4E-T–Y30A. The interaction of the wt and mutant 4E-T protein with eIF4E was examined by Far-Western analysis (top panel) using a [^{32}P]-labeled eIF4E probe. (C) LUC and CAT activity were measured 16 h after transfection. The LUC/CAT activity ratio is expressed as a percentage of the control (pcDNA3) set at 100%. The experiments were performed four times in duplicate.

A



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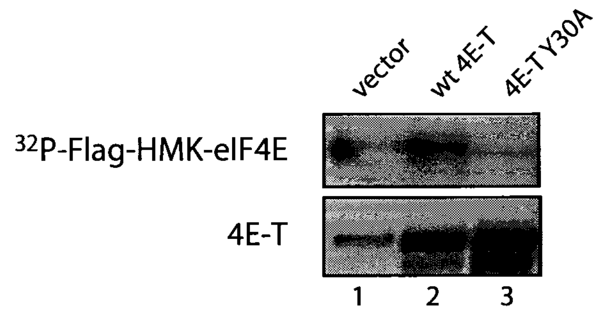

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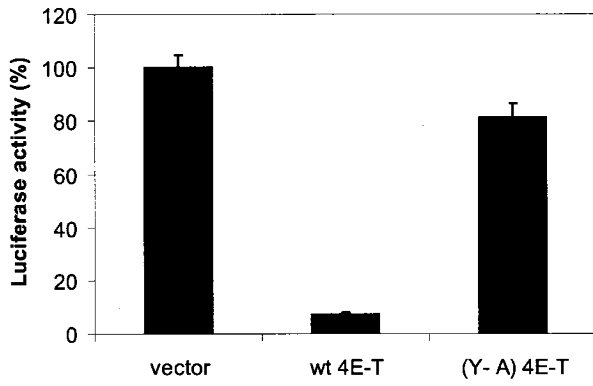
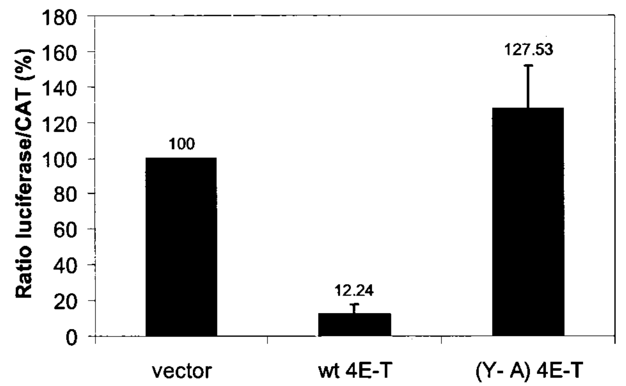
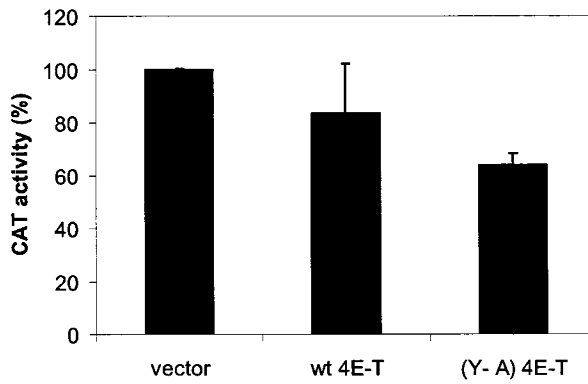






products largely are untranslated (~0.4-1% of uncapped compared to capped RNA: unpublished data). All constructs were expressed to similar levels (Fig.3.2 B, bottom panel, compare lanes 2 and 3). The binding of 4E-T to eIF4E was demonstrated by Far-Western blotting with [^{32}P]HMK-eIF4E (Fig. 3.2 B, lane 2, top panel). The 4E-T mutant, Y30A, which is mutated in the YXXXXLΦ motif, did not bind to eIF4E, as expected (Fig. 3.2 B, lane 3, top panel). LUC expression was decreased significantly by overexpression of wild type 4E-T (Fig. 3.2 C, left, top panel), whereas CAT activity remained relatively unchanged (Fig. 3.2 C, left, bottom panel). In contrast, the Y30A mutant of 4E-T did not affect LUC or CAT expression. Therefore, overexpression of wild type 4E-T, but not the mutant Y30A, strongly inhibited (88%) translation (Fig. 3.2 C). In conclusion, overexpression of 4E-T in HeLa cells results in the repression of translation only when bound to eIF4E.

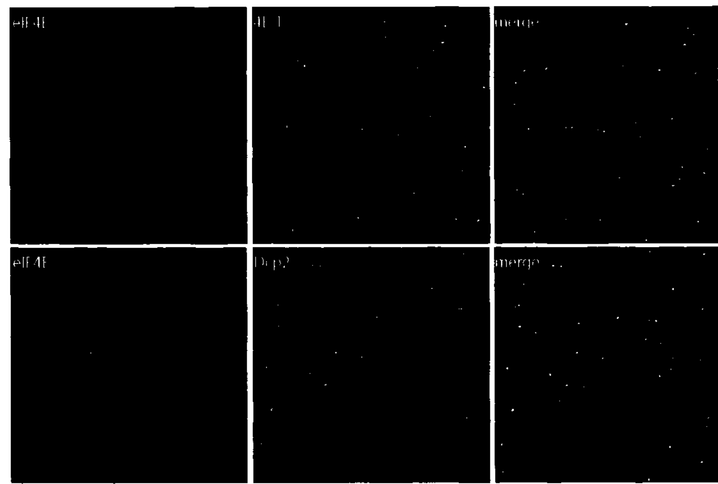
3.3.3 eIF4E localizes to P-bodies

Because 4E-T localizes to P-bodies, and because it interacts with eIF4E, it was pertinent to ask whether eIF4E also localizes to P-bodies, and whether this localization requires 4E-T. To this end, we performed immunofluorescence analysis of endogenous eIF4E in HeLa cells using a monoclonal antibody, which recognizes eIF4E in the cytoplasm (Kimball et al., 2003). Although eIF4E is distributed throughout the cytoplasm, it is concentrated in foci reminiscent of P-bodies (Fig. 3.3 A). This staining pattern is observed in two different HeLa cell sublines: HeLa CCL2 and HeLa S3 (Carter et al., 1995), but with qualitative differences (Fig. 3.3 A and B-E, respectively), and in mouse

Figure 3.3. eIF4E colocalizes with 4E-T, Dcp1a and Dcp2 in P-bodies.

(A) HeLa cells (CCL2) were fixed and the localization of eIF4E was determined by indirect immunofluorescence with mouse anti-eIF4E monoclonal antibody and Alexa Fluor 594 anti-mouse IgG. The localization of 4E-T was examined with anti-4E-T and that of Dcp2 with anti-Dcp2 and Alexa Fluor 488 anti-rabbit IgG. The colocalization of these factors appears yellow in the merged image. (B) The colocalization of endogenous eIF4E with 4E-T and Dcp1a was examined with anti-4E-T and anti-Dcp1a in HeLa S3 as above. The left panel demonstrates endogenous eIF4E localization in HeLa S3. Zoomed images (right panel) display colocalization of endogenous eIF4E and Dcp1a in P-bodies. Some of the more distinct P-bodies are indicated by arrowheads. (C) HeLa cells were transfected with GFP-4E-T and the staining of endogenous eIF4E was performed with anti-eIF4E antibody as described above. (D) The intensity of fluorescence of endogenous eIF4E in P-bodies from GFP-4E-T-transfected cells (from panel C) was compared against the intensity of fluorescence of eIF4E from nontransfected cells along the path which is indicated by the arrow. The P-bodies along the path of the arrow are circled in the adjacent panel for comparison for better visibility. The signal was allowed to bleach before quantification in order to avoid a saturated signal. The graphs plot the intensity of fluorescence against the distance (μm) traversed by the arrow. (E) HeLa cells transfected with GFP-4E-T were incubated with Alexa Fluor 594 anti-mouse IgG (M-AF594) without prior incubation with anti-eIF4E antibody to demonstrate the integrity of the green filter.

A



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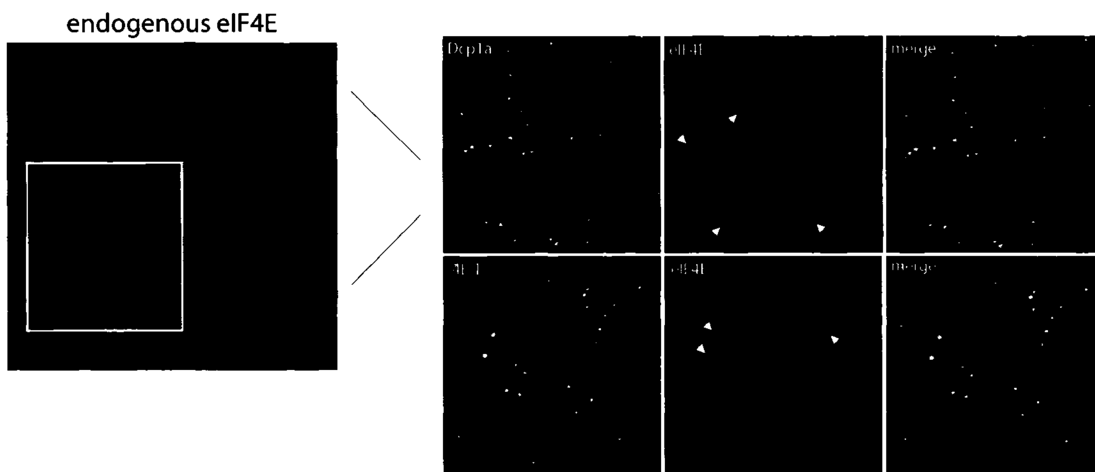

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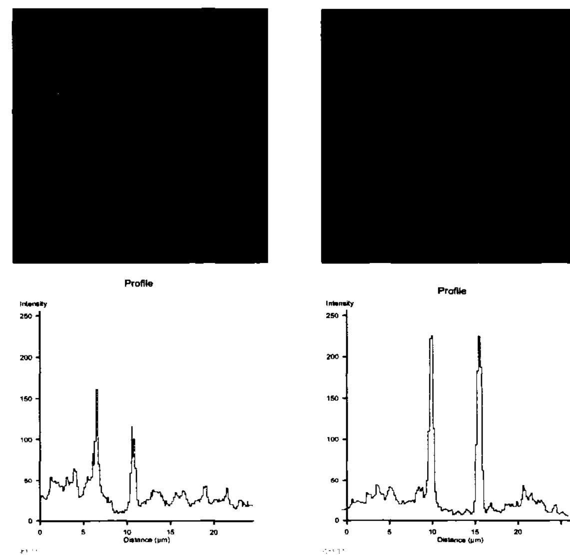

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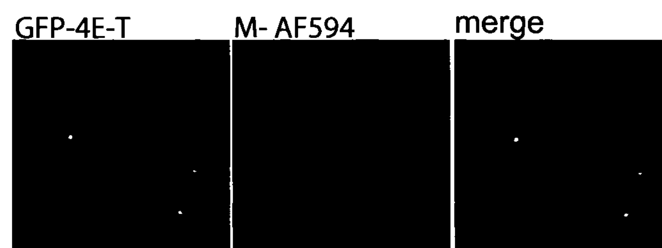


embryo fibroblasts (not depicted). Furthermore, transiently transfected HA-eIF4E also displays a similar cytoplasmic distribution pattern (see below, Fig. 3.4 A).

Double-label immunofluorescence analysis shows that eIF4E colocalized with 4E-T and Dcp2 in P-bodies (Fig. 3.3 A). eIF4E exhibited a more pronounced association with P-bodies in CCL2 (Fig. 3.3 A) as compared to HeLa S3 (Fig. 3.3 B). eIF4E colocalized with Dcp1a and 4E-T in P-bodies, albeit staining of eIF4E in these structures is not as apparent as the staining of 4E-T and Dcp1a in HeLa S3 (Fig. 3.3 B). Strikingly, overexpression of GFP-4E-T resulted in enhanced staining of eIF4E in P-bodies (Fig. 3.3 C). Because localization of eIF4E in P-bodies was more prominent, and the foci appeared larger and brighter in GFP-4E-T overexpressing cells than in parental cells, these data suggest that 4E-T might tether eIF4E to P-body structures. This effect is illustrated in a graph plotting the intensity of the eIF4E signal (red channel) in two P-bodies marked by an arrow in cells overexpressing 4E-T levels (Fig. 3 D, right panel) and in cells expressing endogenous 4E-T levels (Fig. 3.3 D, left panel; the respective P-bodies traversed by the arrow are circled in the adjacent panel for comparison). The signal intensity of eIF4E was approximately two-fold higher in cells overexpressing 4E-T as compared with endogenous 4E-T levels, when P-bodies of weaker intensity were selected from cells overexpressing 4E-T to avoid signal saturation of the plot. Similar results were obtained with a monoclonal antibody against eIF4E from Transduction Laboratories (610270), and with a monoclonal antibody (10C6), which recognizes eIF4E predominantly in the nucleus (Dostie et al., 2000b; Lejbkowicz et al., 1992); unpublished data). The enhanced eIF4E signal is not due to leakage of the FITC signal, since no

significant signal was detected in the red channel when cells transfected with GFP-4E-T were stained with the secondary antibody alone (Fig. 3.3 E).

To determine whether the association of eIF4E with P-bodies requires its interaction with 4E-T, we performed immunofluorescence analysis using wild type HA-eIF4E and a mutant lacking the 4E-T binding site (eIF4E W73A) (Dostie et al., 2000a). Low eIF4E cDNA amounts were transfected transiently into HeLa cells to obtain a predominantly cytoplasmic (Dostie et al., 2000a) and P-body localization (Fig.3.4 A). Cytoplasmic eIF4E levels in HA-eIF4E-expressing cells were similar to endogenous levels. However, a more pronounced cytoplasmic foci staining was observed in ~ 40% of the transfected cells, presumably because more eIF4E is expressed in these cells. In contrast, HA-eIF4E W73A did not localize to P-bodies, and was distributed uniformly between the cytoplasm and the nucleus (Fig. 3.4 A). Therefore, loss of 4E-T interaction [and also interaction with eIF4G and 4E-BPs (Mader et al., 1995)] leads to deregulated cellular localization, which results in diffuse staining throughout the cell. In addition, because the HA-4E-T (Y30A) mutant, which is defective in eIF4E binding (Dostie et al., 2000a), still localizes to P-bodies, these results suggest that 4E-T is required for the association of eIF4E with P-bodies, but not *vice versa* (see also Fig. 3.5 C).

Because eIF4E is part of the heterotrimeric eIF4F complex, it was important to determine whether eIF4A and eIF4G also localize to P-bodies. Because 4E-T and eIF4G are expected to compete for binding to eIF4E, no other eIF4F complex components - with the exception of eIF4E - were expected to localize to P- bodies. To investigate this, the localization of endogenous eIF4GI and eIF4A was studied in HeLa cells transfected with GFP-Dcp1b. Western blot analysis with anti-eIF4A, which recognizes both eIF4AI and

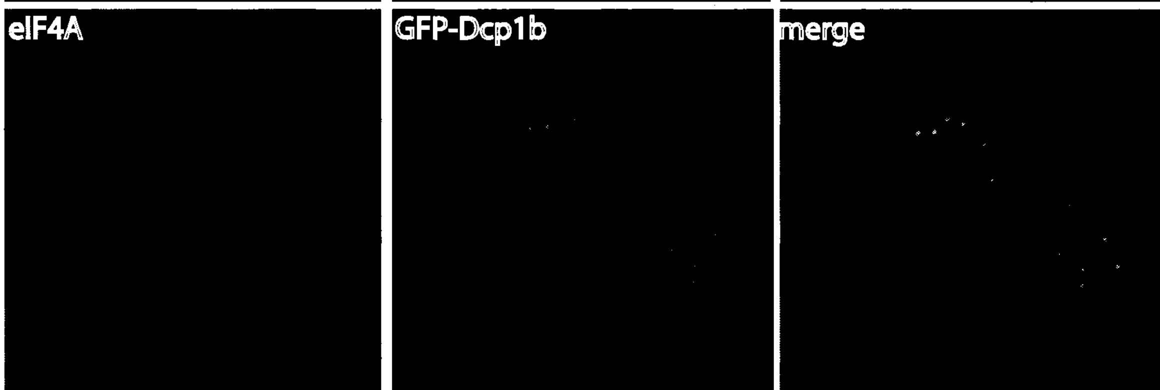
Figure 3.4. eIF4E requires interaction with 4E-T for localization to P-bodies while the other components of the eIF4F complex are excluded from P-bodies.

(A) HeLa cells were transfected with HA-eIF4E wt, HA-eIF4E W73A, or HA-4E-T Y30A. Localization of proteins was studied by indirect immunofluorescence with anti-HA (Covance) and Alexa Fluor 594 anti-mouse IgG. (B and C) HeLa cells were transfected with GFP-Dcp1b and staining of endogenous eIF4GI (B) or eIF4A (C) was detected with anti-eIF4GI and Texas Red conjugated anti-rabbit IgG or with anti- eIF4A and Alexa Fluor 594 anti-mouse IgG. (D) HeLa extract (50 and 100 μg) was resolved by SDS-8% PAGE and a Western blot was performed with rabbit anti-eIF4GI polyclonal antibody. For eIF4A detection, proteins were resolved by SDS-10% PAGE and Western blot was performed with a mouse anti- eIF4A monoclonal antibody.

A

B

C



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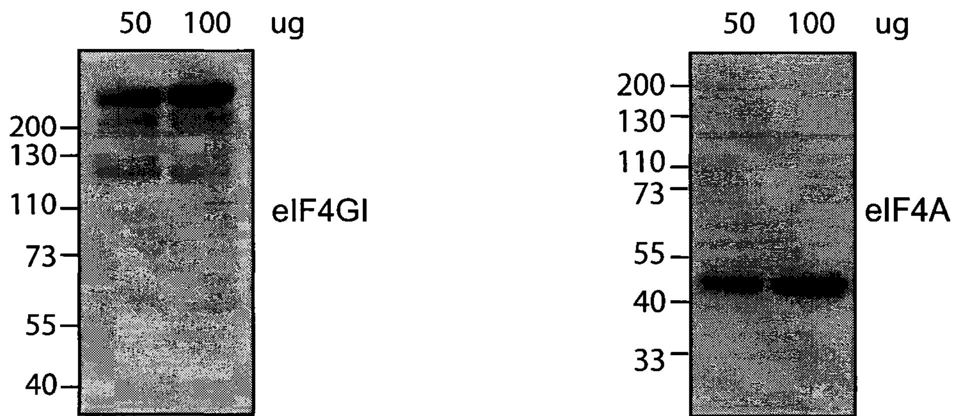


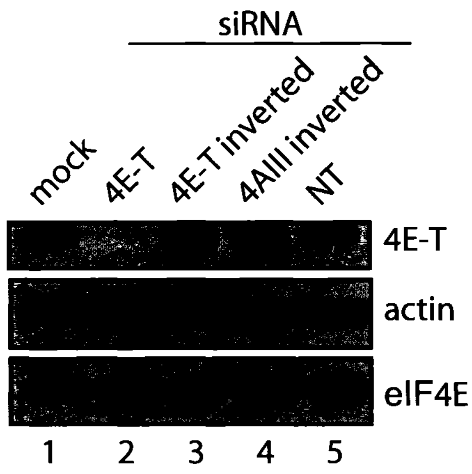
eIF4AII (Edery et al., 1983), and anti-eIF4GI revealed protein bands corresponding to the expected size of the proteins (Fig. 3.4 D). Immunofluorescence analysis with these antibodies shows that eIF4GI and eIF4A do not localize to P-bodies because neither protein colocalizes with the P-body marker, GFP-Dcp1b (Fig. 3.4, B and C). Similar results were obtained using two other anti-eIF4GI antibodies (unpublished data). Thus, eIF4E, but neither eIF4GI nor eIF4A, localized to P-bodies *in vivo*. It is highly likely that eIF4GII behaves in a similar manner, because it exhibits very similar properties to eIF4GI (Gradi et al., 1998).

3.3.4 siRNA against 4E-T results in decreased localization of decapping factors to mammalian P-bodies

To investigate whether 4E-T plays a role in the formation of P-bodies, cellular 4E-T protein levels were reduced by RNA interference (RNAi). Western blotting (Fig. 3.5 A) and immunofluorescence analysis (Fig. 3.5 C) demonstrate that 4E-T was reduced strongly (85%) after treatment with a short interfering RNA (siRNA) against 4E-T, but not by the corresponding inverted sequence (4E-T inverted) (Fig. 3.5 A). Another nonspecific siRNA that was used as a control, 4AIII inverted (Ferraiuolo et al., 2004), failed to affect 4E-T protein levels (Fig. 3.5, A and C). 4E-T depletion caused Dcp1a and eIF4E to disperse throughout the cytoplasm and no longer concentrate in foci (Fig. 3.5 C; cells which were unaffected in their staining in P-bodies are those which had not taken up the siRNA against 4E-T, as confirmed by immunostaining with anti-4E-T antibody: not depicted). The disappearance of P-bodies was not observed in cells that were transfected

Figure 3.5. Depletion of 4E-T from HeLa cells results in disappearance of decapping factors from P-bodies.

(A) HeLa cells were transfected with siRNA against 4E-T or control siRNAs (4AIII inverted or 4E-T inverted). 48 h aftertransfection, cells were split into chamber slides for immunofluorescence analysis (see (C)) and into a 6 cm dish for Western blot analysis. 60 h after transfection, protein cell extracts were prepared and 20 μg of extract was resolved by SDS-10% PAGE. Western blotting was performed with rabbit anti-4E-T and anti-eIF4E antibodies, or mouse anti-β-actin monoclonal antibody (Sigma-Aldrich). 4E-T levels were quantified against β-actin, which served as a loading control, and the level of protein in the negative control (4AIII inverted) transfected cells was set as 100. (B) HeLa extract (20μg) as in (A) was resolved by SDS-10%PAGE and Western blotting was performed with rabbit anti-Dcp1a and anti-Xenopus p54 (Xp54) antibodies, or monoclonal mouse anti-eIF4E (SK) and anti-β-actin antibodies. (C) HeLa (CCL2) cells were transfected with control siRNA (4AIII inverted) or 4E-T siRNA and indirect immunoflorescence was performed 60 h after transfection with rabbit anti-4E-T and anti-Dcp1a antibodies, or monoclonal mouse anti-eIF4E antibody. (D) HeLa S3 cells were transfected with 4E-T siRNA or control siRNA (4AIII inverted). 24 h after transfection, cells were transfected with myc-EYFP-Me31B; 36 h later, extracts were collected for either SDS-PAGE analysis or fixed for immunofluorescence. Cell extracts were resolved by 10% SDS-PAGE and Western blot analysis with rabbit anti-4E-T, or monoclonal anti-myc (9E10) and anti-β-actin was performed. Indirect immunofluorescence was performed to assess the localization of 4E-T, and the localization of myc-EYFP-Me31B was assessed by direct immunofluorescence. The right most panels show the enlarged bordered image.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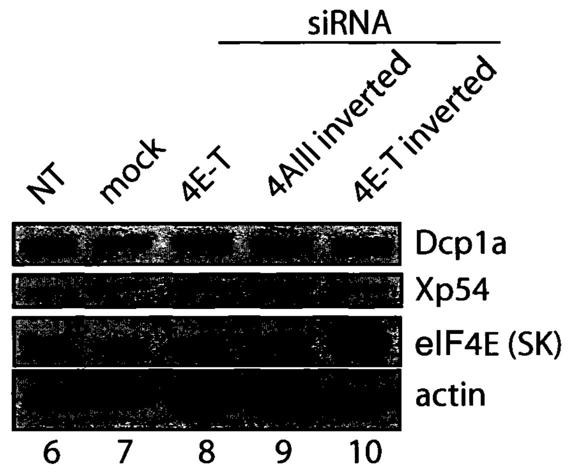
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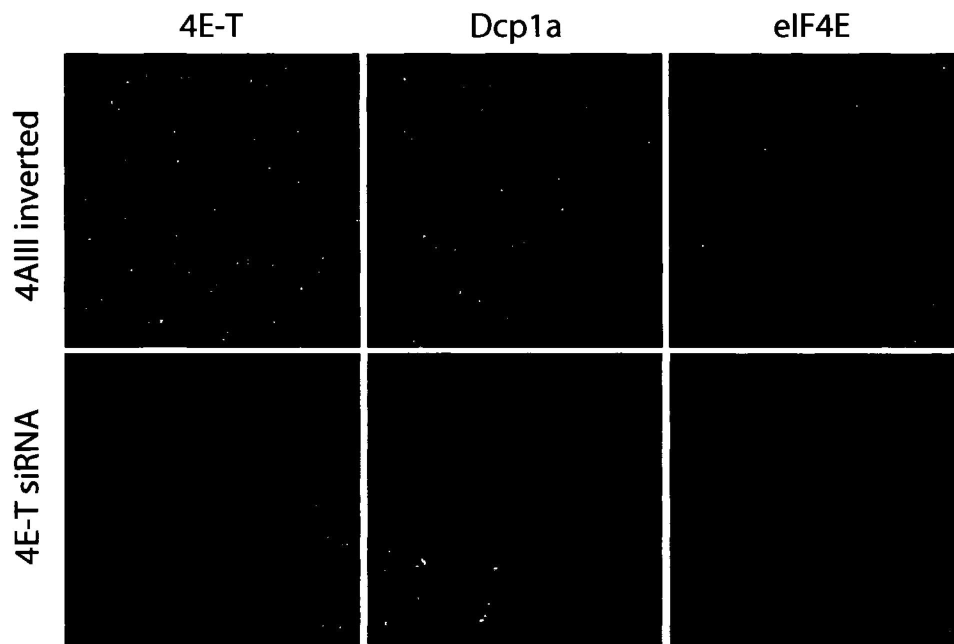
Dcp1a
Xp54
eIF4E (SK)
actin
6 7 8 9 10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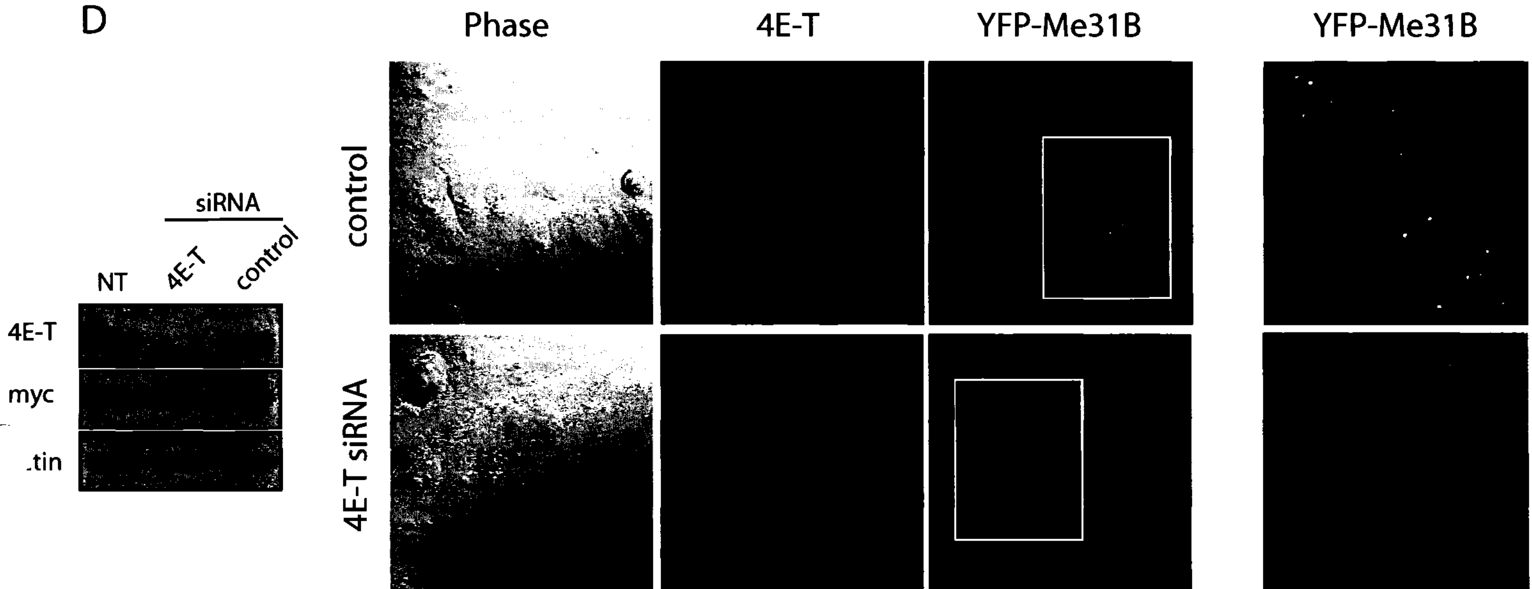
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NT
4E-T
control
4E-T
myc
.tin
Phase
4E-T
YFP-Me31B
YFP-Me31B
control
4E-T siRNA

with the nonspecific control siRNA. No changes in Dhh1p/p54, Dcp1a, and eIF4E protein levels occured in 4E-T depleted cells as compared with nonspecific siRNA controls (Fig. 3.5, A and B).

The effect of 4E-T depletion on Me31B localization also was examined (Fig. 3.5 D). The tagged version of *Drosophila* Me31B was used because the antibody against endogenous p54, used in Fig. 3.5 B for Western analysis, is not suitable for immunofluorescence. HeLa cells transfected with 4E-T or control siRNAs were transfected with the myc-EYFP-Me31B plasmid, and analyzed by Western blotting and double label immunofluorescence (Fig. 3.5 D). 4E-T levels were reduced efficiently in 4E-T siRNA treated cells, and the myc-EYFP-Me31B protein was expressed in cells treated with control or 4E-T siRNAs (left panel). In control cells, myc-EYFP-Me31B was predominantly cytoplasmic and concentrated in P-bodies (right panel). However, when 4E-T levels were reduced, Me31B exhibited a diffuse staining pattern throughout the cytoplasm and reduced or no staining in P-bodies (right panel). Thus, reduction of 4E-T protein levels causes mislocalization of eIF4E and decapping factors away from P-bodies.

3.3.5 Cycloheximide inhibits localization of 4E-T to P-bodies whilst LMB treatment does not affect its localization

In yeast and mammalian cells, cycloheximide treatment releases decapping factors from P-bodies (Cougot et al., 2004; Sheth and Parker, 2003). Cycloheximide inhibits mRNA translation at the elongation step, which prevents mRNA degradation because of its protection by ribosomes (Ross, 1995). To study whether 4E-T localization to P-bodies is dependent on mRNA availability, the effect of cycloheximide on the localization of 4E-T

was examined. HeLa cells were treated with cycloheximide (10μg/ml), and analyzed by immunofluorescence with anti-Dcp1a and anti-4E-T antibodies. A near complete loss of 4E-T and Dcp1a from P-bodies was observed after 30 min of cycloheximide treatment (Fig. 3.6 A); this suggested that the accumulation of 4E-T in P-bodies is dependent on mRNA availability. This effect is not due to a reduction in 4E-T protein levels. Western blot analysis demonstrates that the levels of 4E-T and Dcp1a remain unchanged throughout the cycloheximide treatment (Fig. 3.6 B). Similar results were obtained in HeLa cells transiently transfected with HA-4E-T (Fig. 3.6 C, middle panel).

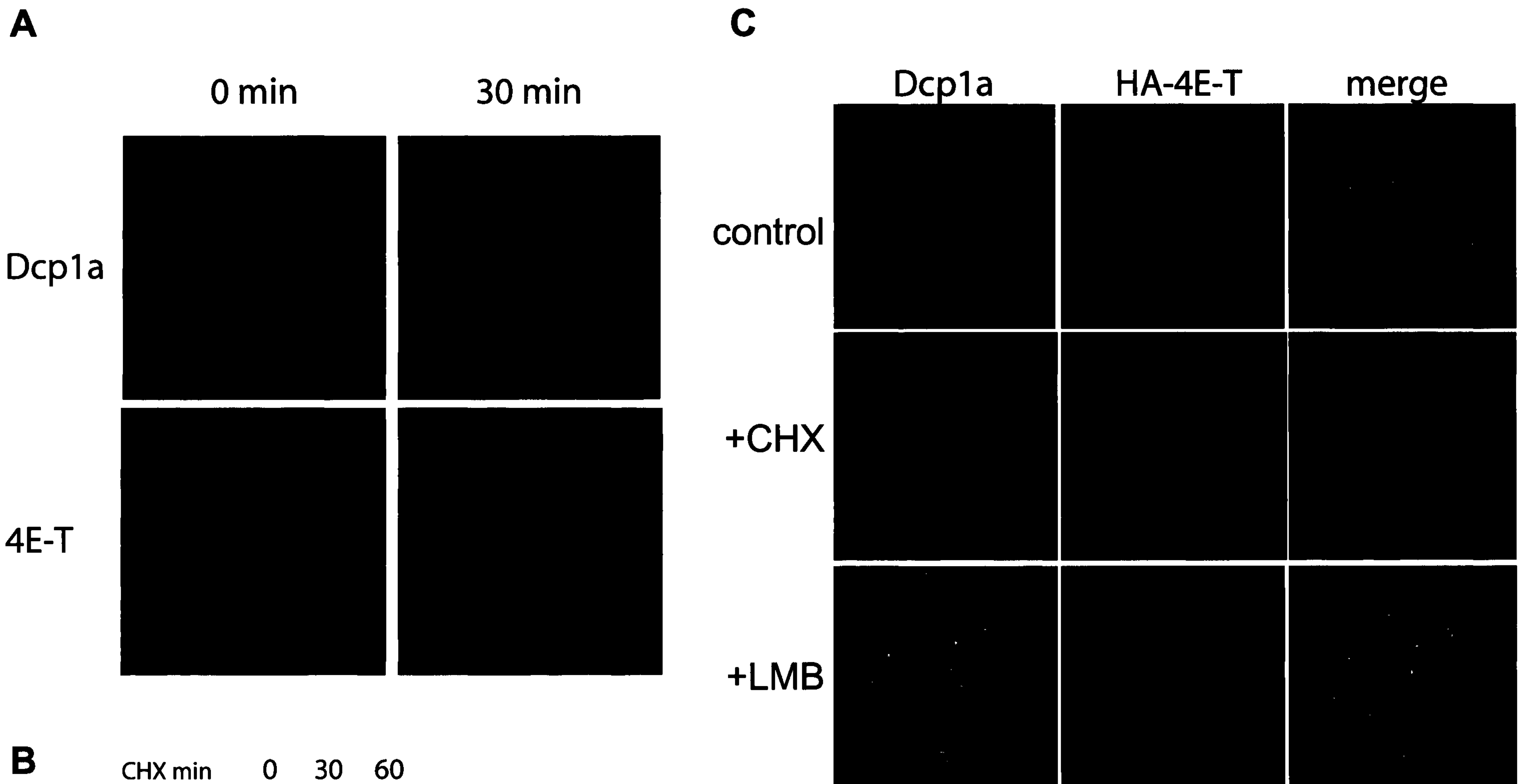
Leptomycin B (LMB) is an inhibitor of chromosome region maintenance-1 (CRM-1)-dependent protein nuclear export. We previously reported that 4E-T is a shuttling protein that exits the nucleus through the CRM-1 pathway (Dostie et al., 2000a). Because Dhh1p/p54 also shuttles between the nucleus and the cytoplasm (Smillie and Sommerville, 2002), it is possible that nuclear export is required for the localization of 4E-T and decapping factors to P-bodies. To test this hypothesis, the effect of LMB on the localization of 4E-T and Dcp1a to P- bodies was studied. LMB treatment resulted in the accumulation of 4E-T in the nucleus, but did not affect its association with P-bodies (Fig. 3.6 C, bottom panel). The distribution of Dcp1a was also unaffected by the drug (bottom panel). Thus, the presence of 4E-T in P-bodies is dependent on mRNA, but is independent of its nucleo-cytoplasmic shuttling.

3.3.6 4E-T knockdown increases mRNA half-life

The results described above suggest that 4E-T might play a role in promoting mRNA degradation. To test this hypothesis, we analyzed the turnover of several mRNAs in 4E-T

Figure 3.6. Effect of cycloheximide and LMB 4E-T localization in P-bodies.

(A) HeLa cells were treated with cycloheximide (CHX; 10μg/ml) for 30 min, and immunofluorescence staining of 4E-T and Dcp1a were examined as described above. (B) HeLa cell extract (20μg) treated with cycloheximide (CHX; 10μg/ml) for 30 and 60 min was resolved by 10% SDS-PAGE and Western blot analysis with rabbit anti-4E-T and anti-Dcp1a, or monoclonal mouse β-actin was performed. (C) HeLa cells were transfected with HA-4E-T. 36 h after transfection, the medium was replaced with fresh medium (control) or medium containing CHX (100μg/ml), and cells were incubated for 3 h before fixation. Alternatively, media containing LMB (5ng/ml) was added to cells and incubated for 5 h before fixation. The colocalization of HA-4E-T was determined by indirect immunofluorescence with anti-HA (Covance) and that of Dcp1a with anti-Dcp1a antibody as described above. The colocalization of HA-4E-T and Dcp1a appears yellow.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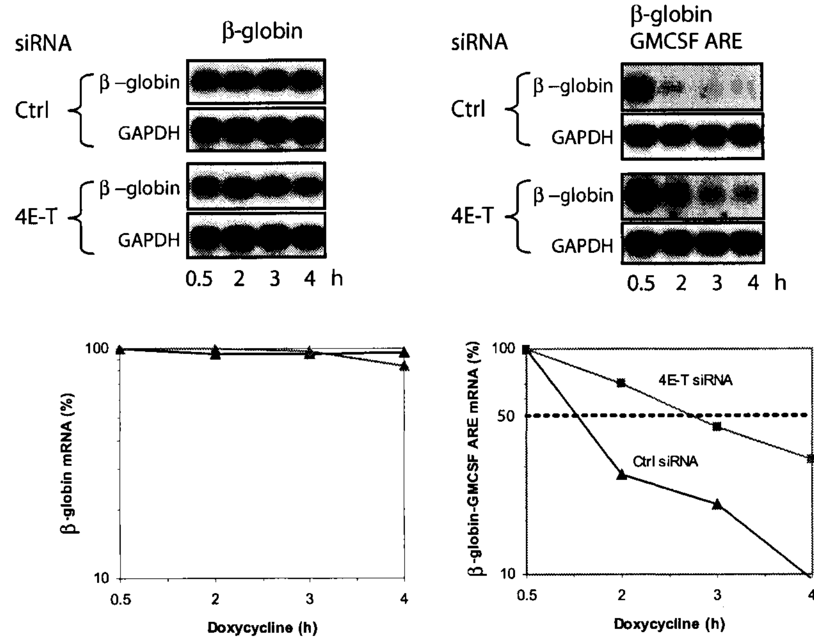
0 min
30 min
Dcp1a
4E-T
B
CHX min
0
30
60
4E-T
Dcp1a
actin
C
Dcp1a
HA-4E-T
merge
control
+CHX
+LMB

depleted and control cells. A β-globin reporter that is under the control of a tetracycline-responsive promoter was fused at its 3' end to one of two distinct adenine/uridine rich elements (AREs) (Stoecklin et al., 2004), that of GMCSF or *c-fos*. The reporter gene - either lacking or containing the ARE - was co-transfected with a plasmid encoding the activator of transcription (pTet-Off) into HeLa cells which were transfected previously with 4E-T or control siRNA. Doxycycline was subsequently added to block transcription and RNA was collected at the indicated times and analyzed by Northern blotting. As expected, β-globin lacking an ARE remained stable throughout the doxycycline treatment, whereas β-globin containing the GMCSF or *c-fos* AREs were rapidly degraded, as was previously reported (Chen et al., 1995) (Fig. 3.7, A and B) . However, 4E-T diminution in HeLa cells resulted in significant stabilization (>2-fold) of both β-globin/GMCSF and β-globin/*c-fos* ARE-containing mRNA, with no effect on β-globin reporter mRNA lacking an ARE (Fig. 3.7, A and B). Western blot analysis demonstrates that 4E-T protein levels were diminished efficiently in HeLa cells that were transfected with the reporter plasmids and in the presence of doxycycline (Fig. 3.7 C). The effect of 4E-T depletion on endogenous *c-fos* mRNA turnover also was analyzed. Similar to transfected β-globin containing the destabilizing element of *c-fos*, endogenous *c-fos* was stabilized in the absence of 4E-T (unpublished data).

The stability of the labile p21 mRNA, which contains an ARE (Lal et al., 2004), also was examined in 4E-T-depleted cells. Western blotting analysis demonstrates that 4E-T RNAi strongly reduced the amount of 4E-T (> 80%, Fig. 3.7 D). p21 mRNA stability was determined by Northern blot analysis after actinomycin D treatment to inhibit *de novo* transcription. p21 mRNA levels were quantified in cells that were treated

Figure 3.7. siRNA-mediated depletion of 4E-T affects mRNA turnover.

HeLa cells were transfected with siRNA against 4E-T or a control (Ctrl) siRNA (4AIII inverted). Protein and RNA were harvested for use in Western (C and D) and Northern (A, B, and E) analysis, respectively. (A and B) HeLa cells were transfected with siRNA against 4E-T or with control siRNA and later cotransfected with pTet-β-globin or pTet-β-globin *c-fos* or GMCSF ARE and pTet Off. Cells were treated with doxycycline (1μg/ml) ~72 h after transfection to block transcription. Total RNA was isolated at 0.5, 2, 3, 4 hours for β-globin/GMCSF ARE assays (A) and 0.5, 1.5, 3, 6 h for β-globin/*c-fos* ARE (B) and analyzed by Northern blot. GAPDH served as a loading control. mRNA half lives were calculated from Northern blots and normalized against GAPDH levels. (C) Protein extracts were collected at 6 h after treatment with doxycycline (1μg/ml) from cells that were transfected with the reporter plasmid and pTetOff and resolved by SDS-PAGE. *Lanes* 1 and 4: glo= β-globin + pTetOff; *lanes* 2 and 5: fos= β-globin/*c-fos* ARE + pTetOff; *lanes* 3 and 6: GM= β-globin/GMCSF ARE + pTetOff. (D) Protein extracts were collected at the latest time point of actinomycin D treatment and resolved by 10% SDS-PAGE. Western blot analysis was done with anti-4E-T and anti-β-actin antibodies. NT=nontransfected. (E) At 48h after transfection, the half lives of p21 mRNA were assessed by using actinomycin D (5μg/ml) for the indicated amount of time. Total RNA (5μg) was resolved on 1.3% formaldehyde gel and analyzed by Northern blotting. 28S levels and 18S levels served as a loading marker. mRNA half- lives were calculated from Northern blots and normalized against ^{32}P-labeled 18S levels and plotted on a graph with the zero time point set at 100.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Ctrl
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GAPDH
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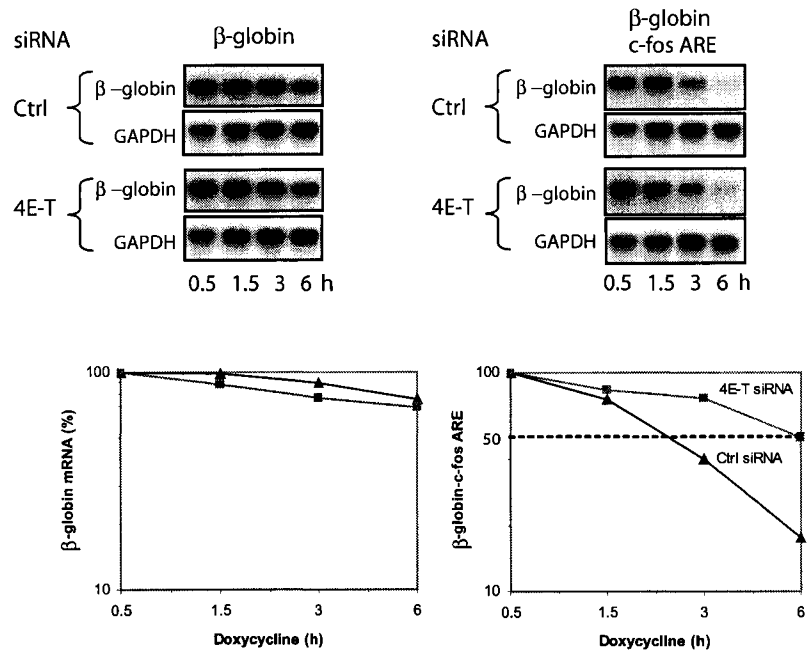
Doxycycline (h)
4E-T siRNA
Ctrl siRNA
β-globin-GMCSF ARE mRNA (%)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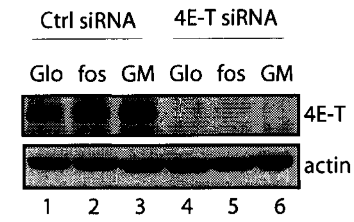
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actin
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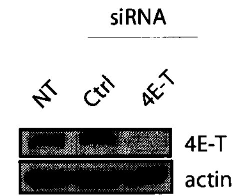
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NT
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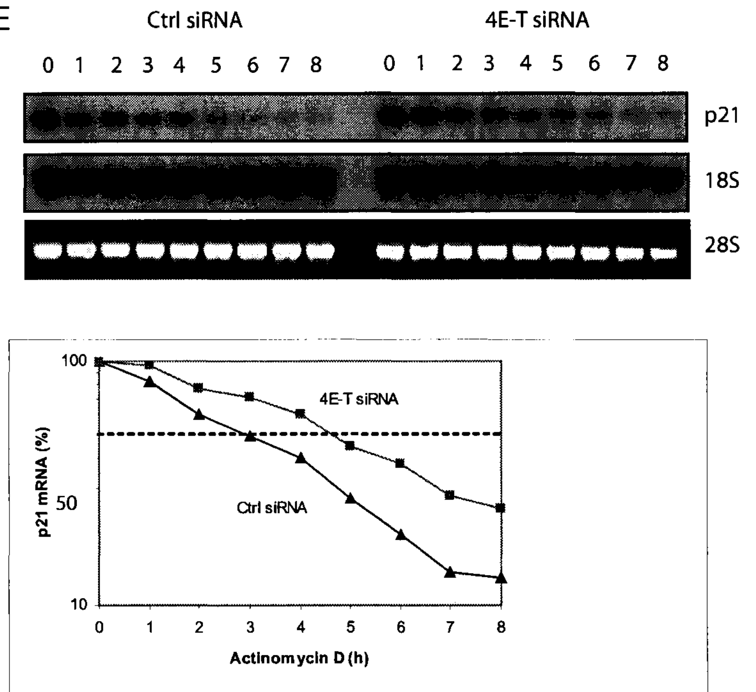
4E-T
4E-T
actin


E
Ctrl siRNA
4E-T siRNA
0 1 2 3 4 5 6 7 8
p21
18S
28S
p21 mRNA (%)
Actinomycin D (h)

with 4E-T siRNAs and in nondepleted cells, and normalized to 18S rRNA levels (Fig. 3.7 E). 4E-T depletion resulted in a modest (~1.5 fold), but significant, increase in the mRNA half-life (Fig. 7 E). The difference in p21 mRNA stability achieved with 4E-T depletion is related inversely to that obtained when the mRNA stabilizer, HuR, is depleted from cells (~ 2-fold decrease) (Lal et al., 2004). Thus, 4E-T promotes degradation of mRNAs that contain an ARE in their 3' untranslated region (3' UTR).

3.4 Discussion

Here we report a new role for 4E-T in mammalian mRNA degradation. We demonstrate that 4E-T inhibits translation, colocalizes with decapping factors in cytoplasmic P-bodies, and decreases mRNA stability. 4E-T interacts with eIF4E through a shared recognition motif (YXXXXLΦ) also found in eIF4G (Dostie et al., 2000a). Similar to other cap-dependent translation inhibitors, 4E-T is likely to inhibit translation by competing with eIF4G for binding to eIF4E and preventing formation of the eIF4F complex (Pause et al., 1994; Poulin et al., 1998). Accordingly, overexpression of 4E-T *wt*, but not of a mutant defective in eIF4E binding, strongly inhibited cap-dependent translation of a reporter mRNA *in vivo* (Fig. 3.2).

The transition of an mRNA from a translationally active mRNP to one destined for decay is believed to be a consequence of translational inhibition, especially in yeast (Teixeira et al., 2005; Tharun and Parker, 2001). Remarkably, 4E-T is present in P-bodies, which are cytoplasmic foci containing factors involved in mRNA decay (Cougot et al., 2004; Ingelfinger et al., 2002; Liu et al., 2004; Sheth and Parker, 2003; van Dijk et al., 2002) (Fig. 3.1). In this study, we also show that eIF4E is localized to mammalian P-bodies (Fig. 3.3). Overexpression of 4E-T in HeLa cells caused a marked increase of eIF4E in P-bodies (Fig. 3.3). This observation suggests that translation inhibition and targeting of eIF4E to P-bodies are causally related. The accumulation of eIF4E in P-bodies requires interaction with 4E-T, because an eIF4E mutant that fails to bind to 4E-T cannot localize to P-bodies (Fig.3.4), and depletion of 4E-T resulted in loss of eIF4E from P-bodies (Fig.3.5). However, the localization of 4E-T to P-bodies does not require

interaction with eIF4E, because a 4E-T mutant defective in binding to eIF4E also localizes to P-bodies (Fig. 3.4).

eIF4GI and eIF4A did not localize to mammalian processing bodies (Fig. 3.4), which is in agreement with studies that demonstrated that mRNA pools from P-bodies are distinct from translating pools (Schwartz and Parker, 1999; Teixeira et al., 2005; Tharun and Parker, 2001). These observations favor a model whereby translation inhibition that results from disruption of the translation initiation complex occurs outside P-bodies, and precedes P-body formation.

As was shown for Dcp1a (Cougot et al., 2004; Sheth and Parker, 2003), cycloheximide treatment of HeLa cells reduced the amount of 4E-T associated with P-bodies (Fig. 3.6). This result indicates that ongoing mRNA translation regulates the association of 4E-T with P-bodies, and suggests that 4E-T is not a permanent P-body constituent, but rather localizes to these structures in an mRNA-dependent manner. Importantly, 4E-T RNAi treatment results in decreased decapping activity in HeLa cells as evidenced by diminished localization of Dcp1a and p54 to P-bodies. Taken together, these data support a model whereby interaction of 4E-T with eIF4E acts as a priming event leading to mRNP remodeling and mRNA decay.

In addition to their role in mRNA decay, P-bodies were suggested to function as mRNA storage sites (Coller and Parker, 2004; Sheth and Parker, 2003). For example, the *Drosophila* decapping factor Me31B, is concentrated in cytoplasmic granules in germline cells where *bicoid* and *oskar* mRNAs are translationally masked (Nakamura et al., 2001). In addition, the Xenopus equivalent, Xp54, is a major constituent of maternal mRNA storage particles where translation is repressed (Ladomery et al., 1997; Minshall and

Standart, 2004). Thus, it is possible that under certain conditions, the presence of eIF4E within P-bodies might permit the transition of a translationallly repressed/stored mRNA to a translationally competent state.

Homology searches using Blast algorithms failed to identify 4E-T yeast homologs (Dostie et al., 2000a), and eIF4E was shown not to localize to P-bodies in yeast (Coller and Parker, 2004), unpublished observations). Therefore, 4E-T might represent a more evolutionarily complex mRNA decay/storage regulation pathway in higher eukaryotes. The *Drosophila* 4E-T homologue, Cup, was reported to mediate translational repression of *nanos* and *oskar* by interacting with eIF4E and 3' trans-acting factors (Wilhelm and Smibert, 2005). Interestingly, the RNA-binding protein Smaug, which interacts with Cup, recently was shown to recruit the CCR4 deadenylase complex in *Drosophila* embryos (Semotok et al., 2005).

An important finding in this paper is that reduction in 4E-T results in an increase in mRNA stability (Fig. 3.7). All the mRNAs tested here are ARE-containing mRNAs, which are believed to be subject to 3'-5' degradation by the exosome, based on *in vitro* decay assays (Chen et al., 2001; Mukherjee et al., 2002). The major deadenylase of AU-containing mRNAs in mammalian cells is believed to be poly (A) ribonuclease (PARN) (Gao et al., 2000; Lai et al., 2003). Therefore, 3'-5'degradation would require destabilization of the interaction between PABP and eIF4G to allow entry and association of PARN with the cap (Wilusz et al., 2001). Therefore, 4E-T may instigate the dissociation of PABP from eIF4G by binding to eIF4E and displacing eIF4G. The decay machinery which processes mRNA through the 5'-3' exonucleolytic pathway, is present in P-bodies. Recent studies have implied that the 5'-3' and 3'-5' pathways

converge at these sites. For instance, mRNA decay enzymes involved in 5'-3'and 3'-5'decay are recruited by the ARE binding proteins, tristetraprolin and butyrate response factor (Lykke-Andersen and Wagner, 2005). Moreover, tristetraprolin, which mediates ARE degradation by recruiting the exosome (Chen et al., 2001), also is found localized in P-bodies (Kedersha et al., 2005). Because the disappearance of P-bodies with 4E-T RNAi was not synonymous with complete stabilization of p21 mRNA, it is possible that this process occurs outside of P-bodies or that its deadenylation/decay occurs by way of a distinct pathway from *c-fos* and GMCSF mRNAs.

In conclusion, the interaction of 4E-T with eIF4E in cells has two consequences: import of eIF4E to the nucleus (Dostie et al., 2000a) and the targeting of eIF4E to sites of mRNA decay. The function of eIF4E in the nucleus is under investigation; a proposed nuclear function of eIF4E is to export a subset of mRNAs from the nucleus (Lai and Borden, 2000; Rousseau et al., 1996). The dual role of 4E-T also might serve to sequester the limiting factor in translation initiation, eIF4E, as a means of maintaining translational homeostasis. It will be important to examine how 4E-T is regulated. It is reported that 4E-T is a phosphoprotein (Pyronnet et al., 2001); therefore it would be interesting to know what pathways regulate its phosphorylation and activity.

While this paper was under review, two reports were published which demonstrated that eIF4E is found in P-bodies (Andrei et al., 2005; Kedersha et al., 2005) and that 4E-T is also concentrated in P-bodies (Andrei et al., 2005)

3.5 Materials and Methods

3.5.1 Plasmids and Cell culture

HA-4E-T, HA-4E-T (Y30A), HA-eIF4E, HA-eIF4E (W73A) (Dostie et al., 2000a) and GFP-Dcp1b (Cougot et al., 2004) were described previously. GFP-4E-T was generated by subcloning the coding region of 4E-T from pcDNA3-4E-T (Dostie et al., 2000a) into pFRED143 (provided by G. Pavlakis, National Cancer Institute, Frederick, MD). Myc-EYFP-Me31B plasmid was a gift from A. Nakamura (University of Tsukuba, Tsukuba, Japan).

Construction of tetracycline-regulated β-globin expression plasmids: The starting plasmid CMV-glo-SPA, which contains the human β-globin gene with the 3'-UTR and polyadenylation site replaced with a multiple cloning site and a strong synthetic polyadenylation element (SPA) was described previously (Das Gupta et al., 1998). The first generation tetracycline regulated globin construct pTet-O-βAc-glo-SPA was prepared by digesting CMV-glo-SPA with HindIII, end-filling with Klenow fragment DNA polymerase, then digesting with NcoI. The globin-SPA fragment was gel purified and cloned into pGTetOβAcLuc3 (provided by Jose Garcia-Sanz, Universidad Autãnoma, Madrid, Spain) in which the LUC insert was removed by digesting with BamHI followed by end-filling with Klenow fragment DNA polymerase plus digestion with NcoI. The *c-fos* ARE was recovered from the plasmid pBBB+ARE^{cfos} (provided by A.-B. Shyu, University of Texas Medical School, Houston, TX) by PCR amplification with primers BBBARE1 (5'-CGCTCTAGACAGAAGGTGGTGGCTGGTGTG) and BBBARE2 (5'-GCGTCTAGACTCAAGGGGCTTCATGATGTC). The GMCSF ARE was similarly recovered by PCR from pBBB+ARE^{GMCSF} using primers BBBARE1 and BBBARE3 (5'CCCTCTAGAGCTGGTTATTGTGCTGTCTCA). The PCR products were digested with XbaI and inserted into XbaI-digested Tet-O-βAc-glo-SPA to create plasmids pTet-O-βAc-glo-ARE[*cfos*]-SPA and pTet-O-βAc-glo-ARE[GMCSF]-SPA. These were used to prepare the second generation plasmids used in this study. The region spanning the tetracycline operator and β-actin promoter elements of each of these plasmids was removed by digestion with Nhe1 and Nco1, and replaced with the region

spanning the tetracycline operator elements and minimal CMV promoter regions of pTREmyc. The latter was prepared by PCR amplification of pTREmyc using primers BM127 (5'-ATGCCATGGTGTCTAGCACGCG) and BM128 (5'-CCGCTAGCCACGAGGCCCTTTCGTCTCG). The final plasmids are designated pTet-CMV_{min}-glo-SPA, pTet-CMV_{min}-glo-ARE[*cfos*]-SPA and pTet-CMV_{min}-glo-ARE[GMCSF]-SPA.

HeLa S3 cells from M. Wilkinson (M.D. Anderson Cancer Center, Houston, TX) (Carter et al., 1995) and HeLa CCL2 (American Type Culture Collection) were cultured in DMEM supplemented with 10% FBS. HeLa S3 cells usually were used unless specified otherwise.

3.5.2 Short Interfering RNA (siRNA) Transfections

siRNA annealed duplexes were purchased from Dharmacon and sequences are listed below. HeLa cells were transfected at 30% confluency in 6-well plates with siRNA (300 pmole final) using oligofectamine according to the manufacturer's instructions. siRNA complexes were left overnight and cells were processed 48 to 72 hours later.

4E-T siRNA 5' GAACAAGAUUAUCGACCUA dTdT 3'

4AIII inverted 5' CGACCAGAGCTTAAGGTGA dTdT 3'

4E-T inverted 5' CGUACCGUGGAAUAGUUCC dTdT 3'

3.5.3 Transient Transfections and Immunofluorecence

Transfections of DNA plasmids were performed by the calcium phosphate method as described previously (Dostie et al., 2000a). HeLa cells were transfected at 50% confluency with 5ug (or 8ug for GFP-4E-T) of plasmid DNA, and were processed 36 h after transfection. For transfection of myc-EYFP-Me31B (Fig. 5D), siRNA transfections were performed in 6- well plates as described above; after 24 h, 2ug of myc-EYFP-Me31B was transfected by the calcium phosphate method. 48 h after siRNA transfection, cells were trypsinized into chamber slides and processed for immunofluorescence 12 h later. Immunofluorescence was performed as described previously (Dostie et al., 2000a). In brief, cells were plated onto Lab-Tek chamber slides (Nunc) 16 h before fixation. Cells were fixed with formaldehyde, permeabilized, blocked and incubated with affinity-

purified rabbit anti-4E-T (1/100) (Dostie et al., 2000a), affinity-purified rabbit anti-Dcp1a (1/200) (a gift from J. Lykke-Andersen, University of Colorado, Boulder, CO), and anti-Dcp2 (1/20) (Liu et al., 2004) antibodies, or monoclonal anti-eIF4A (1/100) (Edery et al., 1983), anti-eIF4E (1/10) (Kimball et al., 2003), and anti-HA (1/1000; Covance) antibodies for 2 h at RT. Cells were washed extensively and incubated with either Texas red or Alexa Fluor 488 or 594 conjugated secondary antibody (1/200; Molecular Probes) for 30 min. Nuclei were stained with Hoechst dye 33258 (Sigma-Aldrich). Images were taken from a 63X objective of a Zeiss LSM 510 confocal microscope. Immunofluorescence analysis for each experiment was performed three to five times, and the most representative results are displayed.

3.5.4 Western Blotting

Cells were washed twice with ice cold PBS and pelleted at maximum speed for 10 minutes in a microcentrifuge. Cell pellets were frozen on dry ice and thawed in RIPA buffer (150mM NaCl, 50mM Tris-HCl pH 7.5, 1% Nonidet P-40, 0.5% SDS and a mixture of protease inhibitors (Roche Diagnostics)). Extracts were incubated on ice for 10 minutes and centrifuged for 5 min at maximum speed to recover the supernatant. Proteins were resolved by SDS/PAGE, transferred to nitrocellulose membrane, blocked, and incubated with rabbit anti-4E-T (1/1000) (Dostie et al., 2000a), anti-Dcp1a (1/1000), anti-Xp54 (1/2000) (Smillie and Sommerville, 2002), anti-eIF4E 5851 (1/1000) (Frederickson et al., 1991) polyclonal antibodies or monoclonal anti-eIF4A (1/1000) (Edery et al., 1983), anti-eIF4E (1/100) (Kimball et al., 2003) anti-HA (1/2000) (Covance) and anti-β-actin (1/5000) (Sigma-Aldrich) antibodies.

3.5.5 Vaccinia Virus Infection, Far Western, and Translation Assay

HeLa cells (80% confluency) were infected with recombinant vaccinia virus for 1 hour at 37°C (Fuerst et al., 1986). Cells were rinsed once with serum-free media and transfected with 5ug each of the reporter plasmid (pGEM-LUC-POLIRES-CAT) and pcDNA3-4E-T wt or mutant constructs using lipofectin (15ug; Invitrogen) as previously reported (Pause et al., 1994). After 16 h, cell extracts were collected and analyzed for LUC activity using a LUC assay system (Promega), and activity was measuredwith a luminometer; CAT

expression was analyzed using ELISA (Boehringer). Far Western analysis was performed on transfected cell extracts as described previously (Dostie et al., 2000a)

3.5.6 Northern blotting

Total RNA was isolated using Qiagen RNAesy kit according to the manufacturer's instructions. 5ug of total RNA was separated on a 1.3% agarose/formaldehyde gel, transferred to a Hybond-N membrane (GE Healthcare), and probed with ^{32}P-labeled, random-primed DNA probe to detect p21 (Lal et al., 2004) or ^{32}P-end labeled oligonucleotide, using terminal deoxynucleotidyl transferase (Invitrogen), to detect 18S rRNA (Lal et al., 2004). For β-globin reporter assays, 4E-T or control siRNA was transfected into HeLa cells as described above. 36 h later, pTet-β-globin reporter plasmids and pTet Off (encoding for tTA, transactivator) were cotransfected. 12 h later, cells were split 1:6 and treated 16 to 20 h later with doxycycline (1μg/ml). RNA was resolved on a formaldehyde gel as above and probed with ^{32}P-labeled, random primed DNA probe for detecting β-globin (Stoecklin et al., 2004) or GAPDH.

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CHAPTER 4

GENERAL DISCUSSION

4.1 P-bodies: A Synopsis

4.1.1 Components of P-bodies

P-bodies were initially considered as mRNA decay centers but recent data supports the idea that P-bodies are more than just "junkyards", they are also recycling factories. P-bodies contain translationally repressed mRNPs that are bound by proteins which can either reinitiate translation (eIF4E) (Andrei et al., 2005; Ferraiuolo et al., 2005; Kedersha et al., 2005), mediate 5'-3'general decay (Dcp1/2, Lsm1-7, Xrn1, Hedls, Edc3, 4E-T) (Cougot et al., 2004; Fenger-Gron et al., 2005; Ferraiuolo et al., 2005; Ingelfinger et al., 2002; Liu et al., 2004; Sheth and Parker, 2003; van Dijk et al., 2002), officiate specialized pathways of decay (SMG5, SMG7, Upf1) (Unterholzner and Izaurralde, 2004), or mediate RNA silencing (Ago1, Ago2, GW182, p54) (Chu and Rana, 2006; Jakymiw et al., 2005; Liu et al., 2005a; Liu et al., 2005b; Sen and Blau, 2005). In addition to proteins, mRNA targets have been localized to P-bodies. mRNAs targeted by miRISC were identified in P-bodies in mammalian cells (Liu et al., 2005b). Silenced mRNAs were shown to return to the translating pool and away from P-bodies in both yeast and mammals (Bhattacharyya et al., 2006; Brengues et al., 2005; Teixeira et al., 2005). Furthermore, mRNAs targeted by the NMD pathway as well as mRNA targets of the 5'-3'pathway of normal mRNA decay were localized in yeast P-bodies (Brengues et al., 2005; Sheth and Parker, 2003; Sheth and Parker, 2006; Teixeira et al., 2005). Moreover, mRNAs targeted for decay by siRNA/RISC were also reported to be in P-bodies in human cells (Jakymiw et al., 2005). Therefore, it appears that components of

the three major eukaryotic mRNA decay pathways (deadenylation-dependent decay, specialized decay, and RNAi) converge to these focal points called P-bodies.

The appearance of an mRNA will dictate its fate. Depending on the composition of proteins/RNA that it is swathed by, it can either be engaged in translation and unravelled into protein, or stored for later activation and translation; alternatively, it can be degraded by decay enzymes. Translation and mRNA decay for the most part are antagonistic processes. The mRNA is in a balancing act between the translation apparatus and proteins/RNA which "tip" it into decay (Coller and Parker, 2005). The rate of mRNA turnover is influenced by translation. In the case of NMD, the message is read by a scanning ribosome and when a termination codon is detected out of context, decay enzymes are recruited which carry out the destruction of the mRNA. Translation rates in turn are influenced by miRNAs. Essentially, P-bodies represent another level of translational control because through repression and decay mRNAs are sequestered from the translating pool in P-bodies (Bruno and Wilkinson, 2006).

4.1.2 Conservation of P-bodies
Districts of Translational Control and mRNA Turnover Across Species

P-bodies are conserved from yeast to mammals (Cougot et al., 2004; Sheth and Parker, 2003). NMD targets are degraded within yeast P-bodies (Sheth and Parker, 2006) and since NMD components are found in mammalian P-bodies, it is likely that this process is conserved in mammals. NMD exists across organisms; however, whether the mechanism and regulation of decay occurs in similar bodies throughout phyla, has yet to be determined. P-bodies, or the equivalent of P-bodies which consist of districts within the

cell which regulate translation/decay, exist in *Xenopus* and *Drosophila* and control translation rates of maternal mRNAs (Coller and Parker, 2004). It remains to be determined whether the mechanisms involved in the translational repression/derepression in localized "districts" across different organisms is conserved, as this will help to shed light on some characteristics of translational silencing and release in human cells. Me31B is involved in masking/silencing of maternal mRNAs until fertilization in *Drosophila* (Nakamura et al., 2001). Me31B is the *Drosophila* homolog of yeast Dhh1p and human p54 which are repressors of translation and are required for P-body assembly in yeast and mammals (Chu and Rana, 2006; Coller and Parker, 2005; Sheth and Parker, 2003). Furthermore, the *Xenopus* homolog of p54 (Xp54) is also a key component of stored mRNA in oocytes and embryos (Smillie and Sommerville, 2002). In *Drosophila,* characterization of the translational regulation of *oskar* mRNA has revealed that Bruno, a protein which binds the Bruno Responsive Element in the 3'UTR of *oskar* mRNA, represses the translation of *oskar* prior to its localization at the posterior pole by two methods: (*i*) it concomitantly interacts with the eIF4E binding protein Cup to directly inhibit recruitment of the 43S preinitiation complex. Interestingly, Cup is the putative *Drosophila* homolog of 4E-T (see section 1.2.4.3.); (*ii*) alternatively, Bruno mediates oligomerization of *oskar* mRNA into large (50-80S) RNP "silencing particles," which are unattainable to the translation apparatus (Chekulaeva et al., 2006). Incidentally, eIF4E and Cup are components of these silencing particles and so is the DEAD box RNA helicase, Me31B (Chekulaeva et al., 2006). It would appear then that the Bruno-mediated silencing particles are similar in function to P-bodies; however, it is still unclear if they are related to the masking *oskar* particles which are derepressed after fertilization

or if they are separate entities (Tekotte and Davis, 2006). P-body-like structures exist in *C. elegans* as well. Recently, AIN-1, the *C. elegans* homolog of GW182 was reported to localize in foci which resemble processing bodies (Ding et al., 2005). Furthermore, it was shown to interact with RISC components and to target the Argonaute protein ALG-1 to P-bodies when overexpressed (Ding et al., 2005). Therefore, miRNA mediated translational silencing/mRNA degradation in P-bodies seems to be conserved in other organisms. Finally, in zebrafish, mi430 induces maternal mRNA cleavage reminiscent of miRNA-mediated cleavage of ARE-mRNA in somatic cells (Weigel and Izaurralde, 2006). Whether this occurs in discrete foci is still unknown. Further analysis in other organisms may illuminate the mechanism of miRNA function and may unravel the means behind mRNA decay, as well as translational silencing and activation with regards to P-bodies. This will ultimately cast some light on P-bodies as translational control centers and elucidate their influence on mRNA decay pathways such as NMD.

4.1.3 Other Granules

In summary, some mRNAs are immediately engaged by the translation apparatus and assembled into polysomes. Once translation is completed, the mRNAs are deadenylated, polysomes are lost, and the mRNA may be degraded or stored. In some cases, translation is prematurely interrupted and the mRNA is prompted for rapid decay. In other cases, mRNAs are not immediately translated, but are transported or stored until their later activation by developmental signals or environmental stimuli. Cytoplasmic granules refer to all nonorganellar mRNP aggregates which play important roles in the regulation of gene expression. RNA granules are visible cytoplasmic entities which regulate

translation/mRNA stability (reviewed in (Anderson and Kedersha, 2006)). They exist in germ cells (germ cell granules), in somatic cells (P-bodies and stress granules), and in neurons (neuronal granules) (Anderson and Kedersha, 2006). The previous section focused on the similarities between P-body components and function across phyla. Here, the differences between P-bodies and other granules are discussed briefly. The release and activation by developmental cues of maternal mRNAs in *Drosophila*, *Xenopus* and worm granules are examples of germ cell granules (Anderson and Kedersha, 2006). Other granules include stress induced mRNP aggregates analogous to P-body structures, called stress granules (Anderson and Kedersha, 2006). Stress granules consist of stalled inactive mRNP translation complexes which can either be released and reinitiate translation when stress is alleviated, or can be shuttled into a P-body for degradation. Stress granules differ from P-bodies in several respects: stress granules are formed as a result of eIF2α phosphorylation and contain small ribosomal subunits, as well as initiation factors, unlike P-bodies (with the exception of eIF4E). Furthermore, mRNAs are not degraded in stress granules. Lastly, stress granules and P-bodies are morphologically distinct (Anderson and Kedersha, 2006). Neuronal granules also harbor ribosomal subunits, in this case the small and large subunits. These granules are said to contain "pretranslational" silenced mRNAs that are transported and released at synapses where they are translated after a stimulus is provided (Anderson and Kedersha, 2006). Neuronal granules differ from stress granules and P-bodies in that they contain highly specific mRNAs (as do germ cell granules), whereas P-bodies and stress granules contain a variety of mRNA cargo. P-bodies contain nonsense-containing mRNAs, ARE-mRNAs, deadenylated mRNAs and mRNAs targeted by miRNA/siRNA. In essence, all RNA

granules contain nontranslating (silenced) mRNA (for review see (Anderson and Kedersha, 2006). The mechanisms contributing to their formation and disintegration as well as the signalling pathways which impact upon them requires further investigation. Notably, not all mRNAs are degraded in P-bodies. Components of the exosome are not present in yeast P-bodies which suggests that mRNA decay can occur away from P-bodies (Coller and Parker, 2005).

This work has contributed to a better understanding of the NMD pathway, upstream of degradation in P-bodies, and has identified a novel component of the EJC, eIF4AIII, which is critical for the detection of a nonsense codon by the translation apparatus. Furthermore, this work has identified 4E-T as a novel component of P-bodies which is required for repression of translation (in an eIF4E-dependent manner), mRNA decay and P-body formation in human cells, and has also revealed the presence of initiation factor eIF4E in P-bodies.

4.2 eIF4AIII: Analysis of eIF4AIII function and future directions

Given the clinical importance of NMD, efforts have been made to uncover the mechanism of PTC recognition in mammalian cells, the factors involved and the regulation and localization of PTC-containing mRNA decay within cells. We and others have identified eIF4AIII as a novel component of the EJC (Chan et al., 2004; Ferraiuolo et al., 2004; Palacios et al., 2004; Shibuya et al., 2004) essential for NMD (Ferraiuolo et al., 2004; Palacios et al., 2004; Shibuya et al., 2004). Since eIF4AIII is related to eIF4AI (~67% identity), it can interact with eIF4G1, and also possesses helicase activity

stimulated by eIF4B, we proposed that eIF4AIII substitutes for eIF4AI in the pioneer round of translation, during NMD (Chapter 2) (Ferraiuolo et al., 2004). However, our studies localized eIF4AIII at the exon-exon junction and it was reported by others to interact with EJC components (Ballut et al., 2005; Chan et al., 2004; Gehring et al., 2005; Palacios et al., 2004; Shibuya et al., 2004; Tange et al., 2005) (Fig. 4.1). Shibuya *et al*, on the other hand, proposed that the function of eIF4AIII in mammalian NMD is to bind mRNA directly and act as a scaffold for other EJC factors (Shibuya et al., 2004). This was based on the observations that eIF4AIII binds spliced RNA directly at the position of the EJC (-24) and that antibodies against eIF4AIII inhibit EJC assembly on the mRNA, generating mRNA specific cleavage fragments normally protected from RNase H digestion by the EJC (Shibuya et al., 2004). It was therefore suggested that eIF4AIII acts as a "placeholder" upon whom other EJC factors assemble (Shibuya et al., 2004). Since then, the minimal stable core complex of the EJC was isolated from HeLa cells and found to consist of eIF4AIII, Y14, Magoh, and MLN51 (Tange et al., 2005). The EJC core complex also was recapitulated *in vitro* using purified proteins produced in *E. coli* (Ballut et al., 2005). The *in vitro* core complex is able to assemble onto ssRNA when ATP is bound to eIF4AIII, and encompasses a 8 nucleotide (nt) footprint on the RNA similar to that observed from EJC deposition on pre-mRNA during splicing (Ballut et al., 2005; Le Hir et al., 2000a). Furthermore, it was demonstrated that the core complex remains stably associated with RNA as a result of inhibition of eIF4AIII ATPase activity by Y14/Magoh (Ballut et al., 2005). eIF4AIII contains all the characteristic motifs conserved amongst DEAD-box proteins, as well as eight motifs which are specific to eIF4AIII. Subsequent to the studies mentioned above, mutational analysis of these motifs revealed that DEAD-

box conserved motifs contribute significantly to EJC assembly and NMD (Motifs Ia, VI), as do eIF4AIII specific motifs (region H) (Shibuya et al., 2006). Therefore, there is substantial evidence which supports the initial proposition put forth by Shibuya *et al*, that eIF4AIII functions at the EJC as a stable clamp upon which other EJC factors assemble during splicing (Shibuya et al., 2004). The newly reported crystal structure of the core complex (eIF4AIII, Barentsz, Y14, Magoh with ATP analog and RNA) validates the idea that eIF4AIII represents a new class of DEAD-box proteins which function as "placeholders" (Andersen et al., 2006; Bono et al., 2006). These studies demonstrate that eIF4AIII enfolds an ATP molecule and binds a 6nt stretch of the RNA. Furthermore, it is affirmed from these studies that the ATPase activity of eIF4AIII is inhibited as a result of the interaction of core proteins (Y14, Magoh and Barentsz) at the interface between the two domains of eIF4AIII, essentially securing the interaction of eIF4AIII with RNA (Andersen et al., 2006; Bono et al., 2006). In effect, mutations within eIF4AIII which disrupt binding with other proteins in the complex (C-terminal region containing region H), ATP-binding (motif VI) or RNA binding (motif Ia) all have an effect on EJC formation and NMD function (Andersen et al., 2006; Bono et al., 2006; Shibuya et al., 2006).

Two recent studies raise interesting, yet contradictory, findings. It was reported that nonsense-mediated degradation of immunoglobin-μ can occur independent of the EJC, and that degradation of nonsense-containing mRNA depends on the distance of the PTC from the 3'UTR, reminiscent of PTC recognition in yeast. In this case, siRNA against eIF4AIII had no effect on degradation of the PTC-containing mRNA (Buhler et al., 2006). This result dispels the idea that eIF4AIII functions as an initiation factor in the

pioneer round of translation during NMD and that it plays an essential role in PTC recognition at the EJC. However, whether EJC-independent degradation is a global process in mammalian cells or if it specific to certain mRNAs, such as immunoglobin-μ, is not known.

Gehring *et al* demonstrated that two alternative NMD pathways exist in human cells (Gehring et al., 2005) (see section 1.3.4.6.). Two distinct mRNP minimal complexes were deduced, which have differential requirements for Upf2; tethering assays were employed to examine the distinct requirements for EJC components to elicit NMD. One pathway is dependent on eIF4AIII/Y14/Magoh/Barentsz/Upf3b to induce NMD but is unaffected by Upf2 depletion. The other NMD pathway is activated by RNPS1/Upf3b/Upf2 but is unaffected by the depletion of eIF4AIII or Barentsz (Gehring et al., 2005). However, it is not yet clear if the RNPS1-dependent mRNP is associated with eIF4AIII and other EJC factors at the exon-exon junction but these remain inactive or whether they are absent. It was suggested that RNPS1 acts as an alternative eIF4AIII-independent clamp for EJC factors in this pathway (Gehring et al., 2005). However, RNPS1 may have bypassed the need for eIF4AIII in a tethering assay. If eIF4AIII functioned as an initiation factor in the first round of translation, one would imagine that RNAi against eIF4AIII would affect degradation mediated by tethered RNPS1. Since tethering assays are employed at the 3'end of a *normal* (not containing a PTC) transcript, deductions can be made of the nature of these factors with regards to PTC recognition and not to their functions in translation. At the very least, the fact that alternative NMD pathways exist which are independent of eIF4AIII (Buhler et al., 2006; Gehring et al.,

2005), raises the possibility that eIF4AIII has additional functions, other than its role in NMD, in the cell (see below).

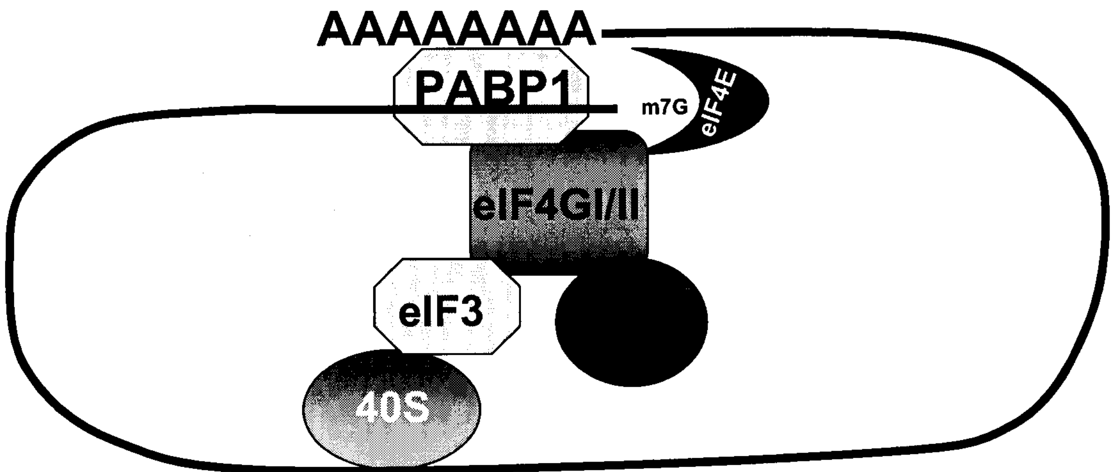
Notably, mutation of Walker A and Walker B motifs which are required for RNA-dependent ATPase activity of eIF4AIII, had no effect on EJC formation or NMD (Shibuya et al., 2006). Inhibition of ATP hydrolysis is required to maintain EJC composure (Andersen et al., 2006; Ballut et al., 2005; Bono et al., 2006). In accordance with this, incubation of HeLa cells stably expressing a *TCR-β* gene containing a mutation which results in a PTC, with the small molecule translational inhibitor pateamine, inhibited NMD (M.A. Ferraiuolo, unpublished data). Pateamine was shown to stimulate eIF4AI RNA binding and ATPase activity (Bordeleau et al., 2005). In addition to eIF4AI, a column coupled to pateamine was able to pull down eIF4AIII (Bordeleau et al., 2005). Therefore, presumably the activation of eIF4AIII ATPase activity is targeted by pateamine, resulting in EJC disassembly and therefore abrogation of NMD (see below).

What then might be the function of eIF4AIII ATPase activity? The unwinding activity of eIF4AIII, like eIF4AI, is stimulated by eIF4B *in vitro* (Li et al., 1999). Furthermore, eIF4AIII interacts with the middle fragment of eIF4G1 (Li et al., 1999) (Fig. 4.1). Unlike eIF4AI, however, eIF4AIII does not form a 48S preinitiation complex in a ribosome binding assay from extracts reconstituted with general translation factors and (intronless) mRNA (Li et al., 1999). In addition, RNAi against eIF4AIII did not affect general translation rates measured by ^{35}S-Methionine incorporation, nor did it affect poliovirus protein production, a system which is more eIF4AI sensitive (see Appendix, Figs. A1 and A2). In *Xenopus*, eIF4AIII overexpression does not stimulate

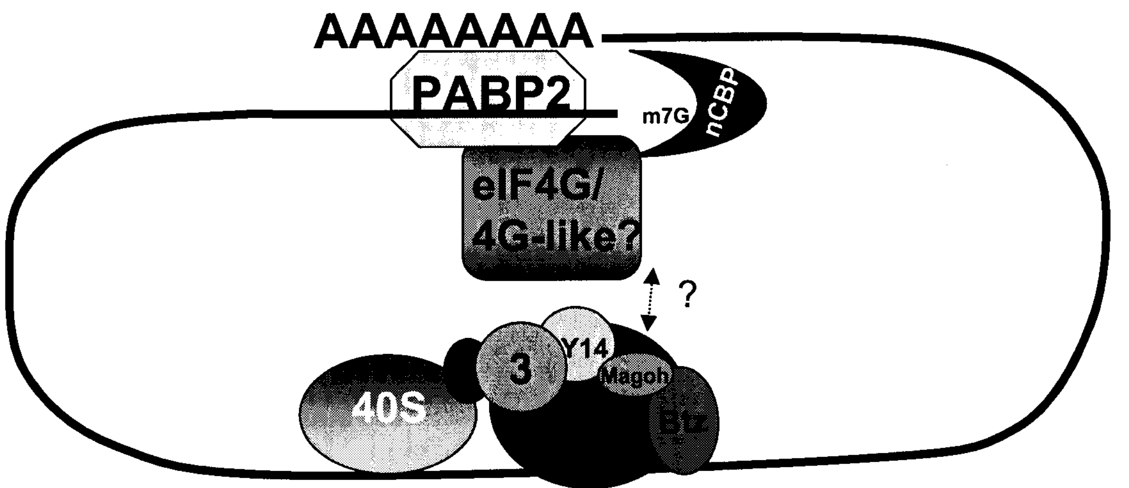
Fig. 4.1 Model for the function of eIF4AIII

(A) *Translation initiation of bulk mRNA in the cytoplasm.* eIF4AI/II interacts with eIF4GI/II, which also binds eIF4E, the ribosome associated factor eIF3, and PABP, leading to the circularization of the mRNA, to initiate bulk cytoplasmic translation round. (B) *Translation initiation of the pioneer round.* CBP20/80 substitutes for eIF4E and PABP2 may substitute for PABP1 during the pioneer round of translation when NMD occurs. eIF4AIII associates with the EJC, but eIF4AIII has the ability to interact with the middle portion of eIF4G and with eIF4G-like. The nature of this interaction may be to promote translation initiation during NMD or may be a separate role from its function at the EJC; alternatively, the interaction with eIF4G-like may be to cause disruption of the closed loop and elicit mRNA decay enzymes.

bulk



pioneer



overall protein synthesis; it does, however, regulate the translation of selective mRNA involved in epidermis formation (Weinstein et al., 1997). Notably, the closest homolog of human eIF4AIII is from *Xenopus* (Li et al., 1999); incidentally, NMD does not occur in *Xenopus* oocytes (Conti and Izaurralde, 2005; Lejeune et al., 2002). Taken together, it cannot be ruled out that eIF4AIII functions as a translational regulator. Accordingly, it has been reported that splicing enhances translation in mammalian cells (Kunz et al., 2006; Nott et al., 2004; Wiegand et al., 2003; Wilkinson, 2005). The observed increase in translation of spliced mRNAs is specifically attributed to the EJC (Kunz et al., 2006; Nott et al., 2004; Wiegand et al., 2003; Wilkinson, 2005). EJC components tethered within the ORF of a reporter luciferase gene, or tethered 6nt away from the translation termination codon of a reporter, stimulated its translation (Nott et al., 2004; Wiegand et al., 2003). The increased translational yield of spliced mRNA is correlated with enhanced polysome association. Interestingly, Upf3 proteins enhance translation when tethered within the ORF of Renilla luciferase, independent of its ability to interact with EJC factors, demonstrating that NMD and translation are separable activities of EJC factors, which may involve different protein complexes and the recruitment of different factors (Kunz et al., 2006). The fact that eIF4AIII can bind to eIF4G suggests a role in translation which may or may not be autonomous from its role in NMD. To dismiss the idea that eIF4AIII functions as translation factor during NMD, one experiment using a PTC-containing reporter which has a hairpin structure in the 5'UTR could be undertaken. It was demonstrated that a hairpin structure in the 5'UTR of TCR-β containing a PTC, inhibited its degradation (Wang et al., 2002a). Using this reporter, overexpression of eIF4AIII should alleviate inhibition and restore NMD. A mutant of eIF4AIII which

abrogates helicase activity or overexpression of eIF4AI wild type should have no effect, if eIF4AIII indeed substitutes for eIF4AI in the pioneer round. Alternatively, to determine whether eIF4AI is excluded from the pioneer round, pateamine could prove useful. Patemaine inhibits NMD (M.A. Ferraiuolo, unpublished data); if this is an effect of its action on eIF4AI, then RNAi against eIF4AI and incubation with pateamine should reduce the inhibitory effect of the drug on NMD.

Alternatively, the interaction of eIF4AIII with eIF4GI observed in mammalian cells may be explained by the presence of MIF4G domains in Upf2 (see section 1.2.3.2) (Mendell et al., 2000). Perhaps Upf2 recruitment to the EJC occurs through interaction with eIF4AIII which disrupts its interaction with eIF4G at the 5'end, disrupting the closed loop, exposing the 5'end and eliciting decay enzymes. However, whether eIF4AIII at the EJC interacts with eIF4GI at the 5'end and whether eIF4AIII interacts with Upf2 in a mutually exclusive manner with eIF4GI has not been verified. Furthermore, one must reconcile the fact that tethered eIF4AIII bypasses the need for Upf2 to degrade the mRNA (Gehring et al., 2005). Identification of an eIF4G-like protein from mass spectrometry analysis of purified splicesomes raises the possibility that eIF4G-like, rather than Upf2, competes for interaction with eIF4G for eIF4AIII (Jurica et al., 2002; Rappsilber et al., 2002; Zhou et al., 2002). Indeed eIF4G-like contains MIF4G and MA-3 domains and interacts with eIF4AIII, but not eIF4AI, in HeLa cells; this interaction persists in micrococcal nuclease treated extracts (L. Huck, unpublished data). Proteomics has identified that OIP2, a protein with 3'-5' exoribonuclease activity, interacts with eIF4G-like (L. Huck, personal communication). Therefore, an eIF4G-like interaction with eIF4AIII may induce a transition in the mRNP which allows for the

recruitment of decay enzymes. Alternatively, eIF4G-like may substitute for eIF4G in the pioneer round of translation.

In *Drosophila*, where exon-exon junctions do not determine PTCs, eIF4AIII is required for proper *oskar* localization to the posterior pole (Palacios et al., 2004). eIF4AIII is a component of *oskar* mRNP complexes where it may play a role in transporting and silencing translation of *oskar* until it is localized to its proper end. *Drosophila* Mago, Y14 and Barentsz are all necessary for proper *oskar* mRNA localization (Hachet and Ephrussi, 2001; Mohr et al., 2001; Palacios et al., 2004; van Eeden et al., 2001). Moreover, Barentsz is a component of mRNPs in the dendrites of hippocampal neurons (Macchi et al., 2003). Collectively, this suggests that EJC components could also function in mRNA localization or regulate the expression of specific mRNAs in humans (Bono et al., 2006).

Clearly the ATPase function of eIF4AIII and its ability to bind to eIF4G/eIF4G-like requires further characterization. From one perspective, the role of eIF4AIII in NMD may be considered as a translational inhibitor since its presence at the EJC inhibits the production of truncated protein. From another perspective, eIF4AIII may indirectly stimulate translation by enhancing the local concentration of eIF4G and perhaps eIF4AI/II onto spliced mRNPs (Chan et al., 2004). Since siRNA against eIF4AIII did not affect ^{35}S-Methionine incorporation, it may regulate the translation of selective mRNAs, like its *Xenopus* counterpart. Furthermore, it remains to be determined whether the role of human eIF4AIII in translation, if any, is distinct from its function in NMD. The studies performed by Shibuya *et al* and the newly reported crystal structure of eIF4AIII have solidified the concept that eIF4AIII represents a novel class of DEAD-box proteins

which act as "placeholders" on the RNA (Andersen et al., 2006; Bono et al., 2006; Shibuya et al., 2006). Since the binding to RNA occurs in a sequence independent manner, it will be interesting to see if eIF4AIII acts as a general "placeholder" upon which other mRNP complexes are assembled. One intriguing possibility is that it may perform a function in RISC loading onto the mRNA. Interestingly, a 6nt stretch on the mRNA is essential for miRNA-mRNA complexes to form (Sarnow et al., 2006). Incidentally, eIF4AIII binds a 6nt stretch of the RNA template used in the crystal structure studies, and the EJC encompasses a 8nt stretch during pre-mRNA splicing (Andersen et al., 2006; Ballut et al., 2005; Bono et al., 2006; Le Hir et al., 2000a).

4.3 4E-T Future Directions

4E-T is an eIF4E-binding protein which is unique amongst its members. It regulates eIF4E function in cap-dependent translation by affecting its localization and sequestering it from the translating pool, either by carrying it into the nucleus or by sequestering it to P-bodies (Dostie et al., 2000a; Ferraiuolo et al., 2005). The role of eIF4E in the nucleus is not quite clear. It has been proposed to mediate the transport of growth-promoting mRNAs which contain extensive secondary structure in their 5'UTR (Rousseau et al., 1996). However, the molecular mechanisms underlying eIF4E release from 4E-T in the nucleus, eIF4E storage with splicing factors in speckles, and the signalling pathways and interactions regulating its release from these nuclear factories to engage in selective transport is not known. The nuclear receptor and auxiliary factors involved in the transport pathway have yet to be characterized as well. To date, PML is the only reported protein shown to regulate the nuclear function of eIF4E (Cohen et al., 2001; Lai and

Borden, 2000). It is suggested that it inhibits eIF4E-mediated transport by reducing its affinity for the cap (Cohen et al., 2001; Lai and Borden, 2000). 4E-T may play a defining role in the nuclear function of eIF4E. eIF4E is released from splicing factories by cap analogs as well as in cells overexpressing the SR protein kinase, Clk/Sty (Dostie et al., 2000b). eIF4E, however, does not contain SR repeats and is likely not a direct substrate of the kinase. 4E-T, on the other hand, contains SR repeats which are conserved in mammalians (Fig. 1.7). Consequently, it is conceivable that 4E-T may regulate eIF4E function through this motif. An *in vitro* kinase assay to test whether 4E-T is a direct target of the Clk/Sty kinase, and the effect of mutations within this motif on eIF4E localization and function should be investigated.

4E-T is a phosphoprotein (Pyronnet et al., 2001). 4E-T phosphorylation correlates with decreased binding to eIF4E, at least in mitosis-arrested cells (Pyronnet et al., 2001). Accordingly, hypophosphorylation of 4E-T correlates with decreased translation rates under prolonged hypoxia, presumably due to its increased interaction with eIF4E (Koritzinsky et al., 2006). Under steady state conditions, 4E-T is phosphorylated ((Pyronnet et al., 2001) and M. Jaramillo, unpublished data). The signalling pathway/s and kinase/s influencing its phosphorylation, the residues phosphorylated, and the effect of phosphorylation on 4E-T functions (i.e. the nuclear import of eIF4E and 4E-T/eIF4E localization in P-bodies) at steady state, and under various stresses, is currently under investigation. 4E-T may be subject to phosphorylation/dephosphorylation in both cellular compartments (nucleus and cytoplasm), and by different enzymes. To date, the phosphorylation of 4E-T appears to be sensitive to stimulation by serum and its phosphorylation is inhibited by the MEK

inhibitor U0126 (M. Jaramillo, unpublished data). Therefore, 4E-T is influenced by the ERK pathway. Identification of the sites phosphorylated by these kinases will aid to decipher the role of this signalling cascade on the reported functions of 4E-T. A putative phosphorylation site by ERK is serine 587. This residue was identified by proteomic analysis of phosphporylated sites in the developing mouse brain (Ballif et al., 2004) and is conserved in other species (Fig. 1.7).

The murine homolog of 4E-T, Clast4, is also post-translationally modified by phosphorylation (Villaescusa et al., 2006). The function of 4E-T phosphorylation in this study was not documented; however, several interesting observations were reported, including the identification of an alternatively spliced isoform of 4E-T, which is missing the arginine stretch and the NLS present in the longer isoform. EST database search revealed a human EST sequence corresponding to this shorter isoform. It would be interesting to know if it is expressed differentially from the long form amongst various tissues, whether the short form of 4E-T is produced as a result of an autoregulatory response, and whether it exclusively functions in mRNA decay (since 4E-T localization in P-bodies is insensitive to LMB treatment). Also, the importance of the arginine rich region in the isoform1 (4E-T), which is missing in isoform2, may allude to a role in the nuclear function of isoform1. Secondly, a two hybrid screen identified a subunit of eIF3 as an interactor of murine Clast4 (unpublished observations by (Villaescusa et al., 2006)). Similarly, two subunits of eIF3 coimmunoprecipitated with 4E-T from HeLa cells and were identified by mass spectrometry analysis (J. Dostie, unpublished data). This raises the interesting possibility that 4E-T binds to eIF4E and eIF3 instead of eIF4G and represses translation of certain mRNAs for later activation by eIF4G. A role of 4E-T in

silencing mRNAs is in accordance with its localization in P-bodies (see below). Furthermore, the *Drosophila* 4E-T homolog, Cup, plays a role in translational repression of *nanos* and *oskar* mRNA and is present in silencing particles (see below; (Chekulaeva et al., 2006; Wilhelm and Smibert, 2005)).

Clearly, 4E-T plays a role in deadenylation-dependent mRNA decay (Chapter 3). What might be the molecular mechanism behind the role of 4E-T in decay? Because 4E-T interacts directly with eIF4E and this interaction is necessary for the reduced expression of a luciferase reporter (Fig. 3.2), it presumably directly interferes with recruitment of the 43S pre-initiation complex and inhibits translation. This interaction may precede deadenylation of the mRNA and could possibly play an indirect role in recruiting the deadenylase complex once PABP and eIF4G no longer interact. Alternatively, it may play a more direct role by interacting with protein/s bound at the 3'UTR, such as TTP and/or BRF-1, which can trigger recruitment of the deadenylase complex (Lai et al., 2003; Lykke-Andersen and Wagner, 2005). Interestingly, the RNA-binding protein Smaug, which interacts with Cup, recruits the Ccr4 deadenylase complex in *Drosophila* embryos (Semotok et al., 2005). The recruitment of the Ccr4 complex by Smaug is either the result of Smaug/Cup/eIF4E mediated translational repression of *nanos* or occurs simultaneously with repression (Semotok et al., 2005). In mammalian cells, disruption of the translation initiation complex by 4E-T/eIF4E may trigger the recruitment of deadenylase activity by 4E-T interaction with ARE-BPs. On the other hand, 4E-T may bind eIF4E after deadenylation. A stimulus may trigger recruitment of a 3'UTR binding protein which favors recruitment of the deadenylase complex and removal of the poly(A) tail, disrupting the closed loop and resulting in 4E-T tethering to

the cap-bound eIF4E. 4E-T may then elicit mRNP rearrangements which favor aggregation of mRNAs into P-bodies and consequently a decrease in translation. It would therefore be a point of interest to determine if 4E-T associates with polyadenylated mRNA and/or deadenylated mRNA as well as to identify 4E-T interacting proteins (i.e. deadenylases, ARE-BPS etc; see below).

The formation of P-bodies is a process which is clearly dependent on 4E-T since its depletion from HeLa cells resulted in disruption of P-bodies (Fig. 3.5). What are the signals which prompt 4E-T to assemble P-bodies? The effect of 4E-T phosphorylation on P-body assembly is currently being studied (see above). Interestingly, rapamycin negatively affected P-body formation. HeLa cells treated overnight with rapamycin exhibited a decrease in P-bodies (M.A. Ferraiuolo, unpublished data). The effect of rapamycin on the phosphorylation of 4E-T, and on its interaction with eIF4E, remains to be established. Furthermore, the effect of such a treatment on the phosphorylation of eIF4E as well as other P-body components must also be assessed. Stressing HeLa cells with arsenite or heat shock caused the expected formation of stress granules but 4E-T did not colocalize with stress granule markers, rather it was present in close proximity to stress granules, as was reported for other P-body components (M.A. Ferraiuolo and R. Mazroui, unpublished data). This may denote that 4E-T plays primarily a role in mRNA decay and not in translational repression. To gain insight into the relationship between cellular translation and P-body formation, infections with several viruses are being tested for their effect on P-bodies. In yeast, P-bodies are sites of viral assembly (Beliakova-Bethell et al., 2006). In mammalian cells, P-bodies have not only been implicated in viral assembly, but also in host-cell defense mechanisms against viruses (Wichroski et al.,

2006). Analysis of the viral effect on P-bodies could reveal which host factors play a role in P-body assembly/disassembly and could indicate the factors involved in viral assembly and host-cell defense. Furthermore, since viruses are also subject to regulation by miRNAs, studying viruses could illuminate the role of 4E-T and other host factors in the regulation of gene expression by miRNAs (Sarnow et al., 2006; Schutz and Sarnow, 2006). Interestingly, several have alluded to the possibility of a link between P-bodies and disease (Marx, 2005). Hypoxia is a marker for poor prognosis in human tumors. Under prolonged hypoxic conditions, 4E-T is hypophosphorylated and translation is inhibited (Koritzinsky et al., 2006). The effect of hypoxia on 4E-T/eIF4E interaction, localization in P-bodies and mRNA stability is currently under investigation.

The possibility of 4E-T mediated translational silencing in P-bodies has emerged from this study (Chapter 3). 4E-T may incite repression in several manners. Firstly, 4E-T may induce repression mediated by miRISC. RISC components and mRNA targets of miRNA are localized in P-bodies (see sections 4.1 and 1.3.5). 4E-T is imperative to the formation of P-bodies (Fig. 3.5). Consequently, 4E-T should play a decisive role in miRNA translational repression. However, knockdown of 4E-T resulted in a slight derepression of translation (<2-fold) of a luciferase reporter fused to the natural 3'UTR of K-Ras (which has several let-7 miRNA binding sites) when compared to a control siRNA (M.A. Ferraiuolo, unpublished data), and had no effect on a reporter fused to the synthetic let-7 miRNA binding sites (L. Huck, unpublished data). The observed effect on the K-Ras reporter fusion was not significant to conclude that 4E-T plays a crucial role in miRNA silencing, similar to the results obtained from depleting the Cup-like protein in *Drosophila* S2 cells (Rehwinkel et al., 2005). Recently, it was demonstrated that

knockdown of Lsm1 in HeLa cells disrupts P-bodies but like 4E-T has no affect on miRNA-mediated repression (Chu and Rana, 2006). Based on these results (and ours), this would suggest that miRNA-mediated translational repression does not require P-body structures and the localization of mRNA targets and RISC components in P-bodies is the *effect* of translational repression and not the *cause* of translational repression (Chu and Rana, 2006). The role of Lsm1 and 4E-T in the miRNA response cannot entirely be ruled out. It is possible that they exert functions in miRISC mediated repression other than let-7 miRISCs. In this regard, other reporters should be tested. Furthermore, to entirely rule out the *causal* effect of P-bodies on miRISC repression, the localization of GW182 in 4E-T depleted cells should be tested. A function of GW182 in RNA silencing has been established (Behm-Ansmant et al., 2006; Ding et al., 2005; Jakymiw et al., 2005; Liu et al., 2005a; Rehwinkel et al., 2005) Furthermore, miRNA processing is required for formation of GW-bodies (Pauley et al., 2006). Therefore, RNAi may specifically take place in GW182-containing bodies or may move from P-bodies to GW bodies for the last step in RNAi biogenesis (Pauley et al., 2006). In contrast to 4E-T and Lsm1, depletion of p54 disrupts P-bodies and alleviates repression induced by miRNA (Chu and Rana, 2006). Depletion of p54 caused an increase in general translation rates, in addition to release of miRNA-mediated repression (Chu and Rana, 2006; Coller and Parker, 2005). In addition, p54 was isolated from active RISC complexes and interacts directly with Ago1 and Ago2 (Chu and Rana, 2006). Depletion of Lsm1 had no effect on p54 interaction with Agos (Chu and Rana, 2006). However, p54 depletion did not affect siRNA mediated silencing by RISC (Chu and Rana, 2006). Therefore, p54 is specific to the miRNA response. Intriguingly, 4E-T may play a specific role in the siRNA response

as opposed to the miRNA response. Preliminary results have suggested a role of 4E-T in siRNA-mediated decay. siRNA against 4E-T, but not a control siRNA, inhibited gene silencing by siRNA against eIF4E or lamin A/C (M.A. Ferraiuolo, unpublished data). Furthermore, depletion of 4E-T inhibited Ago2 ("slicer") localization in P-bodies (M.A. Ferraiuolo, unpublished data). Whether 4E-T knockdown inhibits degradation of a siRNA reporter has yet to be tested. Moreover, it would be interesting to see the effect of 4E-T knockdown on p54 interaction with Ago1 and Ago2 and other RISC components.

. Secondly, since 4E-T inhibited cap-dependent translation, it is possible that it mediates selective mRNA repression by interaction with proteins bound to the 3'UTR, other than RISC, such as is reported for its *Drosophila* homolog. 4E-T may act as an adaptor molecule between eIF4E at the 5'end and a protein bound at the 3'end, similar to the way Cup is sandwiched between eIF4E and Bruno. Alternatively, 4E-T may interact with eIF4E at the 5' end and interact directly with an RNA element in the 3'UTR much like the 4E-HP/Bicoid/BRE (Bicoid Responsive Element) interaction (Cho et al., 2005; Wilhelm and Smibert, 2005). Some candidate proteins at the 3'end that may interact with 4E-T are: ARE-BPS, which have known functions in the regulation of translation (Barreau et al., 2005), Bruno human homologs (Good et al., 2000) and CPEB (cytoplasmic polyadenylation element (CPE) binding factor), the latter of which is complexed with 4E-T in *Xenopus* oocytes (N. Standart, personal communication) and which is localized in mammalian P-bodies (Wilczynska et al., 2005). Interestingly, *Xenopus* p54 (Xp54) and CPEB interact in oocytes in large complexes which also contain eIF4E; furthermore, tethered Xp54 represses translation in *Xenopus* oocytes (Minshall and Standart, 2004). An interaction of 4E-T with p54 was also detected in *Xenopus*

oocytes, which is independent of RNA, therefore, 4E-T may be in a complex with Xp54 and CPEB and repress translation by preventing the binding of eIF4E with eIF4G in *Xenopus* oocytes (N. Standart, personal communication). It would be of interest to determine if 4E-T interacts with p54 *in vivo* in mammalian cells, and whether general translation is affected by this interaction. The idea that 4E-T interacts with specific RNA elements in the 3'UTR is suggested by the presence of an arginine rich stretch within 4E-T and a serine rich stretch which may function as RNA binding domains (Fig. 1.7). The ability of 4E-T to interact with RNA directly should be examined.

The latter point raises a third possibility in which 4E-T may mediate repression. This is inspired by studies performed in *Drosophila*, in which *oskar* mRNA silencing occurs by Bruno-mediated RNA oligomerization (Chekulaeva et al., 2006). Although RNA oligomerization is independent of a Cup/eIF4E interaction, Cup is present in these large RNP particles (Chekulaeva et al., 2006). Surprisingly, it was also observed in this study that Cup recruits eIF4E to the mRNA independent of the cap (Chekulaeva et al., 2006). Therefore, examining the role of these putative RNA-binding elements, discussed above, within 4E-T may prove informative. Furthermore, the high proline content of this protein implies a role in multiprotein associations, which may be needed for mRNP aggregation. Lastly, it should be reiterated that the speculated modes of translational repression mediated by 4E-T here, derives from the fact that 4E-T is present in P-bodies (which are "silencing centers") and that its overexpression inhibits cap-dependent translation in HeLa cells. 4E-T can hypothetically regulate gene expression in two manners: translation repression and mRNA degradation. Whether the two are separable roles of 4E-T requires further analysis; although we have demonstrated a role of 4E-T in

mRNA decay, translation repression and mRNA decay are not necessarily mutually exclusive properties of 4E-T, as was demonstrated for GW182 (Behm-Ansmant et al., 2006). It is conceivable that the biological context or stimuli and the nature of the resultant mRNA-bound proteins will dictate the pathway by which 4E-T incites a decrease in gene expression, i.e. mRNA decay versus translational repression or both.

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From: "Millerd, Tiffany" <TMillerd@nas.edu>
To: mferra1@po-box.mcgill.ca
Subject: RE:
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Dear Ms. Ferraiuolo,

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Please feel free to contact us with any additional questions you might have.

Best regards,
Tiffany Millerd for
Diane Sullenberger
Executive Editor
PNAS

-----Original Message-----
From: mferra1@po-box.mcgill.ca [mailto:mferra1@po-box.mcgill.ca]
Sent: Thursday, July 27, 2006 7:58 PM
To: PNAS Permissions
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To The PNAS Permissions Editor,

attached is an official request for permission to include the published manuscript in my doctoral thesis.

Regards
Maria Ferraiuolo

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