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An examination of the effects of 4E-T-mediated re-localization of eIF4E on eIF4E function

par

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An examination of the effects of 4E-T-mediated re-localization of eIF4E on eIF4E function

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Résumé

Le facteur d'initiation de la traduction chez les eukaryotes eIF4E (4E) est un puissant oncogène en raison de sa capacité à faciliter l'export et/ou la traduction de certains transcripts, dont beaucoup sont eux-mêmes des oncogènes. 4E intéragit avec un grand nombre de protéines régulatrices dont la protéine 4E-T (pour 4E-Transporter). La capacité de 4E-T à modifier la localisation subcellulaire de 4E pourrait offrir un mécanisme permettant de modifier le potentiel oncogène d'une cellule. La surexpression de 4E-T dans des cellules d'ostéosarcome conduit à l'augmentation du nombre et de la taille des P-bodies, dans lesquels 4E colocalisent avec 4E-T mais pas avec la version tronquée 4E-T/Y30A. Cependant, les différentes expériences menées, permettant d'analyser les taux de transcription, la quantité de protéine, les profiles polysomiques ainsi que la distribution nucléo-cytoplasmique, montrent que la surexpression de 4E-T n'a pas d'effet sur la fonction de 4E. L'observation d'un enrichissement cytoplasmique et d'une charge réduite de ribosomes sur les transcripts codant les protéines cycline D1 et ODC (profile polysomique) dans la lignée 4E-T suggère un role de 4E-T dans la séquestration cytoplasmique de certains transcrips par un mécanisme qui reste encore à déterminer.

Mots-clés : 4ET, eIF4E, P-body

Abstract

The eukaryotic translation initation factor eIF4E (4E) is a potent oncogene due to its ability to facilitate export and/or translation of certain transcripts, many of which are, themselves, oncogenes. 4E has many protein-binding partners including the 4E transporter protein (4E-T). The ability to alter the subcellular localization of 4E via 4E-T could offer a mechanism by which to alter the oncogenic potential of a cell. Overexpression of 4E-T in osteosarcoma cells leads to formation of larger and more numerous P-bodies that can effectively colocalize with 4E than either a vector control (E) or a less efficient 4E-binding version of 4E-T (Y30A). However, the overexpression of 4E-T did not affect the 4E's function as determined by examining global levels of transcription, polysome profile, cytoplasmic/nuclear distribution, or protein levels of most of the transcripts tested. The observation that there was cytoplasmic enrichment and reduced loading of ribosomes on cyclin D1 and ODC transcripts (polysome profile) in the 4ET line suggests a possible 4ET-mediated cytoplasmic sequestration of certain transcripts by an as yet to be determined mechanism.

Keywords : 4E-T, P-body, 4E

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To the memory of my father

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Chapter 1. General Introduction

This study explores the effect of overexpression of eukaryotic translation initiation factor transporter EIF4ENIF1 (referred to as 4E-T in this work) on its relationship with the eukaryotic translation initiation factor eIF4E (4E). A review of the literature concerning RNA export and translation as well as the current knowledge to date about 4E and 4E-T follows.

Transcript generation and movement in the eukaryotic cell

The central dogma of molecular biology in its most general form states that DNA is transcribed to messenger RNA (mRNA) and mRNA is translated to protein (F. Crick, 1970; F. H. Crick, 1958) (Figure 1.1). If we assume that proteins are the active species in a cell and that the variety and abundance of proteins within a cell is important in determining health versus disease status, then the pool of RNA available to be translated to protein becomes of prime importance. The pool of RNA within a cell is regulated by rates of transcription, mRNA nuclear export, and mRNA sequestration (or in mathematical terms, where k= rate; RNA pool = $k_{Transcription}$- $k_{mRNA\ nuclear\ export} - k_{mRNA\ degradation}$ - $k_{mRNA\ sequestration}$) (Figure 1.1). Crudely, protein abundance within a cell can be expressed as $k_{Translation} - k_{Protein\ degradation}$ (Figure 1.1).

Transcription rate is influenced by promoter strength and presence of regulatory elements for the gene in question. DNA is initially transcribed in the nucleus by RNA polymerase II to precursor mRNA. Shortly after transcription initiation, a 7-methyl guanosine dinucleotide cap structure (m^7GpppN, where N is any nucleotide) is added to the 5' end of the RNA. Introns are contranscriptionally removed from the precursor mRNA, RNA editing occurs, and the cleavage and polyadenylation of the 3' end take place. A variety of RNA-binding proteins interact with the precursor mRNA during these processes and afford a level RNA quality control (known as RNA surveillance) within the nucleus (reviewed by Moraes, 2010). While undergoing proper processing, the mRNA also needs to fold into a favorable tertiary structure—folding is thought to occur via the kinetic energy conformation-based model (Schroeder, Grossberger, Pichler, & Waldsich, 2002). If there are errors in the nascent mRNAs and/or their tertiary structure, they may be subject to degradation via 3'to 5' exosome complexes either in the nucleus or the cytoplasm.

Export of mRNA from the nucleus to the cytoplasm is controlled in a process known as gatekeeping (reviewed by Dimaano & Ullman, 2004; Saguez, Olesen, & Jensen, 2005; Vinciguerra & Stutz, 2004). Gatekeeping occurs at the nuclear pore and works in at least two ways: 1) it restricts export of transcripts that are incompletely matured; and 2) it promotes export of mRNAs that are associated with the appropriate proteins. Mammalian RNA export through nuclear pores can be facilitated by association with various RNA-binding proteins depending on the type of RNA (as reviewed by Kohler & Hurt, 2007). tRNA export is mediated by Exp-t protein, while miRNA export is mediated by Exp-5. The assembly of CBC, PHAX and CRM1 aids snRNA export. rRNA export involves the proteins Mex67, Mtr2, Arx1, Nmd3, and CRM1. tRNA, miRNA, snRNA, and rRNA export all require Ran GTPases. mRNA export involves CBC, ALY/Yra1, TAP/Nex67, and p15/Mtr2. The translation initiation factor eIF4E (4E) can also facilitate the export of certain mRNAs, which possess a specific secondary structure in their 3' untranslated region (UTR). A more detailed discussion about 4E's export function follows in the eIF4E section of this introductory chapter.

Aberrant transcripts must be removed in order to prevent translation of abnormal proteins (reviewed in Isken & Maquat, 2007). In mammals, when an mRNA possesses a premature termination codon (PTC), nonsense-mediated decay (NMD) can occur. Classical NMD requires a splicing event downstream from a PTC while fail-safe NMD requires a splicing event upstream of a PTC. If the transcript lacks a termination codon, then non-stop decay can occur. If the transcript causes translation elongation to stall, then and non-go decay can occur.

Normal transcript can be removed via binding of microRNAs (miRNAs). miRNAs are small, endogenous, non-coding ~22 nucleotide RNAs that regulate gene expression of approximately 60% of genes in the genome by interfering with translation or stability of target transcripts (reviewed in Jackson & Linsley, 2010). They bind through partial complementarity, mostly involving an 8-nucleotide seed sequence to 3' UTR of target mRNAs. As the miRNAs do not require perfect complementarity for target recognition, a single miRNA may regulate multiple mRNAs.

Normal mRNA turnover helps the cell achieve its biochemical equilibrium. Integration of physiological signals determines when a transcript is to be degraded. Rate of degradation of a specific transcript is controlled by *cis*-acting elements in the mRNA and *trans*-acting factors that bind these *cis* elements. In this way, the specific RNA-binding proteins that interact with a given mRNA will affect the mRNA's stability (and consequently abundance) in the cytoplasm. Examples of *cis* elements include the AU-nucleotide rich elements (AREs) found in the 3' UTRs of mRNAs (reviewed in Kedersha & Anderson, 2002). HuR and tristetrapolin protein (TTP) are examples of *trans* factors that bind to AREs. HuR is a member of a superfamily of elav-related proteins that can bind AREs. HuR is predominantly nuclear, but shuttles between the nucleus and the cytoplasm. When HuR is bound to an ARE, it stabilizes the mRNA. In contrast, TTP is a predominantly cytoplasmic ARE binding protein that targets certain mRNAs (e.g. tumour necrosis factor-α, granulocyte-macrophage colony-stimulating factor, and interleukin-3) for degradation. Interestingly, both HuR and TTP are found in both stress granules and processing bodies (P-bodies)—to be discussed in the P-bodies and Stress Granules section of this introductory chapter.

Appropriate cytoplasmic localization of certain mRNAs for proper function represents another type of pre-translational mRNA quality control (Gray & Wickens, 1998; Isken & Maquat, 2007). Transcript localization is important for the spatial regulation of genes involved in protein secretion, synaptic plasticity, cell motility, and embryonic axis formation (reviewed in Holt & Bullock, 2009; Mohr & Richter, 2001). Selection of mRNA destined for localization appears to be determined by binding of *trans* factors binding to *cis*-elements in the 3' UTR (Mohr & Richter, 2001). These ribonucleoprotein (RNP) complexes are linked to cytoskeletal elements (microfilaments or microtubules) along which the RNPs travel. Molecular anchors are required to fix transcripts at their final subcellular destinations. While in transit, the transcripts are translationally silenced. Holt and Bullock (2009) suggest that transcript localization offers certain advantages over targeting protein product: 1) increased efficiency afforded by producing multiple protein molecules from a single mRNA molecule; 2) prevention of ectopic protein activation during translocation; 3) facilitating assembly of macromolecular protein complexes; and 4) decentralizing control of gene expression by permitting local control of translation on demand in response to a signal.

The rate of ribosome loading onto the transcript is important in determining the overall protein abundance in a cell. Eukaryotic translation involves initiation, elongation and termination. Translation initiation mostly occurs via 4E in a cap-dependent manner (Sonenberg, 1988). A schematic drawing of the elements involved in cap-dependent translation initiation is depicted in Figure 1.2. Certain transcripts that possess complex 5' UTRs appear to be more reliant on 4E for translation initiation—so-called 4E-sensitive transcripts (discussed in more detail in the 4E section).

Other translation initiation factors can either display increased or decreased expression and/or phosphorylation status changes in certain cancers (reviewed in Silvera, Formenti, & Schneider, 2010). Uncoupling of translation regulation from inhibition by cellular stresses such as hypoxia and nutrient deprivation is often seen in human cancers (reviewed in Silvera, et al., 2010). Alterations in translation regulation can also be associated with specific types of cancer and/or different stages of disease or transformation.

Cap-independent translation initiation can occur via internal ribosome entry sites (IRESs) (reviewed in Gray & Wickens, 1998; van der Velden & Thomas, 1999). Other transcripts such as those encoding for certain ribosomal proteins and translation elongation factors make use of 5' terminal oligopyrimidine (5'TOP) sequence for translation initiation (van der Velden & Thomas, 1999).

Protein degradation is influenced by posttranslational modifications such as ubiquitiniation and sumoylation. However, the focus of this M.Sc. work explores the observed effects of 4ET overexpression on $k_{mRNA\ nuclear\ export}$, $k_{mRNA\ sequestration}$, and $k_{Translation}$ for 4E target RNAs.

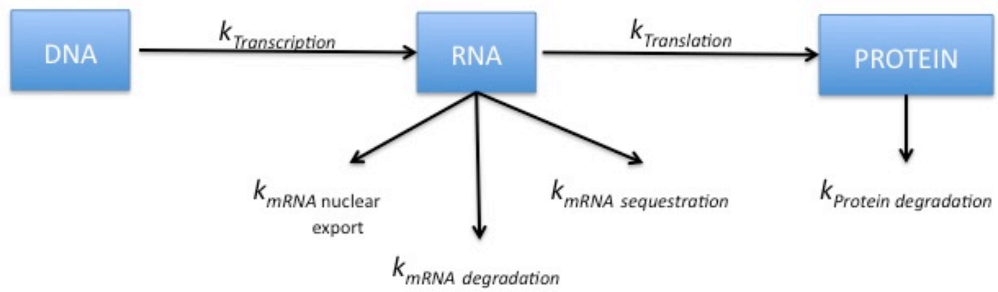


Figure 1.1 The central dogma of molecular biology showing some rate processes, which affect cellular RNA and protein levels. (adapted from (de Sousa Abreu, Penalva, Marcotte, & Vogel, 2009)

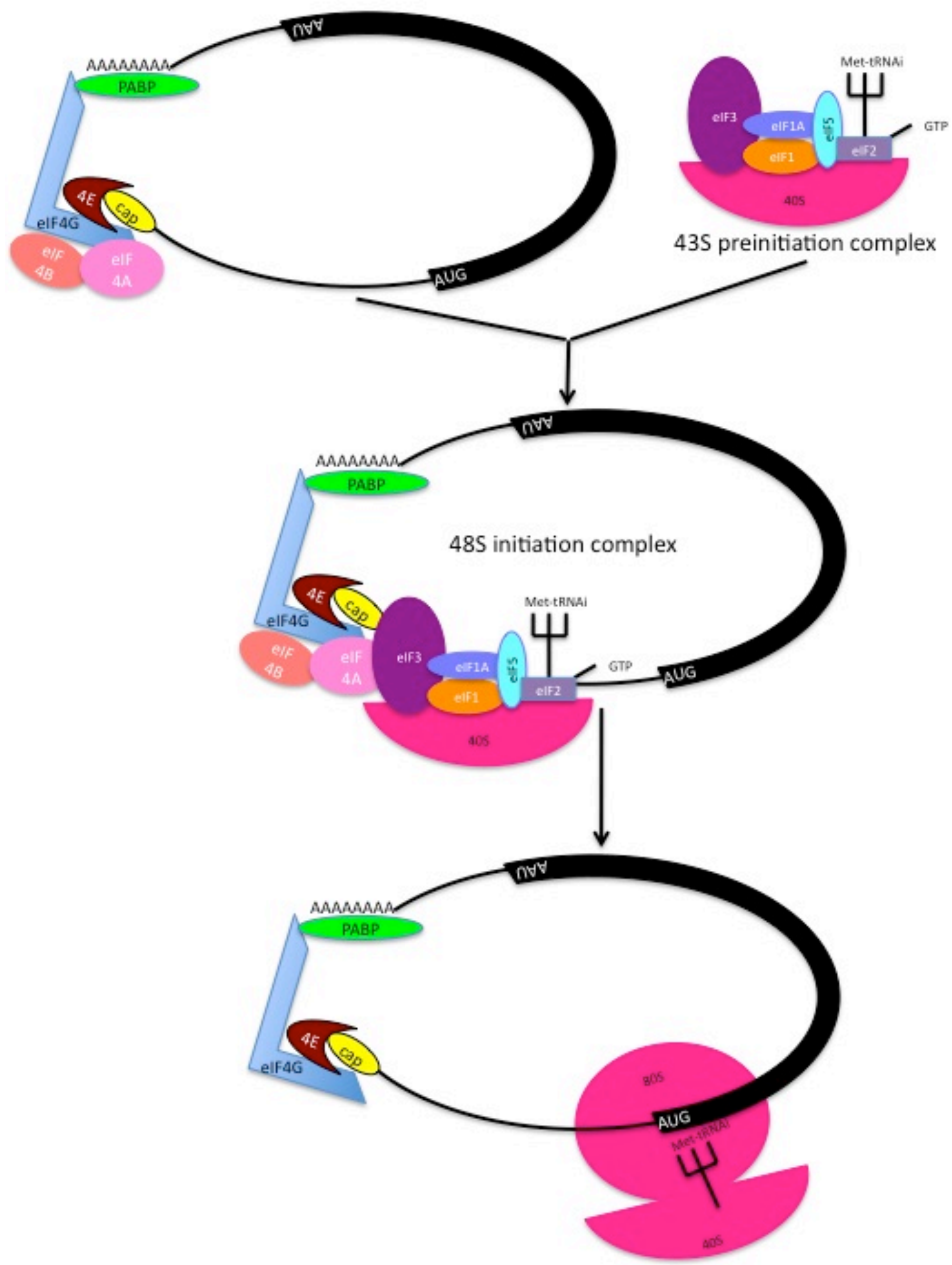


Figure 1.2. Preininitiation and translation initiation complex. eIF2-GTP-tRNA = ternary complex. eIF4A + eIF4E + eIF4G = eIF4F. PABP = polyA binding protein. 40S + 80S = ribosome.

eIF4E

The eukaryotic translation initiation factor eIF4E (4E) regulates gene expression post-transcriptionally at the levels of mRNA translation and mRNA export (Culjkovic et al., 2008; Culjkovic, Topisirovic, Skrabanek, Ruiz-Gutierrez, & Borden, 2005; Sonenberg & Gingras, 1998). It has recently been reported that there are 3 classes of 4E (Joshi, Cameron, & Jagus, 2004; Rhoads, 2009) a comparison of the 4E members is presented in Table 1.1). 4E (human eIF4E-1) overexpression leads to oncogenic transformation in cell culture (Graff et al., 1995; Lazaris-Karatzas & Sonenberg, 1992) and tumour enlargement and increased metastases in xenograft mouse models (Ruggero et al., 2004). Interestingly, a newly identified member eIF4E1b shows oocyte specificity and strong binding to the 4E transporter protein 4ET (Evsikov & Marin de Evsikova, 2009; Standart & Minshall, 2008; Villaescusa et al., 2006). Unlike the other classes of 4E, eIF4E1b acts a a translational repressor of dormant maternal mRNAs (Evsikov & Marin de Evsikova, 2009).

Molecular basis for 4E mediated transformation

The oncogenic potential of 4E arises from its biochemical functions: mRNA export, translation initiation, and potentially mRNA stability. In the nucleus, 4E exports certain transcripts. An approximately 50-nucleotide sequence denoted the 4E sensitivity element (4E-SE) is found in the 3' UTR of transcripts whose export is effected by 4E (Culjkovic, et al., 2005). In both the nucleus and the cytoplasm, 4E associates with the 5' end of mRNAs via the cap structure (Sonenberg, 1988). In the cytoplasm, 4E, along with eIF4A and eIF4G, forms the 5'-cap binding protein complex and promotes cap-dependent translation initiation (Rhoads, 1988; Rozen et al., 1990)(Figure 1.2). Translation efficiency differs between mRNAs depending on the complexity of the secondary structure in their 5' UTRs. 4E overexpression increases translational efficiency of mRNAs with structured 5’ UTRs. These transcripts are considered 4E sensitive (4E-sensitive transcripts or 4E-STs). Examples of 4E-SE containing transcripts and 4E-STs are presented in Table 1.2.

Recent studies describe 4E as a focal point in an "RNA regulon" that governs cell proliferation and survival (Culjkovic, Topisirovic, & Borden, 2007). A regulon is an organizational unit that synchronizes the expression of specific genes post-transcriptionally that are involved in a common pathway (Keene & Lager, 2005). 4E coordinately promotes both the export of mRNA and translation of several genes involved in cell cycle progression and survival. The coordination occurs via co-factors binding elements in the 3' and 5' UTRs. Families of 4E-STs with similar secondary structures will be coordinately regulated.

4E is a clinically relevant oncogene which is elevated in 30% of cancers including breast cancer, colon cancer, head and neck squamous cell carcinoma, and several types of leukemia and lymphoma (Culjkovic & Borden, 2009; Graff & Zimmer, 2003). 4E is a diagnostic marker for metastases, increased tumour invasion and is correlated with poor prognosis. Targeting 4E in leukemia patients led to clinical benefit (reviewed in (Assouline et al., 2009; Graff & Zimmer, 2003).

Modes of Regulation of 4E

4E can be regulated at the level of its transcription, at the level of its subcellular localization, and at the level of its interaction with other proteins.

Controlling the expression of 4E mRNA

In some human 4E-related cancer cases, the elevated level is due to gene amplification of 4E (Sorrells, Meschonat, Black, & Li, 1999). In other cases, increased stability of the 4E transcript leads to its observed increased level (e.g. stabilization by the protein HuR (Topisirovic, Siddiqui, & Borden, 2009)).

Control of 4E through interactions with partner proteins

4E activity can be modulated by the presence or absence of 4E binding proteins. Proteins that interact with 4E fall into three broad categories (refer to Table 1.3): 1) proteins containing a conserved eIF4E-binding site (YXXXXLΦ, where X is any residue and Φ is a hydrophobic residue (Mader, Lee, Pause, & Sonenberg, 1995)) to interact directly with the dorsal surface of 4E; 2) proteins containing a RING domain (such as promyelocytic leukemia protein (PML) (Volpon, Osborne, Capul, de la Torre, & Borden, 2010)); and 3) proteins that do not contain YXXXXLΦ or a RING domain (such as invasion inhibitory protein 45 (IIp45) (Borden lab, unpublished)).

Table 1.1. Classes of eIF4E. (adapted from (Evsikov & Marin de Evsikova, 2009; Joshi, et al., 2004; Rhoads, 2009)

	Class I	Class IB	Class II	Class III
NM*	NM_001968.3	NM_001099408.1	NM_004846.2	NM_173359.4
Chromosomal location*	4q21	5q35.2	2q37.1	3p14
mRNA length*	4749 bp	1974 bp	1014 bp	6185 bp
Amino acid length*	217 residues	242 residues	245 residues	118 residues
Protein weight*	25 kDa	27.4 kDa	28 kDa	13.2 kDa
Cap binding tryptophans and/or substitutions**	W43 W56 W73 W103 W113 W130 W160	?	W43→Y, F, L W56→Y, F	W56→C, Y
Tissue distribution* (mRNA)	Ubiquitous, most in testes and muscle	growing oocyte	Ubiquitous, most in testes	Heart, skeletal muscle, lung, and spleen
Ability to bind cap	Yes	weak	Yes	Yes (ability to discriminate between GTP and m7GTP < Class I)
Ability to bind eIF4G	Yes	weak	No	Yes
Ability to bind 4EBPs	Yes	Unknown; binds 4ET strongly	No	Yes
Ability to rescue eIF4E- *S. cerevisiae*	Yes	unknown	No	No
Phylogenetic distribution	All eukaryotes	tetrapods	All metazoans	Chordates
Examples	*C. elegans* IFE-1 *C. elegans* IFE-2 *D. rerio* eIF4E1-B *S. pombe* eIF4E-2 *X. laevis* eIF4E-1B	*M. musculus* eIF4E1b	*A. thaliana* nCBP *C. elegans* IFE-4 *D. melanogaster* 4EHP *H. sapiens* 4E-HP *M. musculus* eIF4E-2	*M. musculus* eIF4E-3

*in humans
**tryptophan position is relative to eIF4E-1 in humans

Table 1.2. Transcripts that contain a 4E-SE and/or are 4E-sensitive. (adapted from (Culjkovic, Topisirovic, Skrabanek, Ruiz-Gutierrez, & Borden, 2006)

mRNA containing 4E-SE	4E-sensitive transcript	4E-sensitive transcript containing 4E-SE
Cyclin D1	VEGF	ODC-1
Cyclin E1		Cyclin B1
Cyclin A2		Pim-1
Mdm2		cMyc
Nbs1		
Fbox1		
CGGbp1		
P54nrb/NONO.1		
Selenoprotein S		

Table 1.3. 4E binding partners. (adapted from (Rhoads, 2009).

Binding motif	Sequence (if known)	Name
YXXXXLΦ	LLLDKRLRSEC	CYFIP1
	YDRKFL	4EBP1
	YVDSFLL	HOXA9
	YAPTPLL	PRH
	YDREFLL	EIF4G
	YSNPDLV	EMX2
	YTKKELL	4ET
	YFWPLLV	LRPPRC
	YIPPHLR	DDX3
	YTIDELF	p20
	TEADFLL	Maskin
	YRKEELE	Lipoxygenase 2
	YTRSRLM	Cup
	YIRPYLP	Bicoid
	RLQSTLKRIG	BTF3
	LKLPFLK and YEAVELL	Gemin5
	YPTEKGL, YQIDKLVKT, and YVPPRLV	Neuroguidin
AA 59-93 of TuMV VPg		Viral VPg proteins
		PGL-1
NHL domain		Brat
RING domain		PML
		Z
Unknown		IIP45

Homeoproteins that contain the conserved 4E-binding motif are tissue-specific factors that modulate 4E activity. PML and Z RING proteins interact with eIF4E via their RING domains leading to a 100X reduction in affinity of 4E for the m^7G cap (Cohen et al., 2001; Kentsis et al., 2001; Volpon, et al., 2010).

The best-characterized regulator of 4E is 4E-binding protein 1 (BP1). BP1 uses its YXXXXLΦ site to bind 4E and prevent its access to eIF4G and the rest of the translation machinery. Phosphorylation of BP1 reduces its interaction with eIF4E that leads to increased translation activity of 4E. BP1 and phospho-BP1 levels are modulated in human cancers including M4 AMLs. BP1 levels can be highly elevated in AML (Borden lab, unpublished) and breast cancers (Armengol et al., 2007) which is thought to reflect a change in translational status resulting from a change in local environment from high oxygen to anoxic.

Controlling 4E activity by modulating its subcellular distribution

As stated above, 4E is found both in the nucleus and in the cytoplasm. In the cytoplasm, 4E is found not only with actively translated transcripts, but also in processing bodies (P-bodies) (Andrei et al., 2005). P-bodies may act as a temporary storage depot for RNAs but may also be the site of RNA degradation. It is thought that the presence of 4E with RNAs in P-bodies is associated with a sequestration of such mRNAs.

Re-distribution of 4E can occur by multiple methods. Transduction of IκB-SR leads to re-organization of 4E by reducing the amount of 4E in the nucleus and increasing its amount in the cytoplasm as well as re-organization of remaining eIF4E nuclear bodies into structures that are morphologically indistinguishable from normal cells (Topisirovic, Guzman et al., 2003). IκB-SR transduction also leads to reduced 4E dependent mRNA export. Treatment with the m^7G cap analogue, m^7GpppG leads to disruption of 4E nuclear bodies and re-distribution of 4E in the cytoplasm (Cohen, et al., 2001; Dostie, Lejbkowicz, & Sonenberg, 2000). A chemical analogue of the m^7G cap, ribavirin, also leads to an increased fraction of 4E in the cytoplasm and reduced amount of 4E-dependent mRNA export (Assouline, et al., 2009; Kentsis et al., 2005; Tan, Culjkovic, Amri, & Borden, 2008). Some AML patient specimens can have mislocalized 4E where it is found primarily in the nucleus (Assouline, et al., 2009; Topisirovic, Culjkovic et al., 2003). Upon ribavirin treatment, these patients display a large fraction of 4E in the cytoplasm which is correlated with a positive response (Assouline, et al., 2009).

There are several endogenous proteins that can modulate the localization of 4E. These proteins include PRH, LRP and 4E-T (Ferraiuolo et al., 2005; Topisirovic, Guzman, et al., 2003; Topisirovic et al., 2009). 4E-T uses its conserved 4E-binding site to interact directly with the dorsal surface of 4E. Overexpression of 4E-T leads to relocalization of the majority of nuclear 4E to the cytoplasm, where a subset is founding processing bodies (P-bodies). The molecular mechanism for this re-distribution is as yet unknown.

4E-T

Dostie et al (2000) first described the 4E transporter protein 4E-T as a nucleocytoplasmic shuttling protein that contains a 4E-binding site, one bipartite nuclear localization signal and two leucine-rich nuclear export signals (see Figure 1.3). A number of transcripts for 4ET have been reported (summarized in Table 1.4). 4ET amino acid sequence is highly conserved across chordate species as is its 4E binding motif (boxed region) (Figure 1.4), which suggests that 4ET function and ability to bind 4E is evolutionarily conserved (and therefore of some functional significance). No 4ET orthologue has been identified in yeast. 4E forms a complex with the importin αβ heterodimer only in the presence of 4E-T. Dostie et al (2000) suggest that 4E-T mediates the nuclear import of 4E via the importin αβ pathway by a piggyback mechanism.

At steady state, 4E-T is predominantly cytoplasmic and appears to be concentrated in P-bodies. Ferraiuolo et al (2005) demonstrated that 4E-T colocalizes with mRNA decapping factors in bona fide P-bodies. 4E-T controls mRNA half-life, because its depletion from cells using short interfering RNA increases mRNA stability. The 4E-T binding partner, 4E, also localized to P-bodies when 4E-T was overexpressed. Ferraiuolo et al (2005) suggest that 4E-T interaction with 4E is a priming event in inducing messenger ribonucleoprotein rearrangement and transition from translation to decay.

4E-T appears to be involved in hypoxia-induced mRNA translation inhibition (Koritzinsky et al., 2006). Hypoxia inhibits mRNA translation initiation biphasically via two distinct pathways: the first phase occurs rapidly, reaching a maximum at 1–2 h and is due to phosphorylation of eIF2α; continued hypoxic exposure activates a second, eIF2α-independent pathway that maintains repression of translation. This phase is characterized by disruption of eIF4F and sequestration of 4E by its inhibitors 4E-BP1 and 4E-T.

Tissue distribution of 4ET mRNA from microarray studies is summarized in Figure 1.5 from data found at www.biogps.gnf.org. The dataset is an atlas of tissue expression (GeneAtlas) on an Affymetrix U133A array, using the gcrma algorithm to process the data. The Y-axis represents normalized, background-subtracted, and summarized (probes to probeset) intensity of the probeset. Highest expression of 4ET transcript appears to be in the testis, dendritic cells, retina, brain, and various blood cell lineages. No 4ET knockout mouse exists, but several knockout cell lines are available (MGI and International Mouse Knockout Consortium). In mice, P-bodies are also found in oocytes but they disappear upon oocyte growth while subcortical ribonucleoportein particle domains (SCRDs) form ((Flemr, Ma, Schultz, & Svoboda, 2010; Swetloff et al., 2009). If 4ET expression is similar to that of another P-body protein GW182, then it is variably but widely expressed in species (Eystathioy et al., 2002).

4ET shows different levels of phosphorylation in the cell cycle (Pyronnet & Sonenberg, 2001). 4ET becomes more phosphorylated and shows decreased binding to 4E in mitosis.

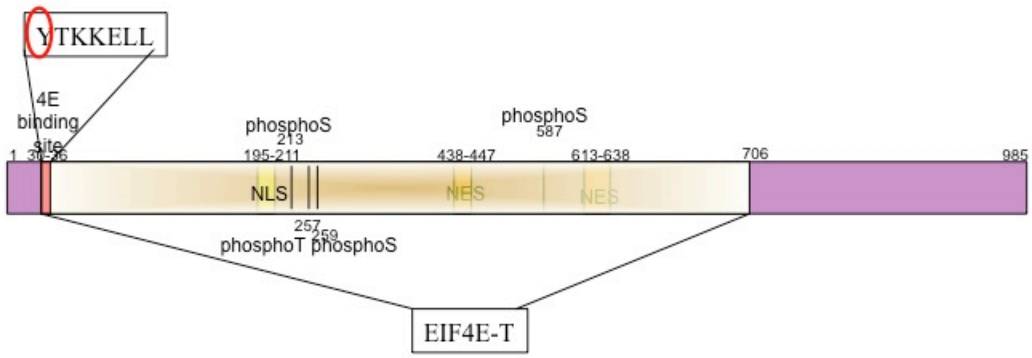


Figure 1.3. Cartoon of the 4E-T protein.

4E-T is shown with the N-terminus on the left and C-terminus on the right. Numbers denote amino acid positions. Putative phosphorylated residues are shown. The EIF4E-T domain is highlighted (domain defined by NCBI). NLS denotes nuclear localization signal. NES denotes nuclear export signal. The 4E-binding site is situated at amino acid positions 30-36. Y at position 30 when mutated to A partially abrogates 4E-binding.

Table 1.4. 4ET characteristics.

Feature			
NM*		019843.2	
Chromosomal position*		22q11.2	
Refseq alternate transcripts	# exons	# amino acids	Protein weight (kDa)
EIF4ENIF-001	17	811	88.2
EIF4ENIF-002	19	985	108.2
EIF4ENIF-003	19	985	108.2
EIF4ENIF-005	17	961	105.5
EIF4ENIF-006	5	188	21.4
EIF4ENIF-008	4	63	7.2
EIF4ENIF-009	4	63	7.2
EIF4ENIF-012	7	239	26.0
EIF4ENIF-201	18	961	105.5
EIF4ENIF-202	18	685	108.2
EIF4ENIF-203	9	63	7.3

*human; 4ET constructs for this work are based on NM019843.2 to produce a HA-tagged protein of approximately 140 kDa.

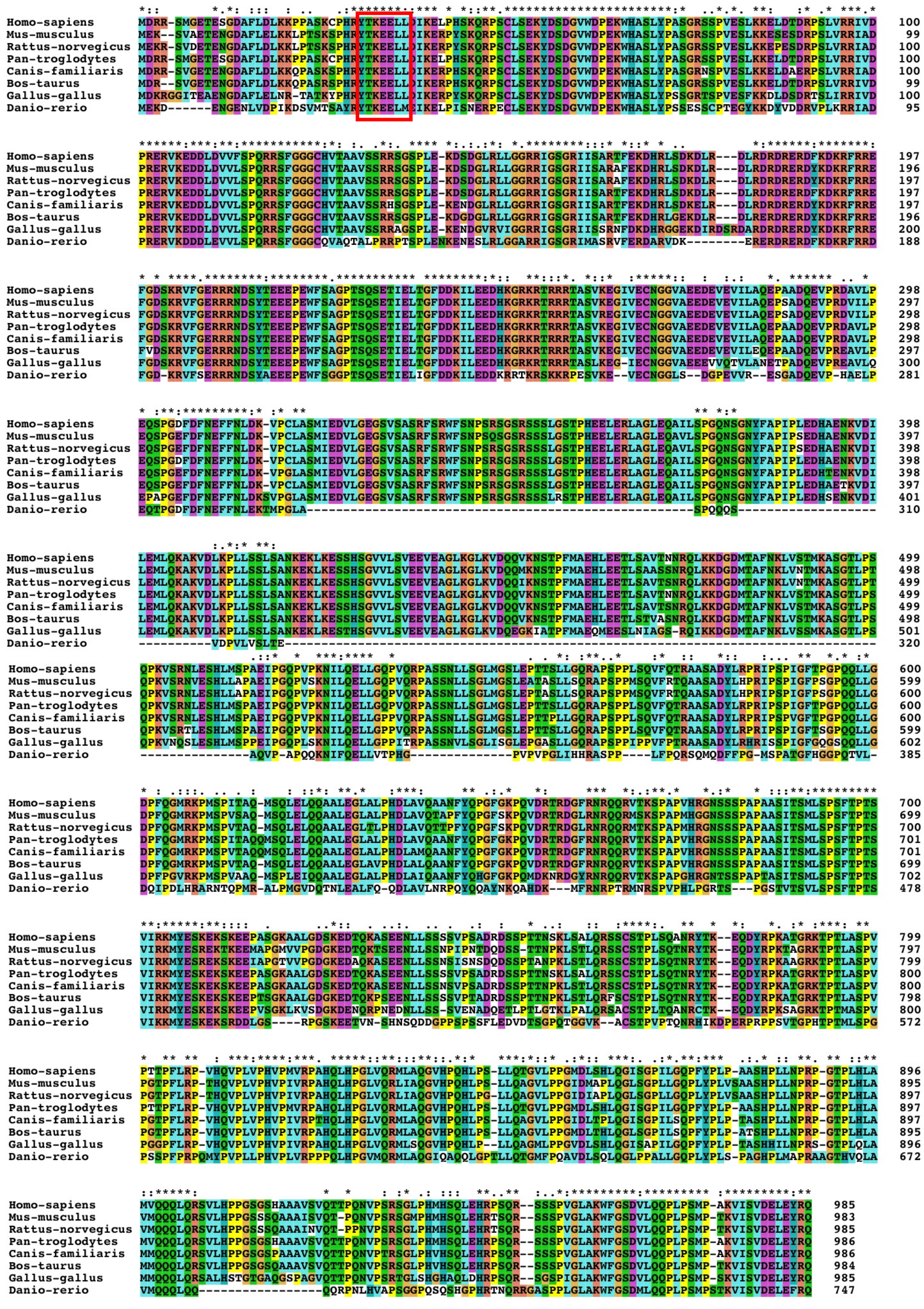


Figure 1.4. Amino acid sequence alignment for 4ET across species. *Homo sapiens* = humans, *Mus musculus* = mouse, *Rattus norvegicus* = rat, *Pan troglodytes* = chimpanzee, *Canis familiaris* = dog, *Bos taurus* = cow, *Gallus gallus*= chicken, *Danio rerio* = zebrafish Boxed region represents 4E binding motif.

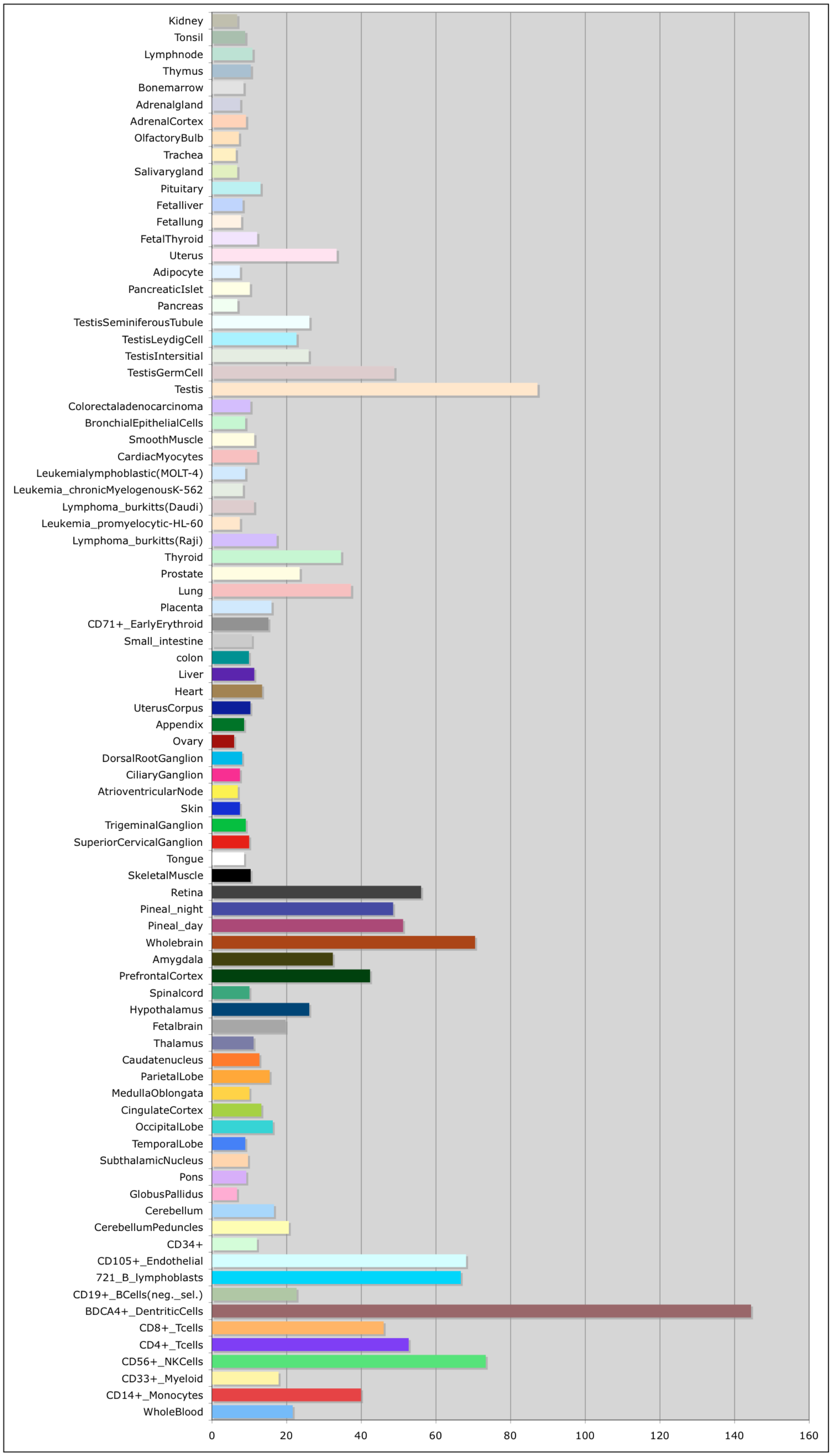


Figure 1.5. Tissue distribution of 4ET mRNA in humans. (from http://biogps.gnf.org)

P-bodies and Stress Granules

As a consequence of stress-induced translational silencing, polysomes disassemble and the circular polyadenylated mRNA protein complexes (mRNPs) can either form stress granules (SGs) or processing bodies (P-bodies, also known as GW182 or GW bodies or Dcp1 bodies) (reviewed in (Anderson & Kedersha, 2009; Kedersha & Anderson, 2007). Both P-bodies and SGs share certain proteins (boldfaced type genes in Tables 1.5 and 1.6). In metazoans, both SGs and P-bodies contain miRNA silencing elements (Leung & Sharp, 2006).

SGs and P-bodies differ in several ways: 1) only P-bodies are present in actively growing cells; 2) SG but not P-body assembly requires stress-induced phosphorylation of eIF2α (Kedersha et al., 2005); 3) SGs contain translational proteins such as eIF3, eIF4A, eIF4G, polyA-binding protein 1 (PABP-1), and small ribosomal subunits; 4) P-bodies contain mRNA decay proteins (refer to Table 1.6).

Multiple proteins are found within SGs and P-bodies (Tables 1.5 and 1.6 respectively). SGs can be roughly classified into three groups: stalled translation complexes; proteins involved in translational silencing or mRNA stability; proteins which regulate splicing, RNA editing and RNA localization. P-body components can also grouped into three classes: RNA-binding proteins and translational repressors; proteins involved in nonsense-mediated decay (NMD); and proteins affecting viral function.

The components of P-bodies and SGs are continually exchanging with the cytoplasm (Andrei, et al., 2005; Gilks et al., 2004; Kedersha, et al., 2005; Z. Yang et al., 2004). SGs and P-bodies are also mobile cytoplasmic structures, which transiently interact with one another (Kedersha, et al., 2005). Kedersha observed that SGs were relatively immobile but fluid in shape whereas P-bodies were fixed in shape but very mobile with intermittent interaction with SGs. The interaction between SGs and P-bodies allows for the possible transfer of mRNPs.

In yeast, P-bodies increase in size but decrease in number with increasing cell density (Teixeira, Sheth, Valencia-Sanchez, Brengues, & Parker, 2005). P-body mRNAs in yeast can leave and re-enter the polyribosome pool (Brengues, Teixeira, & Parker, 2005). In HeLa cells, P-bodies display cell cycle related changes: they assemble in G1, are small in early S phase and become larger during late S and G2 phases, and disassemble prior to mitosis (Z. Yang, et al., 2004). Yang et al. (2004) also found that P-bodies are also larger, brighter, and more numerous in proliferating cells.

Table 1.5. Protein components of SGs. (adapted from (Kedersha & Anderson, 2007)(genes in boldfaced type are also present in P-bodies)

Function	Protein name
RNAi slicer	**Ago2**
Antiviral response	**APOBEC3G**
Translation	Ataxin-2
Cell growth	Caprin-1
mRNA silencing	**CPEB**
Unknown	DIS1
Translation	eIF3
Translation	**4E**
Translation	eIF4G
Translation	**FAST**
Translation	**FMRP** and **FXR1**
RNA decay	FBP and KSRP
Ras signaling	G3BP
RNA stability	**HuR**
Signaling	IP5K
Development	**Lin28**
Transposon	LINE 1 ORF1p
Splicing	MLN51
Translation, stability	PABP-1
mRNA decay	**RCK**
Adhesion	Plakophilin
mRNA decay	**PMR1**
mRNA silencing	Pumilio 2
mRNA silencing	**Rap55**
Transcription	**Rpb4**
Transcription	SRC3
mRNA silencing	**Staufen**
RNP assembly	SMN
mRNA silencing	**TIA-1** and **TIAR**
Signaling	TRAF2
mRNA decay	**TTP** and **BRF-1**
Cold-shock RNA binding protein	YB-1
localization	ZBP1

Table 1.6. Protein components of P-bodies. (adapted from (Eulalio, Behm-Ansmant, & Izaurralde, 2007). (genes in boldfaced type are also present in SGs)

Function	Protein name	Organisms
5'→3' exonuclease	XRN1	Human, mouse
	Kem1	*S. cerevisieae*
miRNA pathway	GW182	Human, *D. melanogaster*
	AIN-1	*C. elegans*
Decapping enzyme	DCP2	Human, *D. melanogaster*
	DCAP-2	*C. elegans, S. cerevisiae*
Decapping enzyme subunit	DCP1	Human, *D. melanogaster, C. elegans*
	DCAP-1	*S. cerevisieae*
Decapping co-activator	Hedls	Human
	Ge-1	*D. melanogaster*
Decapping co-activator	CG5208	*D. melanogaster*
	Pat1	*S. cereviseae*
	Pat1b	Human
Decapping co-activator	EDC3 (LSm16)	Human, *D. melanogaster, S. cerevisiae*
Decapping co-activator complex	LSm1-7	Human, *S. cereviseae*
Predicted decapping co-activator	**RAP55 (LSm14)**	Human
Decapping co-activator, translation regulator	**RCK/p54**	Human
	Me31B	*D. melanogaster*
	CGH-1	*C. elegans*
	Dhh1	*S. cereviseae*
Translation initiation factor	**4E**	Human, rat
Translation repression	4E-T	Human
NMD	SMG7	Human
NMD	SMG5	Human
NMD	UPF1	Human
	Nam7	*S. cereviseae*
NMD	UPF2	*S. cerevisiae*
NMD	UPF3	*S. cerevisiae*
siRNA and miRNA pathways	**Argonaute proteins**	Human, *D. melanogaster, C. elegans*
Deadenylation	CCR4-CAF1-NOT complex	Human, *S. cerevisiae*
Translation regulator	**CPEB**	Human
Fas-activated Ser/Thr phosphoprotein	**FAST**	Human
ARE-mediated mRNA decay	**TTP**	Human
Double-stranded RNA-binding protein, mRNA localization	**Staufen**	*D. melanogaster*
RNA-binding protein, decay of mitochondrial porin mRNA	Rbp1	*S. cereviseae*
RNA polII component	Rbp4	*S. cereviseae*
Suppressor of decapping defects	Sbp1	*S. cereviseae*
SMN complex component involved in U snRNPs assembly	Gemin5	Human
Stress-induced regulatory subunit of Dcs1	Dcs2	*S. cereviseae*
Deoxycytidine deaminase with antiviral activity	**APOBEC3G**, APOBEC3F	Human

Rationale and Relevance:

4E has been validated as a cancer target in myeloid leukemia by our lab in collaboration with several hospitals (Assouline, et al., 2009). The ability to reduce the oncogenic effects of 4E is a potential avenue for cancer therapy. Therefore an understanding of how to manipulate 4E's subcellular localization via 4E-T could be of therapeutic benefit to leukemia.

Hypothesis:

4E-T simultaneously modulates the activities of 4E in the nucleus (mRNA export), in the cytosol (translation), and in P-bodies (mRNA stability/sequestration) thereby reprogramming the proteome of the cell.

Aims:

i) Determine the effects of 4E-T on 4E-mediated mRNA export, translation activity, and sequestration in P-bodies;
ii) Determine if the effects of overexpression of 4E-T are restricted to 4E-STs.

Scientific approach and expected outcomes:

The approach taken was to establish stable U2Os cell lines overexpressing 4E-T and a less efficient-4E interacting form of 4E-T (Y30A mutant) as well as a vector control and perform various analyses using these cell lines (see Figure 1.3).

Changes in 4E activity and localization as a function of 4E-T levels were monitored by Western blot, RT-qPCR, and indirect immunofluorescence microscopy. The ability of 4E-T to modulate the 4E-mediated nuclear export of 4E-SE containing transcripts was assessed using subcellular fractionation and RT-qPCR. Translational efficiency of these 4E-SE transcripts was examined by polysomal analysis and RT-qPCR.

This proposal will investigate the effects of 4E-T mediated relocalization of 4E and whether its biochemical and biological functions are related to its physiological activities in oncogenic transformation. Elucidating the mechanisms involved in 4E control will contribute to our understanding of 4E-mediated oncogenesis. This information may eventually facilitate the development of novel therapaeutic approaches for malignancies with disregulated 4E activity.

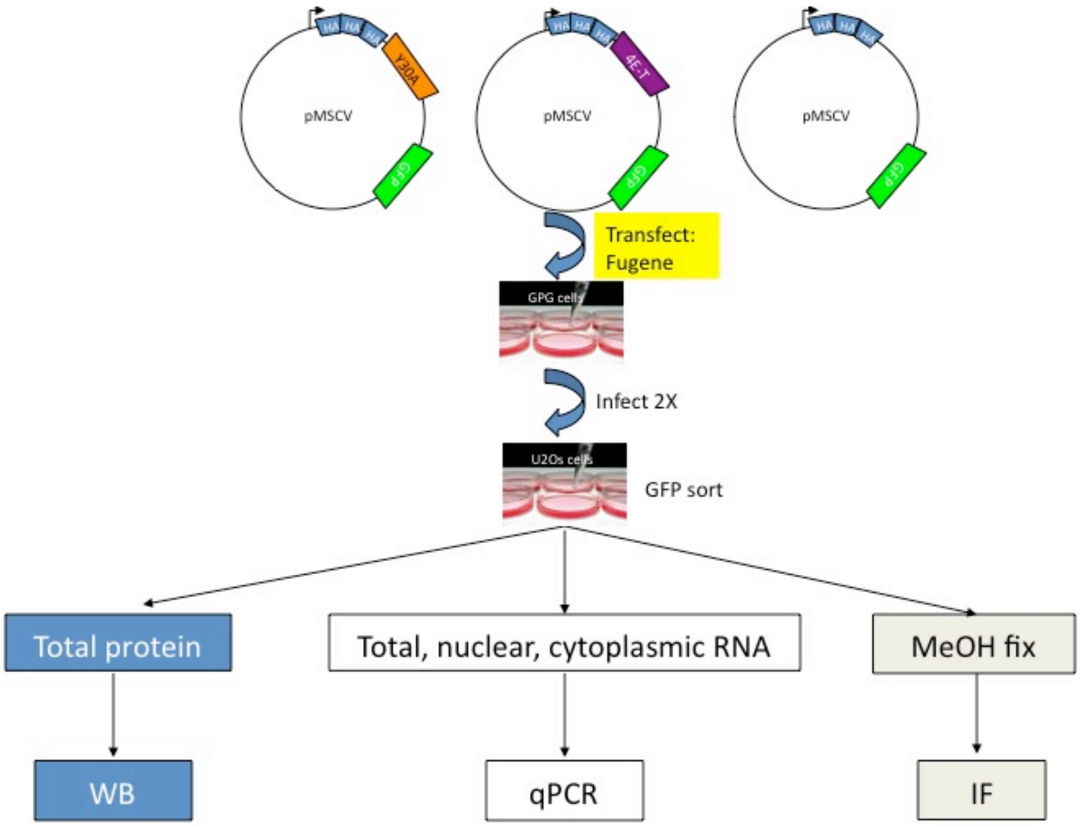


Figure 1.6. Schematic overview of experimental strategy for construction of overexpression cell lines.

WB = Western blot, qPCR = reverse transcription quantitative PCR, MeOH= methanol, IF = indirect immunofluorescence.

Chapter 2. Creation of 4E-T overexpression cell lines

Introduction

In order to test the hypothesis that the manipulation of 4E-T protein levels could alter the subcellular localization and potentially the function of 4E, it was imperative to construct a cell line that reliably overexpressed 4E-T.

After several attempts at transient overexpression and clonal selection strategies, I proceeded with a murine stem cell virus (MSCV) retroviral expression strategy to establish overexpressing cell lines. The strategy for construction of the overexpression cell lines is shown in Figure 1.4. The cell lines overexpressed either: 1) wild type version of 4ET (4ET); 2) a reduced 4E-binding version of 4ET (Y30A); or an empty vector control (E). 4ET and Y30A were triple HA-tagged at their N-terminus. All constructs contained a GFP cassette to allow for GFP-sorting post integration to select for transduced cells.

Materials and Methods

Constructing the 4ET overexpression cell line

293 GPG cells (gift from the Roux lab) were used to generate amphotropic retroviruses to infect human osteosarcoma cell line U2Os (ATCC # HTB-96) using the constructs

pMSCVY30A, pMSCV4ET, and pMSCVE (plasmids described in Dostie et al., 2000; gift from Sonenberg lab, McGill). Briefly, 293 GPG cells grown in DMEM + 10% FBS + Penicillin/Streptomycin (all from Gibco) were transfected with 4 μg of construct using FuGENE 6 (Roche Applied Science) as per manufacturer's protocol. The next day, 293 GPG cells were washed 2 X 3ml PBS and then grown overnight in 8 ml DMEM + 10% FBS + penicillin/streptomycin. Supernatant was collected 24 hrs and 48 hours post transfection and filtered (45 μm filter) to infect U2Os cells in the presence of polybrene (Sigma #H-9268; 4 μg/ml final concentration). Infected U2Os cells were allowed to grow to confluence and then split 1:3. As all constructs contained a GFP cassette, GFP positive cells were collected by FACS (IRIC Flow Cytometry Platform using the BD FACSAria Cell Sorter) to establish the overexpression cell lines.

Total protein lysate collection

Cells were grown to approximately 90% confluence and collected by trypsinization and washed 2X with PBS (pH 7.2; centrifuged 2000 X g 5 minutes, 4 ºC). Cell pellets were lysed in RIPA buffer + KKM (peptstain A (1 mg/ml), aprotinin (2 mg/ml), leupeptin (1 mg/ml), phenanthroline (1mg/ml), and benzamidine (1.6 mg/ml)) and sonicated (Sonic Dismembrator Model 100 (Fisher Scientific) power 3; 1 pulse). Lysed cells were centrifuged (12000 rpm, 4ºC, 20 minutes) and supernatant quantified by bicinchoninic acid (BCA) assay (Thermo Scientific) using the absorbance at 562 nm (Ultrospec 2100 pro UV/visible spectrophotometer, GE Healthcare).

Western blot

12% SDS resolving PAGE gels and 4% stacking gels were used. Gel running conditions were 92 V for 17 minutes and then 192 V for 57 minutes. 20-80 μg of protein lysate were loaded per sample (equal amounts for each gel). Gels were electroblotted (Biorad) to PVDF membrane (Thermo Scientific) overnight at 4ºC in 1X Transfer buffer at a constant current of 200 mA. Membranes were blocked in 5% milk TBST at room temperature for 1 hour. Membranes were incubated with primary antibody (1:500 4ET (Abnova H00056478-B01); 1:500 HA (mouse, Santa Cruz F-7 SC-7392) ; 1:5000 Actin (Sigma A54411)) overnight at 4 ºC with rotation. Membranes were washed (3X TBST, 1X TBS, 10 minutes at room temperature with agitation), incubated with secondary antibody (1:5000 ECL-anti-mouse IgG HRP-linked (GE Healthcare)) for 1 hour at room temperature, and washed again (as described previously). ECL reagent (SuperSignal West Pico, Thermo Scientific) was prepared as per manufacturer's specifications and applied to membranes. Membranes were exposed to X-ray film (AGFA Radiomat B plus full blue, Agfa) and developed in Kodak X-OMAT 2000A processor (Kodak).

RNA collection

Cells were grown to approximately 90% confluence, washed 2X with PBS and lysed with TRIzol (Invitrogen). RNA was collected as per manufacturer's specifications and resuspended in DEPC dH_2O.

RT-PCR and qPCR

RNA was quantified (Nanodrop 2000c; Thermo Scientific). RNA was DNAse treated (Turbo DNAse kit AM 2339, Ambion) and 100 ng of DNased RNA was used to construct cDNA (random hexamers, MMLV; Invitrogen) using manufacturer's protocol. Quantitative PCR (EXPRESS SYBR GreenER qPCR Supermix universal, Invitrogen) was conducted on ABI StepOne Plus real time PCR System (Applied Biosystems). Primers for 4E-T are (forward 5'AGACCTTGAGTGCCGTAACCAACA3'; reverse 5'ATGGCTTTCAAGGTTTCGGCTGAC3'). Primers for actin are (forward 5' GCATGGAGTCCTGTGGCATCCACG3'; reverse 5' GGTGTAACGCAACTAAGTCATAG3'). Relative expression of mRNA is given as $\log_2$ of 2-ΔΔCt values.

Indirect Immunofluoresence

Cells were grown on acid cleaned glass coverslips (Fisher Scientific). Coverslips were washed 3X PBS (pH 7.2); quenched (50 mM ammonium chloride in PBS) 10 minutes at room temperature, air dried, fixed in 100% methanol (-20ºC, 10 minutes), then blocked (10% FBS, 0.1% Tween) for 30-60 minutes at room temperature. Coverslips were incubated with primary antibody in blocking buffer at 4ºC overnight. Conditions for single and double staining are presented in Table 2.1. Coverslips were washed (3X 5 minutes PBS, room temperature), incubated with secondary antibody at room temperature in the dark for 60 minutes (Table 2.1); washed (3X 5 minutes PBS room temperature, in the dark), air-dried, and mounted with DAPI (Vectorshield mounting medium for fluorescence with DAPI, Vector Laboratories). Fluorescence micrographs from several fields were observed at 100X optical magnification with 2X digital zoom on a laser scanning confocal microscope (100X objective and numerical aperture 1.4; Zeiss LSM510, IRIC Bio-imaging Platform). The blue channel was converted to green using the LSM510 software version 3.2 (Carl Zeiss, Inc).

Results

Establishment of overexpression cell lines

Post-infection, GFP-positive U2Os cells were obtained for the establishment of each of the overexpression cell lines (55.4% GFP-positive pMSCVY30A cells, 88.6% GFP-positive pMSCV4ET cells; and 99.6% GFP-positive pMSCVE cells, Table 2.2).

GFP-positive cells were tested for their ability to overexpress HA (by Western blot) and 4E-T (by Western blot and RT-qPCR). Both Y30A and 4ET cell lines overexpress HA and 4E-T protein as compared to the vector (E) control (Figure 2.1A). The overexpression of mRNA for 4ET is observed by RT-qPCR for 4E-T in Y30A and 4ET as compared to E (Figure 2.1B). There are some smaller protein products (both HA and 4ET) seen in the Western blot in Y30A and 4ET as compared to E.

Phenotype of overexpression cell lines

Both the Y30A and 4ET cell lines displayed HA-positive cytoplasmic puncta (Figure 2.2A) and 4E-T positive cytoplasmic puncta (Figure 2.2B).

The overexpressed 4E-T protein seen in the 4E-T overexpression and Y30A overexpression cell lines also expresses HA (Figure 2.3) and colocalizes in cytoplasmic puncta. The E cell line does not show overexpression of 4E-T nor does it show colocalization of 4E-T and HA signal (compare E line to Y30A and 4E-T in Figure 2.3).

Discussion

The presence of GFP-positive U2Os cells post infection suggest that the retroviral strategy to create overexpression cell lines using the pMSCV constructs was successful. The characterization of the GFP-positive cells suggests that Y30A and 4ET overexpress mRNA for 4E-T (Figure 2.1B) that is translated into HA-tagged versions of 4E-T (Figure 2.1A and 2.2) that manifest themselves as cytoplasmic foci (Figure 2.3). Quantification of the level of protein overexpression would require reanalysis of Western blots with densitometry software. The observation of cytoplasmic 4E-T positive foci is consistent with previously reported 4E-T overexpression cell systems (Dostie, Ferraiuolo, Pause, Adam, & Sonenberg, 2000; Ferraiuolo, et al., 2005; Rong et al., 2008; Borden lab, unpublished).

The observation that the level of 4ET overexpression is higher in the 4ET line as compared to the Y30A cell line (in this derivation of the overexpression cell lines and previous derivations of overexpression cell lines using different overexpression vectors) is curious especially given that the construct design (e.g. vector backbone and promoter) were identical. It is possible that the cells do not tolerate the Y30A variant of 4ET as well as wild type version of 4ET and so "adapts" to reduce the level of Y30A to "tolerable" levels. The total level of 4ET transcript appears to be 22X higher and 8X higher for 4ET and Y30A respectively as compared to E (Figure 2.1B). Therefore, the introduced Y30A could be "silenced" at the level of transcriptional initiation or at the level of mRNA stability. In the case of silencing at the level of transcriptional initiation, it could be that Y30A is either integrating into heterochromatic region of the genome more often than the 4ET construct or that the site of its integration is more prone to epigenetic silencing (e.g. DNA methylation). It is also possible that the transcript of Y30A is more rapidly degraded than the 4ET transcript. Polysome analysis of 4ET transcript in the 4ET, Y30A, and E lines would establish whether or not the transcripts were behaving differently with respect to loading of ribosomes.

The lower protein bands seen in the Western blots for 4ET and HA in the Y30A and 4ET cell lines that are not observed in the E line are not likely to be products of alternative transcripts (Table 1.4) as the introduced construct is based on 4ET cDNA. The smaller protein 4ET/HA positive protein products of 4ET and Y30A are probably protein degradation products (Figure 2.1A). Collection of lysates in the presence of a proteasome inhibitor would resolve whether these smaller products are degradation products.

The presence of some HA-signal that does not colocalize with 4E-T in both Y30A and 4ET is unexpected (Figure 2.2A). Since there is HA signal in the nucleus in these cell lines, I suggest that this HA-staining is artefactual and due to the HA antibody used in the double stain cross reacting non-specifically with a nuclear protein since the other panel of 4ET/HA double staining displayed only 4ET/HA positive cytoplasmic puncta (contrast Figure 2.2B with Figure 2.2A).

Given that HA-tagged 4E-T is overexpressed in Y30A and 4ET at the mRNA and protein level and is represented by cytoplasmic puncta (Figures 2.2-2.3), the data strongly suggest that I have established a robust 4E-T overexpression cell lines.

Table 2.1. Antibody concentrations used for indirect immunofluorescence.

Figure	Primary antibody	primary antibody dilution	Secondary antibody*	secondary antibody dilution
4ET single stain (Figure 2.2A)	4ET (mouse; Abnova H00056478-B01)	1:100	Texas red conjugated goat anti-mouse	1:100
HA single stain (Figure 2.2B)	HA (mouse; Santa Cruz F7 SC-7392	1:100	Texas red conjugated goat anti-mouse	1:100
4ET/HA double stain (Figure 2.3A)	4ET (mouse; Abnova H00056478-B01); HA (rabbit; Santa Cruz Y-11 SC-805)	1:100; 1:100	Texas red conjugated goat anti-mouse; Cy5 conjugated donkey anti-rabbit	1:100; 1:100
4ET/HA double stain (Figure 2.3B)	4ET (rabbit; Abcam ab55881); HA (mouse; Santa Cruz F7 SC-7392)	1:100; 1:100	Texas red conjugated goat anti-rabbit; Cy5 conjugated donkey anti-mouse antibody	1:100; 1:100

*all are from Jackson Immunoresearch

Table 2.2. Flow cytometry data from BD FACSAria sorted pMSCV-infected U2Os cells.

Sample	Percentage GFP positive	Number of cells sorted
U2Os pMSCVY30A	55.4%	1 506 450
U2Os pMSCV4ET	86.6%	1 442 280
U2Os pMSCVE	99.6%	1 126 263

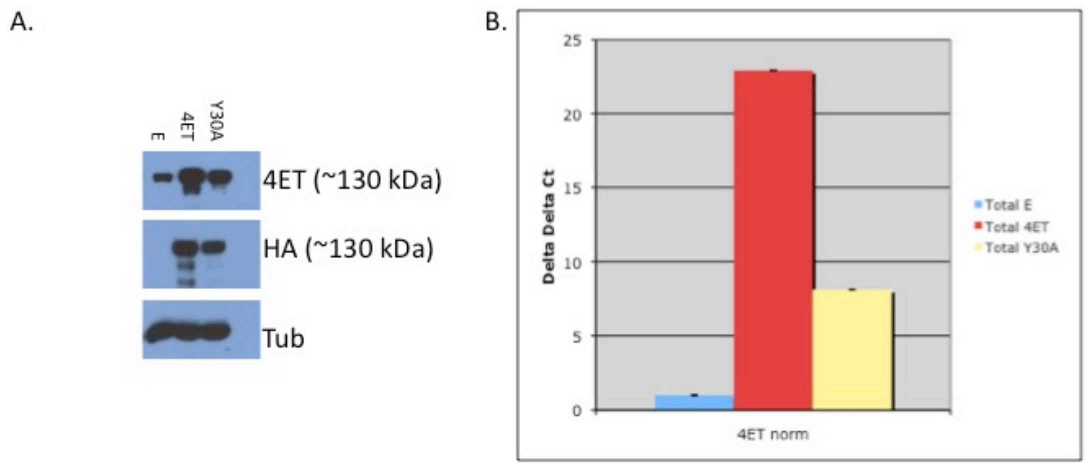


Figure 2.1. 4ET is overexpressed in Y30A and 4ET cell lines as compared to the E control. A) Western blot against 4E-T and HA, tubulin (Tub) shown as a loading control. B) RT-qPCR for 4E-T mRNA normalized by actin and relative to E control.

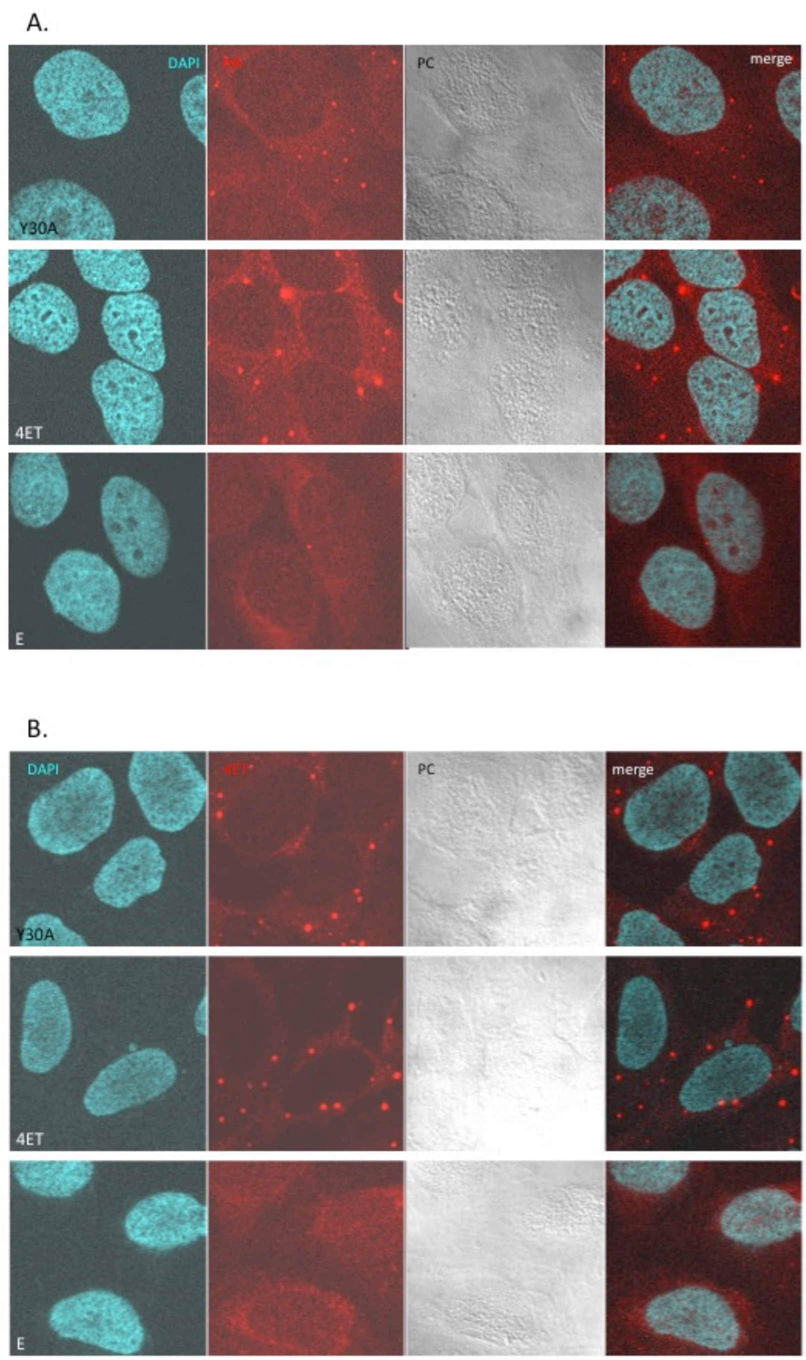


Figure 2.2 Indirect immunofluorescence in the cell lines.

A) HA signal (red) is observed as cytoplasmic puncta in Y30A and 4ET but not E; B) 4ET signal (red) is observed as a cytoplasmic puncta in Y30A and 4ET and as more diffuse staining throughout the cell in E. DAPI (cyan) marks DNA in nuclei, PC (phase contrast).

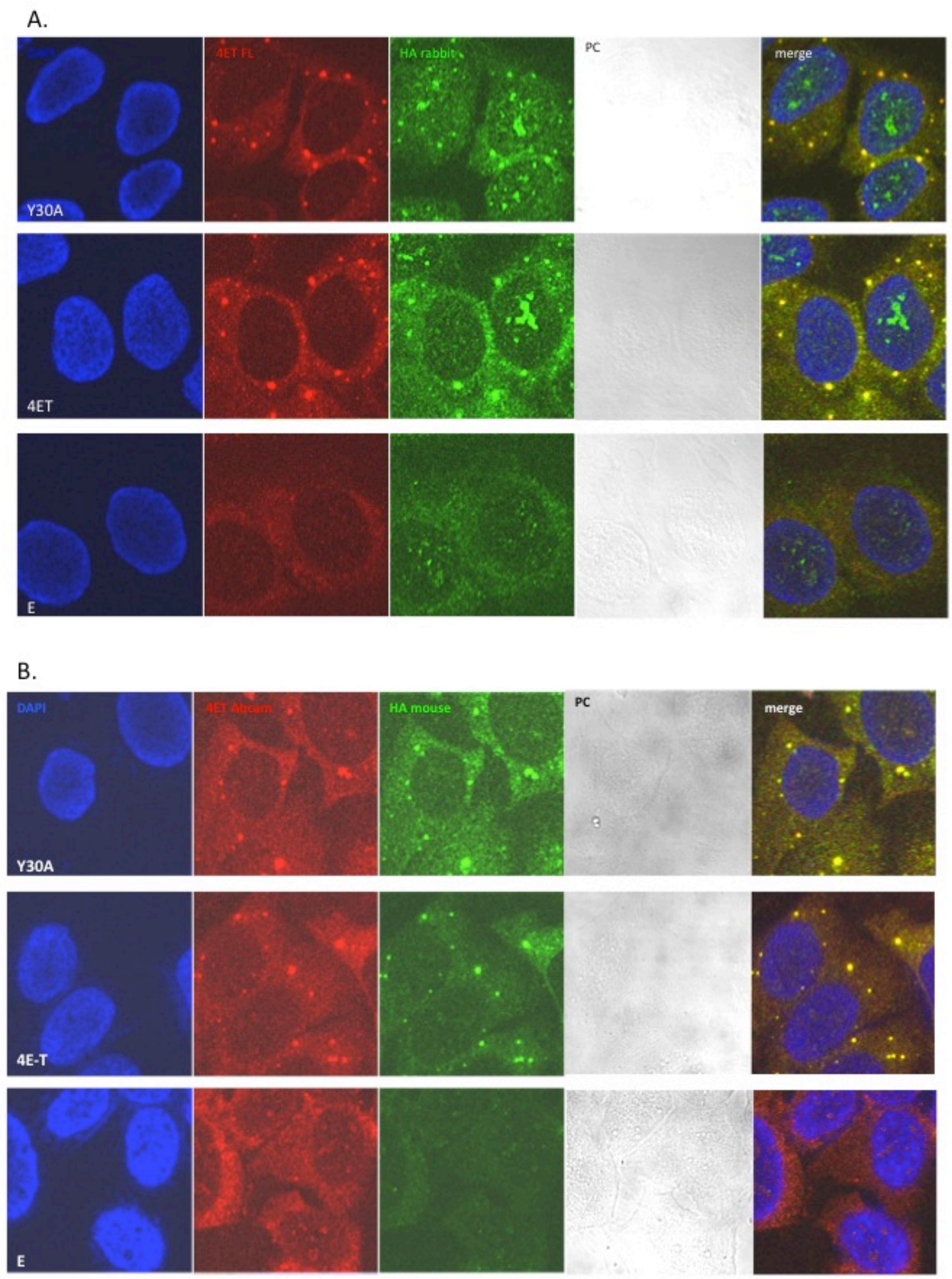


Figure 2.3. Micrographs demonstrating that overexpressed 4E-T is also HA-tagged. A) 4ET FL mouse antibody and rabbit HA; B) Abcam 4ET rabbit antibody and mouse HA.

Chapter 3. Overexpression of 4E-T causes the formation of processing bodies

Introduction

4E-T is a component of cytoplasmic foci known as processing bodies (P-bodies) (Ferraiuolo, et al., 2005). P-bodies are ribonucleotide complexes composed of several proteins (refer to Table 1.6) whose function within cells was discussed in Chapter 1.

Given that overexpression of 4E-T results in cytoplasmic puncta, it is important to determine whether these entities are P-bodies. Decapping protein 1 a (Dcp1a) is a well-established component of P-bodies which are not found in SGs (Cougot, Babajko, & Seraphin, 2004; Kedersha, et al., 2005). Therefore, co-localization of 4E-T and Dcp1a in the cytoplasmic puncta in the 4ET cell line (by immunofluorescence) would support the notion that 4E-T overexpression induces the formation of (large) P-bodies.

Another method to test the formation of P-bodies in the 4ET overexpression cell line is to demonstrate their disappearance (by immunofluorescence) upon treatment with a P-body dispersion drug. Emetine is a drug that can disassemble both P-bodies and stress granules (reviewed in (Kedersha & Anderson, 2007).

Materials and Methods

4E-T overexpression cell lines

U2Os cells overexpressing 4E-T and the mutant non-4E-binding form of 4E-T as well vector control were described in Chapter 2.

Emetine treatment

20 μg/ml emetine (Sigma) was applied to cells grown on coverslips for 1 hour, 1.5 hours, and 2 hours (37 ºC) prior to fixation and immunofluorescence staining. Untreated controls of all cell lines was also collected.

Indirect Immunofluorescence

The indirect immunofluorescence procedure was as described in Chapter 2. Rabbit anti-Dcp1a serum was used to identify Dcp1a-positive entities (gift from Andersen lab; 1:100 dilution).

Results

4E-T overexpression leads to the formation of P-bodies

The Y30A and 4E-T overexpression lines both demonstrate the ability of 4E-T to colocalize Dcp1a in the cytoplasm (Figure 3.1) suggesting that it does not have to bind 4E in order to be part of P-bodies. Since Dcp1a is a marker for P-bodies, it is surmized that the 4E-T-positivie/Dcp1a-positive cytoplasmic puncta seen in Y30A and 4E-T are P-bodies. The vector control (E) does not show large Dcp1a positive cytoplasmic puncta (contrast E with Y30A and 4E-T in Figure 3.1). By Western blot, there does not seem to be a gross increase in the level of Dcp1a protein in Y30A and 4ET as compared to E (Figure 3.2).

Emetine treatment causes the disappearance of P-bodies

The Y30A cell line displayed more diffuse 4ET and Dcp1a staining with emetine treatment starting at 1 hour with almost complete disappearance of 4ET/Dcp1a positive cytoplasmic bodies by 2 hours of emetine treatment as compared to the untreated control (Figure 3.3).

The 4ET cell line showed 4ET bodies that resisted dispersal with emetine treatment. However, these bodies appeared to become smaller and surrounded by more diffuse 4ET staining with increasing incubation time with emetine as compared to the untreated control (Figure 3.4). The Dcp1a staining was completely colocalized with 4ET staining in the untreated control and was observed as cytoplasmic puncta surrounded by more diffuse Dcp1a staining with increasing incubation time with emetine (Figure 3.4). After 2 hours of emetine treatment the Dcp1a signal was no longer colocalized with 4ET signal (contrast bottom panel of Figure 3.4 with top panel of Figure 3.4).

Discussion

Given that both the Y30A and 4ET cell lines were able to produce cytoplasmic puncta that were positive for 4ET and Dcp1a (Figures 3.1, 3.3 and 3.4) which were not observed in the vector control line E, the data suggest that overexpression of 4ET (whether the wild type form or less effectively-4E binding form) do induce the formation of P-bodies. These data are consistent with data presented by Ferraiuolo et al., 2005 who suggest that localization of 4ET to P-bodies does not require interaction with 4E. The dissolution of 4ET bodies with increasing incubation times with emetine in the Y30A cell line also supports the notion that P-bodies were present in this cell line. The overexpression of 4ET does not appear to affect the protein levels of Dcp1a as seen by Western blot (Figure 3.2).

The persistence of 4ET bodies in the 4ET cell line may be a function of the level of 4ET overexpression in this cell line as compared to either Y30A or E (see Chapter 2 Figure 2.1). However, the dissociation of Dcp1a from 4ET at 2 hours of emetine treatment and the more diffuse 4ET staining around the 4ET puncta present with emetine treatment suggest that the P-body dissassembly drug was able to partially dissociate P-bodies seen in the 4ET line.

A similar study of overexpression of another P-body protein (Pat1b) by Ozgur et al. (2010) suggest an alternate explanation for resistance to diassembly in the presence of another translation elongation inhibitor (cyclohexamide). When Pat1b protein was overexpressed, the induced P-bodies resisted disassembly in the presence of cyclohexamide (even after 6 hours of treatment) which they interpreted as meaning that Pat1b plays a role in stabilizing P-bodies ((Ozgur, Chekulaeva, & Stoecklin, 2010). Therefore, 4ET could also be important for the stability of P-bodies. When 4ET was depleted in a cell line with endogenously high levels of 4E and P-bodies (FaDu cell line), P-bodies disappeared which also suggests that 4ET is important for stability of P-bodies (Supplemental Figure S.1).

Small 4ET/Dcp1a positive bodies are seen in the untreated E line. In the presence of emetine, E shows only diffuse staining of both 4ET and Dcp1a (Figure 3.5).

As emetine is a drug that blocks protein synthesis in eukaryotic cells by binding to the 40S subunit of the ribosome (Jimenez, Carrasco, & Vazquez, 1977), it is unlikely to itself directly cause the degradation of Dcp1a or 4ET protein. However, in order to demonstrate that emetine treatment was affecting only the localization of Dcp1a and 4ET, one would have to repeat the experiment and perform Western blot analysis for Dcp1a and 4ET. Given that Ferraiuolo et al., 2005 demonstrated that Dcp1a or 4ET protein levels were unchanged in Hela cells when subjected to 0, 30 and 60 minutes of cyclohexamide, it is expected that emetine treatment will have similar effects.

Together, the data strongly suggest that overexpression of both the mutant and wild type forms of 4ET induce the formation of P-bodies but that those P-bodies formed in the 4ET cell line are more resistant to disassembly with emetine treatment. It is suggested that the inability to completely dissassemble P-bodies in the 4ET cell line could be overcome with either increased incubation time and/or increased concentration with emetine.

Treating Y30A, 4ET, and E cell lines with another P-body disassembly drug such as cyclohexamide (1- 2 hour treatment with 20-50 ug/ml as suggested in (Kedersha & Anderson, 2007); and as observed in yeast in (Brengues, et al., 2005) might also be performed to lend further credence to the notion that the cytoplasmic 4ET/Dcp1a positive bodies seen in Y30A and 4ET are indeed P-bodies.

As P-bodies contain a large number of protein components (refer to Table 1.6 in Chapter 1), it is also possible (albeit exhaustive) to test for the colocalization of other P-body components with 4ET by immunofluorescence.

The presence of P-bodies changes with the cell cycle. Therefore, increasing the size and/or number and/or half-life of P-bodies could have an effect on the cell cycle. In fact, a recent study showed that overexpression of a splice variant of the tumor supressor gene TFL (transformed follicular lymphoma, $P58^{TFL}$) resulted in the formation of P-bodies and an inhibition of G1 to S phase progression (Minagawa et al., 2009). Cell synchronization experiments (e.g. double thymidine block) combined with BrDU analysis to address cell-cycle associated changes in 4ET and/or P-bodies could be of benefit to address these issues.

I suspect that cell cycle has not drastically been altered in Y30A and 4ET as compared to E as all cell lines did not display growth defects (Wu unpublished observation).

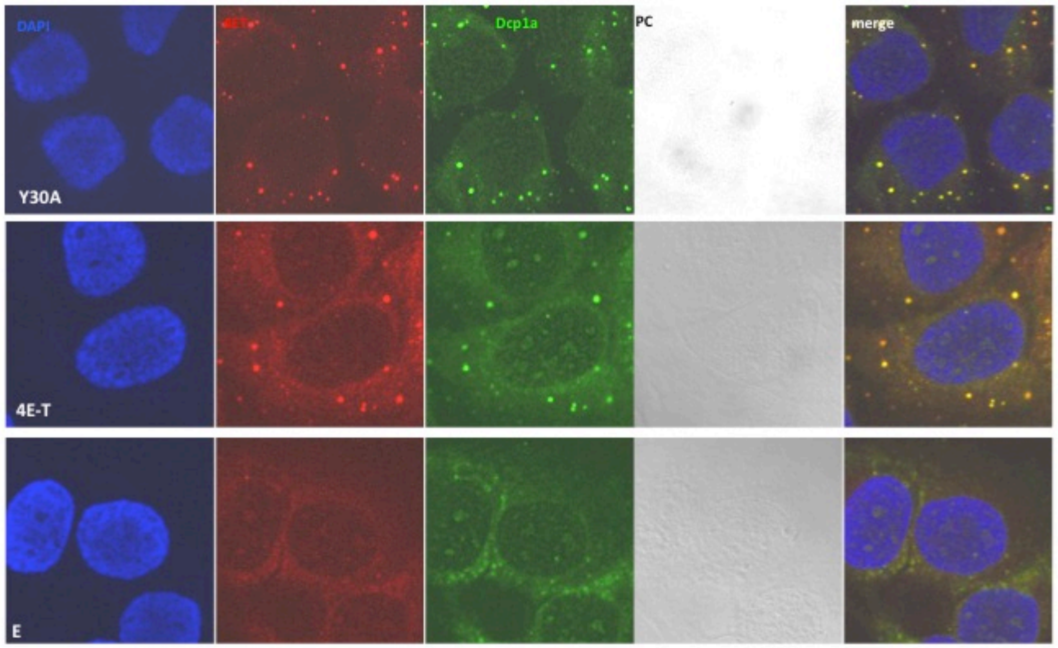


Figure 3.1. Micrographs showing subcellular localization of 4E-T and Dcp1a.

Figure 3.2.Western blot of Dcp1a.

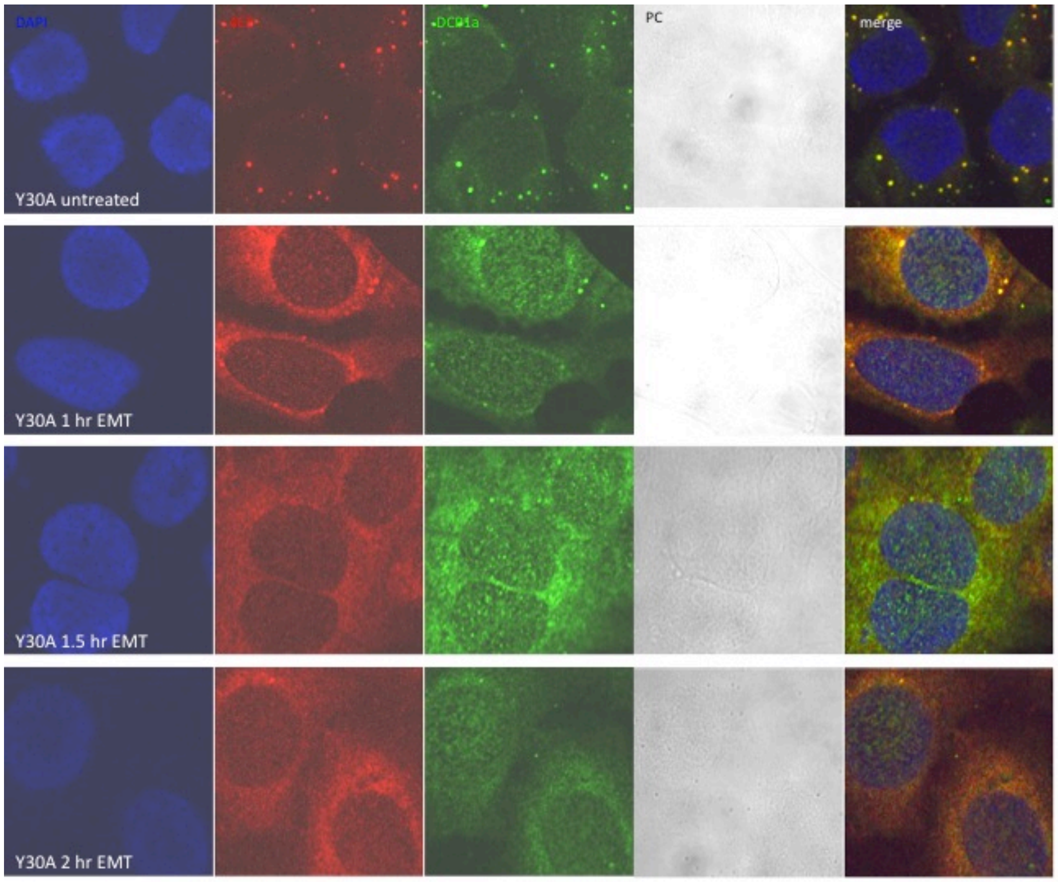


Figure 3.3. Micrographs of Y30A cell line showing disappearance of P-bodies with increasing emetine treatment.

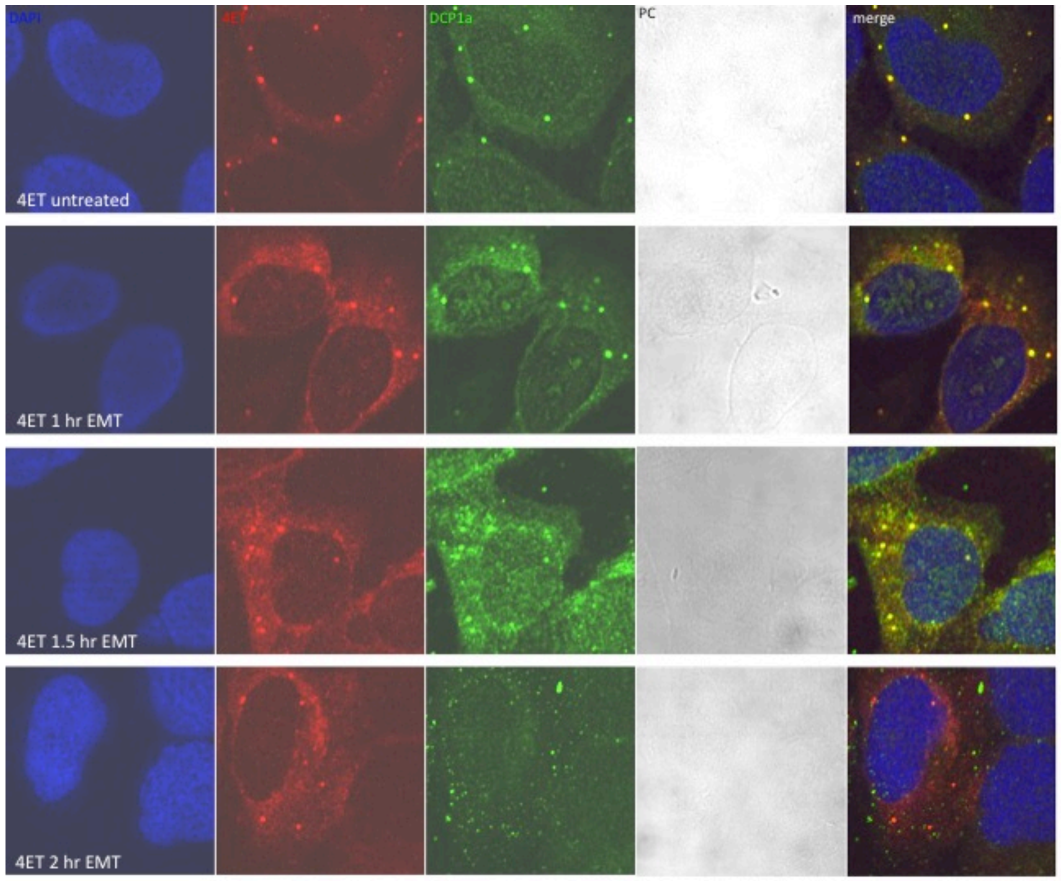


Figure 3.4. Micrographs of 4ET cell line showing disappearance of P-bodies with increasing emetine treatment.

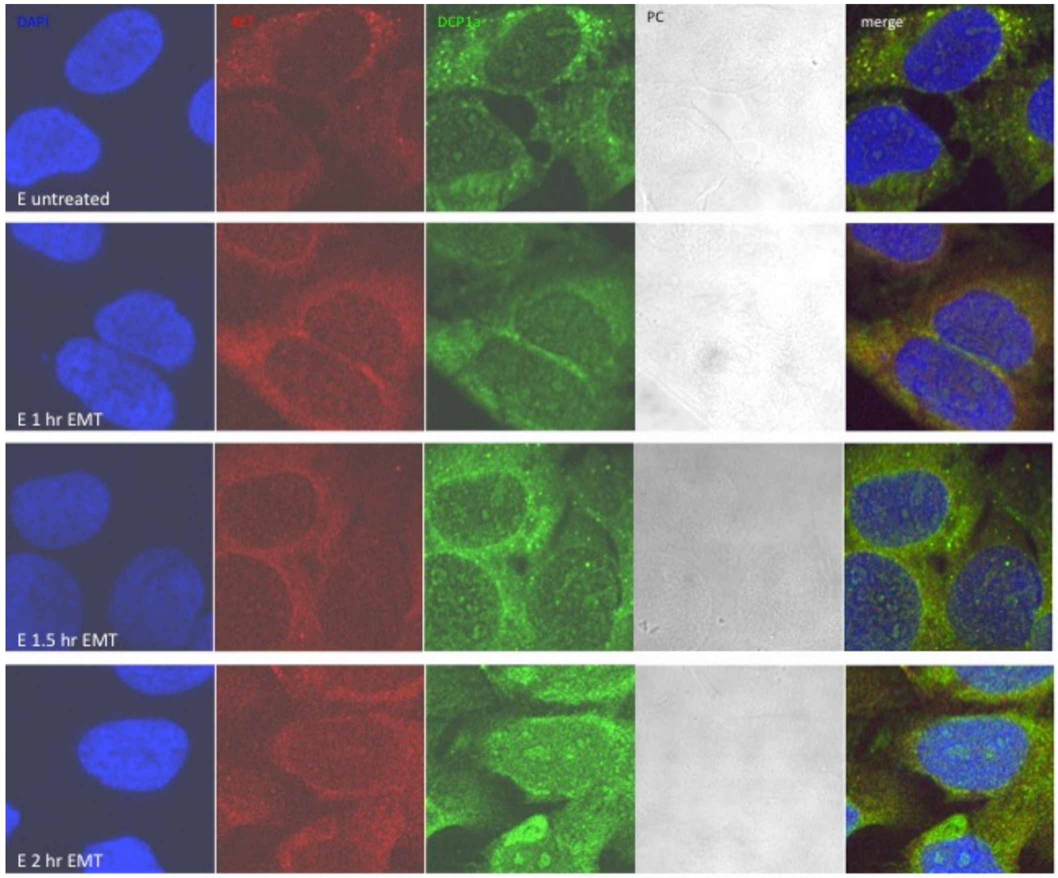


Figure 3.5. Micrographs of E cell line untreated, and treated with emetine for 1 hour, 1.5 hours, and 2 hours.

Chapter 4. 4E-T overexpression relocalizes a proportion of 4E in U2Os cells.

Introduction

Having established cell lines that overexpress 4E-T (wild type overexpression line will heretofore be referred to as 4ET or 4ET line and the mutant overexpression line will be referred to as Y30A or Y30A line) and induce the formation of P-bodies (see Chapters 2 and 3), it is important to investigate the impact of these changes on the cellular levels (mRNA and protein) and subcellular localization of 4E. If the overexpression of 4E-T is able to relocalize a proportion of 4E to P-bodies, then it has the potential of altering the function of 4E.

Materials and Methods

4E-T overexpression cell lines

U2Os cells overexpressing 4E-T and the mutant non-4E-binding form of 4E-T as well vector control were described in Chapter 1.

Indirect Immunofluorescence

The indirect immunofluorescence procedure was as described in Chapter 2. In order to demonstrate co-localization of 4E-T with 4E, a rabbit 4E-T antibody was used (1:100 dilution; Abcam ab55881)

Western blot

Western blot was conducted as described in Chapter 2.

RNA collection

RNA collection was as described in Chapter 2.

RT-PCR and qPCR

RT-PCR and qPCR were performed as described in Chapter 2. 4E forward primer 5' CTGTGCCTTATTGGAGAAT 3' and reverse primer 5' GGAGGAAGTCCTAACCTTT3'.

Results

4E-T overexpression can relocalize endogenous 4E

Overexpression of the wild type form of 4E-T (4ET) as well as the mutant form of 4E-T (Y30A) can relocalize 4E to the cytoplasm. (Figure 4.1A). In the vector control (E), 4E is observed to have less cytoplasmic staining as compared to either 4ET or Y30A (Figure 4.1A). The 4E-T-positive foci in the 4ET overexpression cell line more effectively colocalize 4E than Y30A (Figure 4.1B).

Overexpression of 4E-T does not alter the protein level or mRNA level of 4E

The protein level of 4E does not appreciably differ between Y30A and 4ET and E (Figure 4.2A). By RT-qPCR, 4E mRNA levels are very slightly reduced in 4ET and slightly elevated in Y30A relative to E (Figure 4.2B). However, these slight variations in 4E mRNA had no impact on 4E protein level.

Discussion

Although overexpression of both the mutant and wild type forms of 4E-T are able to relocalize 4E as compared to the vector control (E) (compare 4E staining of E to Y30A and 4ET in Figure 4.1A), the overlap in indirect immunoflourescence signals is more complete in 4ET as compared to Y30A (compare circled regions highlighted in Figure 4.1B). The mutation in Y30A has been demonstrated to inhibit 4E binding by immunoprecipitation (Dostie et al., 2000). However, as the Y30A cell line was able to relocalize some 4E as compared to E (Figure 4.1A and 4.1B), it is possible that the single amino acid mutation may not be sufficient to completely abrogate the 4E-T-4E interaction. Since the consensus sequence for 4E binding partners is YXXXXLΦ, perhaps a 4E-T mutant containing a Y to A as well as L to A would be a better mutant.

It is also possible that 4ET has different affinities for the different classes of 4E family members (refer to Table 1.1 in Chapter 1). As our 4E antibody is unlikely to be able to distinguish between the different classes of 4E, it impossible to determine the specificity of Y30A or 4ET for the various classes of 4E either by Western blot or by immunofluorescence at this time.

It would appear that the mRNA levels and protein abundance of 4E is not significantly different between Y30A and 4ET as compared to E (see Figure 4.2). If anything, the mRNA level of 4E is slightly reduced in 4ET and slightly elevated in Y30A as compared to E while the protein levels (by visual inspection) would appear to approximately the same in all cell lines. To more accurately describe the protein levels of 4E in the cell lines, one would need to use densitometry and quantification software.

Together, these data suggest that the 4ET line is more effective at producing P-bodies that relocalize a proportion of 4E as compare to Y30A without greatly affecting the total level of 4E mRNA or protein levels.

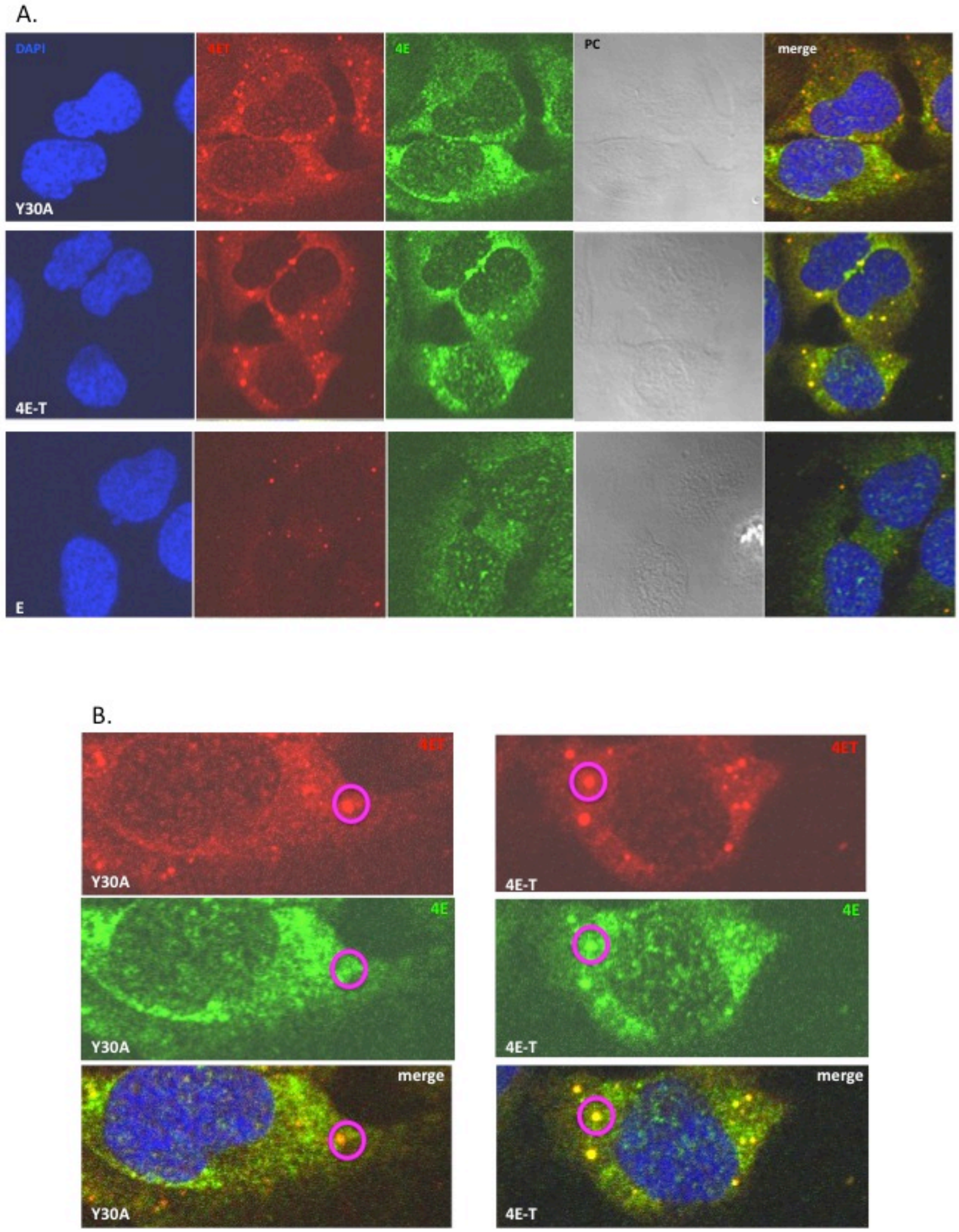


Figure 4.1. Overexpression of 4E-T relocalizes endogenous 4E. A) Both Y30A and 4ET (red) can relocalize 4E (green) more than E. B) 4ET forms more distinct 4E-positive cytoplasmic foci than Y30A. Pink circles are shown around representative 4E-T foci for 4ET and Y30A

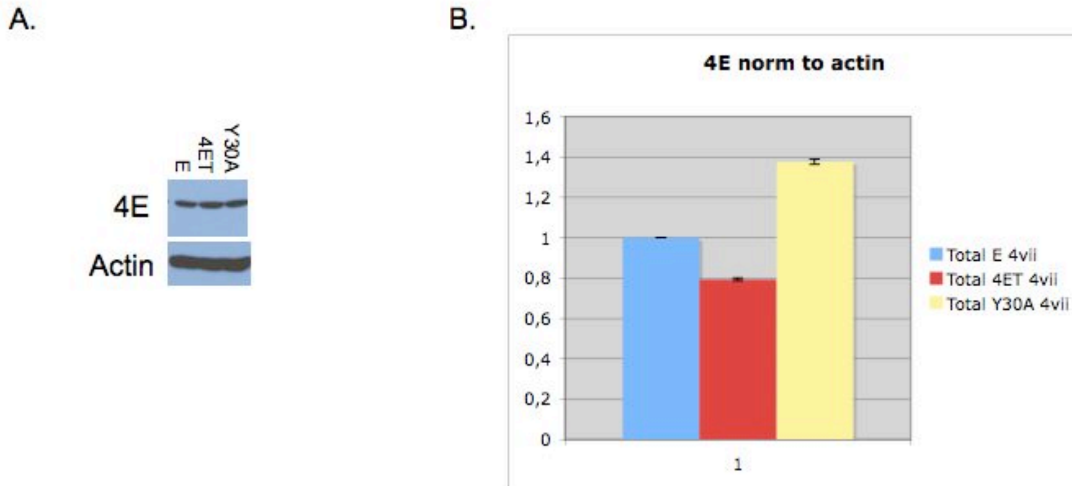


Figure 4.2. 4E-T overexpression does not drastically alter the protein level or mRNA level of 4E. A) Western blot of 4E in Y30A, 4ET, and E; actin shown as a loading control; B) RT-qPCR data for 4E (delta delta Ct as a function of cell line) for Y30A, 4ET, and E.

Chapter 5. Impact of 4E-T overexpression on 4E-SE-containing transcripts and the protein products of 4E-sensitive transcripts.

Introduction

To this point, I have demonstrated that the 4ET line induces the formation of P-bodies that can more effectively relocalize a proportion of 4E protein without altering 4E mRNA/protein levels than the Y30A line (Chapters 3 and 4). In this chapter I investigate the potential impact of 4ET overexpression on 4E function. More specifically, I address the impact of 4ET overexpression on the expression and cytoplasmic to nuclear distribution of 4E-SE-containig transcripts (cyclin D1, Pim1, nibrin, CGGbp, and NONO.1), a translationally sensitive transcripts (VEGF), and 4E sensitive transcripts containing 4E-SE (Pim1 and ODC) by using cellular fractionation and RT-qPCR.

Materials and Methods

4E-T overexpression cell lines

U2Os cells overexpressing 4E-T and the mutant non-4E-binding form of 4E-T as well vector control were described in Chapter 2.

Western blot

Western blot was conducted as described in Chapter 2. Only total protein lysates were analyzed. Targets tested are summarized in Table 5.1.

RNA collection

RNA collection was as described in Chapter 2.

Cellular fractionation

Cells were grown to near confluence (approximately 3 X 10^7 cells), rinsed 2X with cold PBS and collected by trypsinization. Cell pellets were gently resuspended in ice cold Lysis B solution (10 mM Tris pH 8.4, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP40, 1 mM DTT and 200 U/ml RNAseOut (Invitrogen)). Cell suspensions were centrifuged at 1000 X g at 4°C for 3 minutes. Supernatant (cytoplasmic fraction) was resuspended in 1 ml Trizol (Invitrogen) and RNA extraction was performed as described in Chapter 1. The pellet (nuclear fraction) was resuspended in 1 ml Lysis B solution, transferred to a round bottom polypropylene tube and 100 μl of detergent stock (3.3% w/v sodium deoxycholate, 6.6% v/v Tween 40 in DEPC dH_2O) was added under slow vortexing, and then incubated for 5 minutes on ice. Nuclear suspensions were transferred to 1.5 ml eppendorf tubes and centrifuged at 1000 X g at 4°C for 3 minutes. Supernatant was discarded, pellets resuspended in 1 ml Lysis buffer B and recentrifuged at 1000 X g at 4°C for 3 minutes. Supernatant was discarded and cell pellets (nuclear fractions) were resupsended in 1 ml Trizol and RNA extraction was performed as described in Chapter 1.

RT-PCR and qPCR

RT-PCR and qPCR were performed as described in Chapter 1. U6 primers were used to confirm that nuclear fractions were enriched for U6 transcripts (Forward 5' CGCTTCGGCAGCACATATAC 3'; Reverse 5' AAAATATGGAACGCTTCACGA 3'). Primers for tRNALys were used to show that cytoplasmic fractions were enriched for cytoplasmic fractions (forward 5' GCCCGGATAGCTCAGTCGGT 3'; reverse 5' CGCCCAACGTGGGGCTCTCG 3').

Various 4E target genes (4E-SE and/or 4E-sensitive) were analyzed by RT-qPCR (Table 5.1). All qPCR data is reported relative to E and normalized to tubulin. Delta delta Ct values in the range of 0.33 to 3.00 are arbitrarily determined to be equivalent (e.g. not differing from E) such that values of less than 0.33 (e.g. 3-fold reduced) are described reduced and greater than 3.00 (e.g. 3-fold enriched) are described as 3-fold enriched.

Table 5.1. Western blot targets.

Category and gene	Company	Dilution
4E-SE :		
CyclinD1	(H-295) sc-753 Santa Cruz	1 :500
Cyclin E1	(C-19) sc-198 Santa Cruz	1 :200
Mdm2	(Ab-1) OP46-100 ug Oncogene	1 :500
4E-sensitive :		
VEGF	(A-20) sc-152 Santa Cruz	1 :500
Normalization :		
actin	A54411 Sigma	1 :5000

Table 5.2. Primers and targets for RT-qPCR

Category and gene	Forward primer	Reverse primer
4E-SE :		
NONO.1	AGGTCCTTCCTGCTAACCACATT	TCATACTCAAACCCAGCCAGGCTGT
Nibrin	AGCAGCAGACCAACTCCATCAGAA	TCCACAATGAGGGTGTAGCAGGTT
CycD1	CAGCGAGCAGCAGAGTCCGC	ACAGGAGCTGGTGTTCCATGGC
4E-SE and 4E-sensitive :		
Pim1	ACACCTTGGGATGGGATAGGA	GAGAAGCAGCACCTAAAAGAGGC
4E-sensitive :		
VEGF	TGCCAAGTGGTCCCAGGCTG	CGGCTTGAAGATGTACTCTAT
Normalization :		
Tubulin	AGCATCCAGTTTGTGGATTGGTGC	CAAAGGCACGCTTGGCATACATCA

Results

Confirmation of cellular fractionation

OneStep PCR was performed for tRNAlys to confirm that cytoplasmic fractions were enriched for tRNAlys RNA (Figure 5.1A). U6 snRNA RT-qPCR was performed to confirm the quality of the nuclear fractions (Figure 5.1B). Fractionation was efficient for all of the cell lines as seen by nuclear enrichment for U6 and cytoplasmic enrichment for tRNAlys.

Total levels of 4E-SE, 4E-sensitive, and 4E-SE containing 4E sensitive transcripts

The total levels of mRNA for 4E-SE transcripts (NONO.1, Nibrin, CycD1, and CGGbp1), 4E-sensitive transcripts (VEGF), and 4E-SE containing 4E-sensitive transcripts (Pim1 and ODC) do not differ between Y30A, 4ET and E (Figure 5.2A, C, E and G, and Figure 5.3A, C, and E).

4ET line has cytoplasmic enrichment of certain transcripts

Relative to E, 4ET shows a cytoplasmic enrichment for the 4E-SE containing transcripts cyclin D1 and the 4E-SE containing 4E-sensitive transcript ODC. (Figure 5.2D, and 5.3F).

Y30A line has nuclear enrichment of certain transcripts

Relative to E, Y30A has a nuclear enrichment for the 4E-SE transcripts cyclin D1 (Figure 5.2D).

Y30A and/or 4ET show no difference in cytoplasmic or nuclear enrichment for certain transcripts

Both Y30A and 4ET show no difference relative to E for cytoplasmic or nuclear enrichment for 4E-SE containing transcripts NONO.1, nibrin, and CGGbp (Figure 5.2B, F, and H); VEGF (4E-sensitive transcript; Figure 5.3B), or Pim1 (4E-SE containing 4E-sensitive transcript) (Figure 5.3D).

Pim1 (4E-SE containing 4E-sensitive transcript) and CycD1 (4E-SE transcript) are not different with respect to their cytoplasmic/nuclear distribution for Y30A as compared to E (Figures 5.3D, and 5.2D).

Western blot data

Cyclin D1 and Mdm2 protein levels are enriched in Y30A and 4ET relative to E (Figure 5.4). Cyclin E1 and VEGF protein levels were similar in Y30A, 4ET, and E cell lines (Figure 5.4).

Discussion

4ET overexpression does not affect transcription of the examined targets

Anecdotal data suggests that 4ET may be present at the promoter of the transcription factor SCL (Hoang lab, unpublished). Therefore, if 4ET were to promote the transcription of SCL, when 4ET is overexpressed, transcriptional targets of SCL could also be elevated. However, since the total level of mRNA for all targets tested (NONO.1, cyclin D1, Nibrin, CGGbp, VEGF, and Pim1 (Figures 5.2 and 5.3) was unchanged between Y30A, 4ET, and E, either these transcripts are not SCL transcriptional targets or the overexpressed 4ET does not have transcriptional activation activity. Overexpression of a tagged version of 4ET (such as the HA-tagged version of 4ET presented in the present study) in a cell line that has endogenously high levels of SCL followed by chromatin immunoprecipitation (ChIP) against HA could be performed to confirm the presence of 4ET at the SCL promoter. RT-qPCR for SCL and/or SCL transcriptional targets in Y30A, 4ET, and E would further resolve whether 4ET has a link to SCL.

Nevertheless, the total amount of the transcripts tested did not vary upon 4ET overexpression.

4ET overexpression can sequester certain transcripts in the cytoplasm

Although 4ET overexpression does not alter total levels of the 4E targets tested, it does appear to have an effect on the cytoplasmic and nuclear distribution of some of the targets.

Since there is an enrichment in the cytoplasm for cyclin D1 and ODC when 4ET is overexpressed (Figures 5.2D, and Figure 5.3F), it is possible that 4ET with 4E is sequestering these transcripts in the cytoplasm, presumably in P-bodies. To confirm the presence of cyclin D1 and ODC transcripts in P-bodies, one could perform fluorescence *in situ* hybridization against these transcripts combined with immunofluorescence against 4ET and 4E. Since the cytoplasmic enrichment of these targets is not seen in the Y30A line (and is more enriched in the nuclear fraction in the case of cyclin D1), the cytoplasmic mRNA sequestration (without degradation) may be dependent on a strong interaction of 4ET with 4E. Alternatively, the Y to A mutation of Y30A may abrogate the 4ET-mRNA or 4ET-RNA binding protein X-mRNA complex that stabilizes the cytoplasmic accumulation of cyclin D1 and ODC transcripts.

Possible mechanisms for 4ET interaction with cyclin D1 and ODC transcripts

Since there was no difference in cytoplasmic/nuclear distribution for all other 4E-SE containing transcripts tested, the effect of 4ET overexpression is not universal for all 4E-SE containing transcripts.

The apparent cytoplasmic sequestration of certain transcripts in the 4ET line may be a consequence of P-body formation without a concomitant increase in P-body proteins involved in mRNA degradation. As seen in the case of overexpression of other P-body proteins (tristetraprolin (TTP) and BRF-1), the overexpression of 4ET may nucleate submicroscopic P-body subcomplexes that contain certain mRNA ((Franks & Lykke-Andersen, 2007). Franks and Lykke-Andersen (2007) suggest that these subcomplexes aggregate to become microscopically visible because there are not enough mRNA degradation enzymes to degrade the accumulated mRNA. This hypothesis is supported by Western blot data for the degradation enzyme Dcp1a presented earlier (Figure 3.2), where no gross changes in its protein level were observed in Y30A and 4ET as compared to E. Since the mRNA decay enzymes are limiting, I posit that the mRNAs found within the P-bodies (perhaps cyclin D1 and ODC) accumulate in the cytoplasm rather than degrade. As the level of 4ET overexpression in the 4ET is so much greater than 4ET in Y30A, (compare 4ET level in 4ET line to Y30A line in Figures 2.1A) cyclin D1 and ODC transcripts aggregate in P-bodies rather than degrade. Thus, the stability of these particular transcripts in the cytoplasm may be increased. Additional experiments exploring differences in stability of transcripts in cytoplasmic and nuclear compartments (e.g. using actinomycin D) could be performed to address this possibility.

Possible mechanisms for 4ET interaction with cyclin D1 and ODC transcripts

4ET protein could interact directly with an mRNA element common to cyclin D1 and ODC. To help identify the mRNA element could be a common sequence or secondary structure recognized by the 4ET protein, one could perform bioinformatic analysis of cyclin D1 and ODC transcripts. In order to provide more data to support the notion that cyclin D1 and ODC transcripts are associated with 4ET in P-bodies, one could perform RNA immunoprecipitation (RNAIP). As I have created HA-tagged cell lines that overexpress 4ET, it is possible to perform and RNA-IP against HA followed by RT-PCR for cyclinD1 and ODC. A possible protocol for isolation of ribonculeoprotein immunoprecipitation (RNP-IP) is presented by (Hassan et al., 2010). The mRNA element is unlikely to be the AU-rich element (ARE) or the cytoplasmic polyadenylation element (CPE) since these elements are not unique to cyclin D1 and ODC (Table 5.3).

4ET could interact with cyclin D1 and ODC transcripts via its interaction with another protein. Clues to the nature of the relationship between 4ET and certain mRNAs may be gained from studies of 4ET paralogues in other species. In *Xenopus,* eIF4E1b and eIF4E interact with 4E-T (Standart & Minshall, 2008). When complexed with eIF4E1b, the *Xenopus* 4E-T interacts with cytoplasmic polyadenylation element-binding protein (CPEB), that in turn interacts with cytoplasmic polyadenylation elements of certain mRNAs such as cyclin (Standart & Minshall, 2008). The interaction between eIF4E1b and *Xenopus* 4ET is independent of the YXXXXLΦ site, suggesting a separate interaction site than that of eIF4E. In my system, it is impossible to determine if the 4E protein that interacts with overexpressed 4ET is eIF4E or eIF4E1b since our antibody is unable to distinguish between these proteins.

Possibility of identifying additional mRNAs in 4ET-induced P-bodies

Studies by Kedersha et al. (2005) suggest that a single class of mRNA localizes to both SGs and P-bodies. However, as these studies used a MS2-reporter construct, they did not identify endogenous mRNA targets (Kedersha, et al., 2005). AU-rich element (ARE) mRNAs have been observed in P-bodies (Franks & Lykke-Andersen, 2007). Another P-body component, GW182, contains an RNA recognition motif (aa 1528-1600) to which a number of mRNA targets were found to bind (Eystathioy et al., 2002). However, cyclin D1 and ODC were not identified in their study as being more than 2-fold enriched. Eystathioy et. al (2002) employed a 1200 cDNA array, therefore, they were unlikely to identify all possible mRNAs found within P-bodies. Cyclin D1, ODC, Pim1, and VEGF were included on their array but NONO.1, nibrin, and CGGbp were not.

Again, as I have created an HA-tagged 4ET overexpression cell line, it would be possible to perform HA-RNAIP, recover the RNA and perform deep sequencing to identify target transcripts. Since P-bodies are transient structures whose components may also be in flux, it may prove difficult to isolate enough material to perform such an experiment (Leung & Sharp, 2006). Other challenges in P-body isolation could arise due to their molecular mass, low abundance and/or incompletely understood physicochemical properties (e.g. high-density hydrophobic proteins such as GW182). Special precautions should be considered in the IP such as modified protocols described by (Moser, Chan, & Fritzler, 2009) and (Hassan, et al., 2010).

As suggested by Standart and Minshall (2008), the specific inhibition of translation of target mRNAs could be renewed by altering 3’ UTR RNP dynamics—another mechanism adding to the complexity of the mRNA regulon.

4ET overexpression probably has no effect on the translation ability of 4E

Since 4ET overexpression was shown to relocalize a proportion of endogenous 4E protein to P-bodies, it was hypothesized that this pool of 4E would be unavailable to be assembled into translation initiation machinery. Therefore, translation, particularly of 4E-sensitive targets, should be impeded. However, no decrease in protein was observed for VEGF (4E-sensitive transcript) (Figure 5.4). I failed in my attempts to perform Western blots for ODC and Pim1 (other 4E-sensitive transcripts that also contain a 4E-SE).

Since Cyclin E1 (a 4E-SE containing transcript that is not 4E-sensitive) and actin (non 4E-SE containing and non 4E-sensitive) were unchanged in Y30A, 4ET, and E, 4ET overexpression had no effect on the translation of these transcripts.

The observation that cyclin D1 and Mdm2 proteins were elevated in Y30A and 4ET as compared to E was unexpected (Figure 5.4). Elevated cyclin D1 protein in the 4ET line could be a consequence of the cytoplasmic enrichment for this transcript (Figure 5.2D). Paradoxically, Y30A shows an elevated level of cyclin D1 protein despite having a nuclear enrichment of cyclin D1 transcript (Figures 5.4 and 5.2D). Perhaps the loading of ribosomes onto cyclin D1 and Mdm2 transcripts in Y30A and 4ET was more efficient than in E (to be assessed by polysome analysis), or the cyclin D1 and Mdm2 proteins are somehow made more stable in Y30A and 4ET as compared to E. HA-IP followed by Western blot for cyclin D1 and Mdm2 would be an experiment to test the protein-protein interaction hypothesis.

Possible reasons for 4ET overexpression not interfering with 4E function

Although overexpression of 4E-T may relocalize and/or concentrate 4E into cytoplasmic puncta (presumed to be P-bodies; see Chapters 2 and 3), it may not be sufficient to interfere with 4E's cytoplasmic functions. Since P-bodies involve more than 20 proteins (see Table 1.6), the co-expression of one or more other P-body components may be required to more efficiently shift the equilibrium of 4E cytoplasmic localization and function (e.g. to be more involved in P-bodies and less involved with mRNA translation initiation). Perhaps the overexpression of P-body proteins that are known to interact with mRNAs slated for decay in P-bodies (e.g. TTP, BRF, Dcp1a, Dcp1b, Dcp2, Ccr4, Lsm, rck/p54 (Cougot, et al., 2004; Franks & Lykke-Andersen, 2007) need to be co-overexpressed with 4ET process to induce a P-body mediated change in the oncogenic potential of 4E.

It is possible that post-translational modifications of 4ET are required for it to relocalize 4E more efficiently to P-bodies. Following this logic, despite 4ET being overexpressed, the molecular pathways that are responsible for the addition/removal of post-translational modifications that enhance 4ET-4E interaction may not be activated sufficiently to radically change that subcellular localization of 4E to P-bodies. Given that 4ET has several phosphorylation sites (refer Figure 1.1 in Chapter 1) and that its phosphorylation status changes in the cell cycle (Pyronnet & Sonenberg, 2001), it is possible that phosphorylation/dephosphorylation is one such post-translational modification. Therefore, overexpression in different cell lines may yield different effects on 4E-SE transcripts and/or translationally sensitive transcripts.

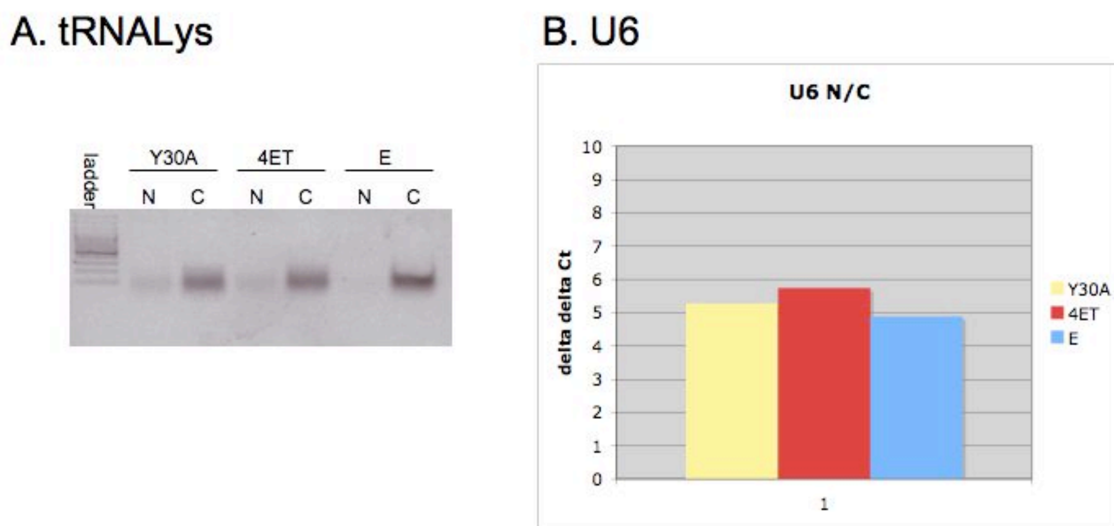


Figure 5.1. Fractionation controls. A) OneStep PCR of the cytoplasmic control tRNAlys; B) RT-qPCR for nuclear control U6.

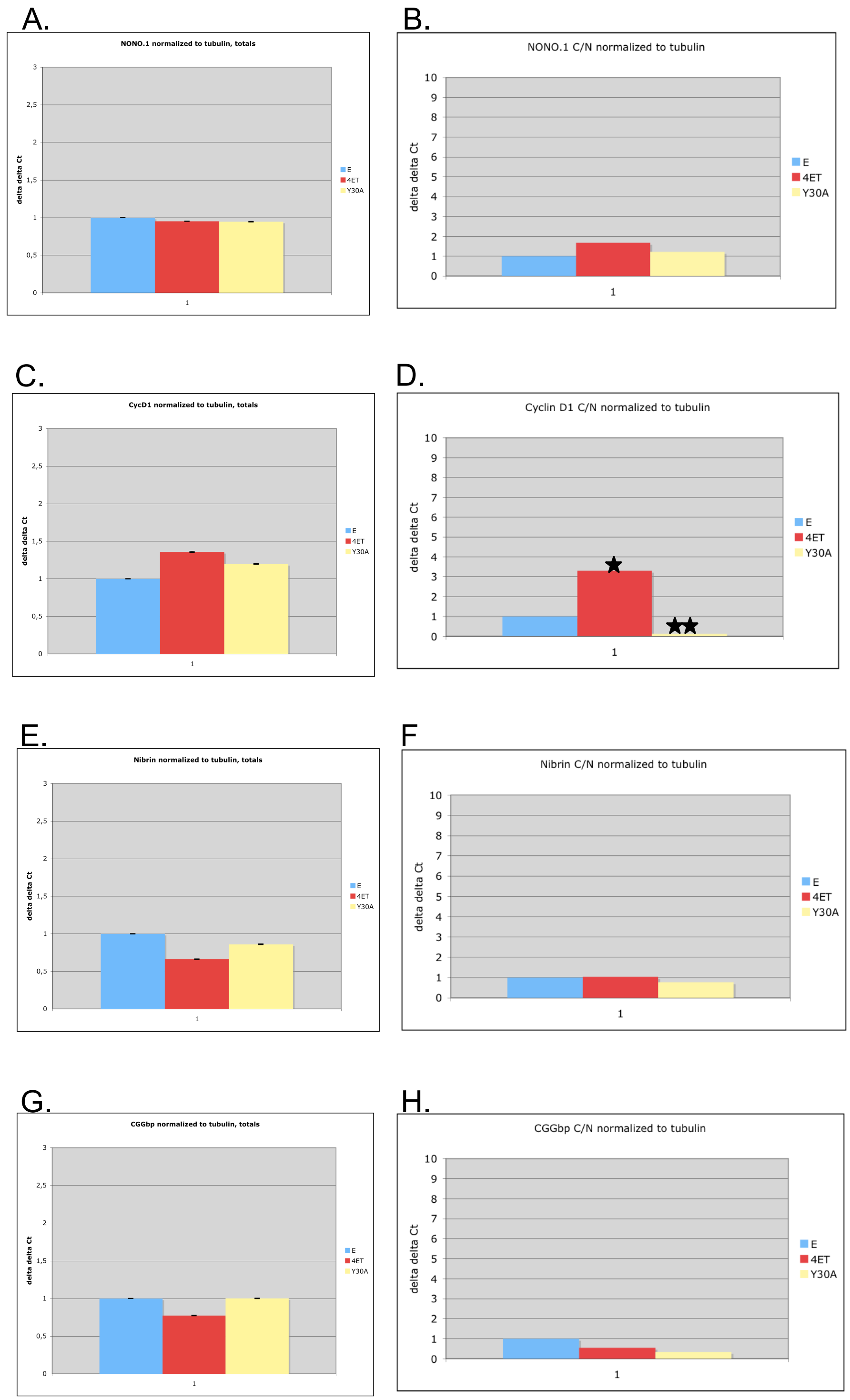


Figure 5.2. RT-qPCR results for 4E-SE transcripts. A, C, E, and G represent total RNA. B, D, F, and H represent cytoplasmic/nuclear (C/N) transcripts. A and B) NONO.1; C and D) Cyclin D1; E and F) Nibrin; G and H) CGGbp; * denotes enriched in cytoplasmic fraction; ** denotes enriched in nuclear fraction.

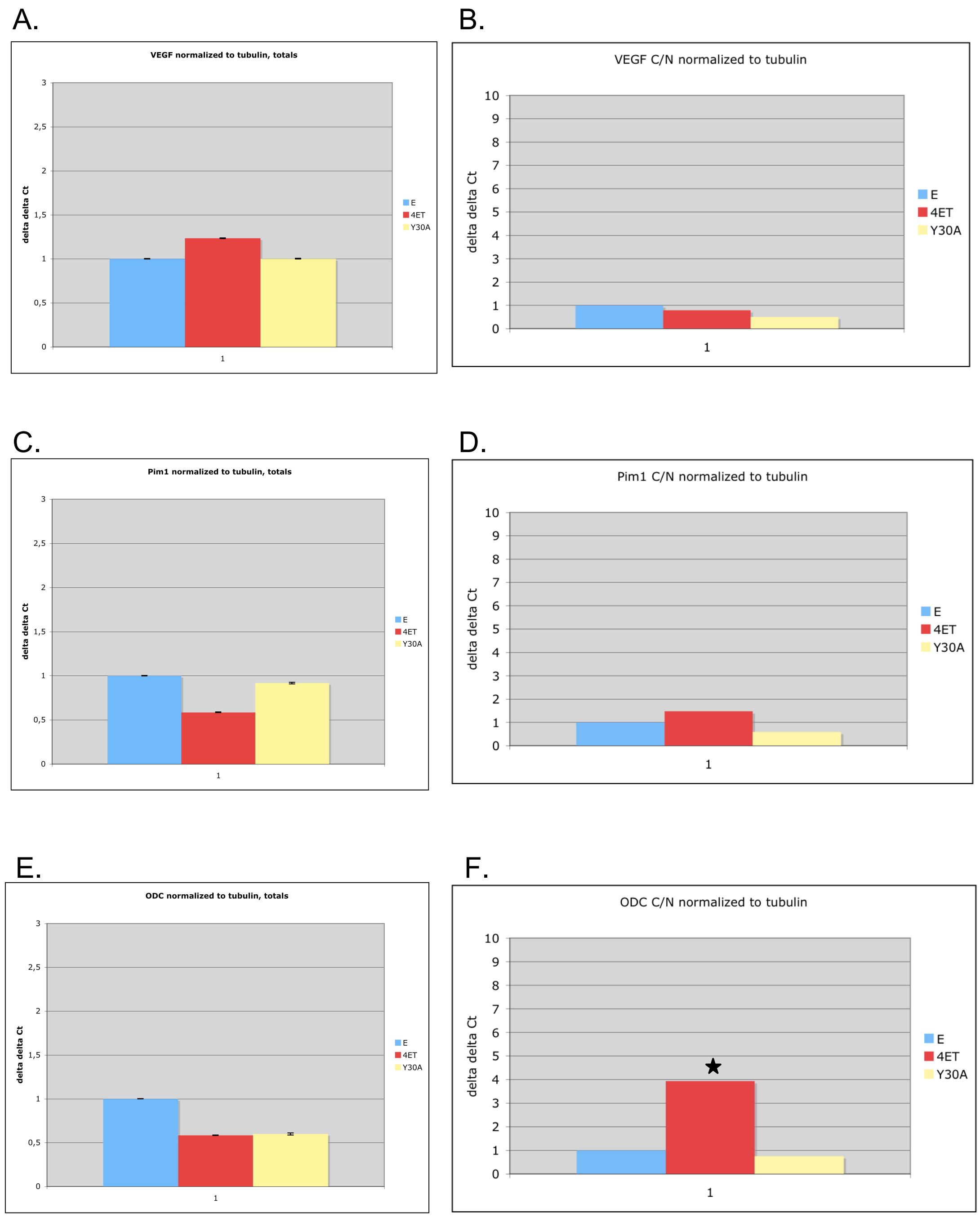


Figure 5.3. RT-qPCR results for 4E-sensitive and 4E-SE containing 4E-sensitive transcripts. A, C, and E represent total RNA; B, D, and F represent C/N RNA. A and B) VEGF (4E-sensitive); C and D) Pim1 (4E-SE containing 4E-sensitive) E and F) ODC (4E-SE containing 4E-sensitive) ; * denotes enriched in cytoplasmic fraction.

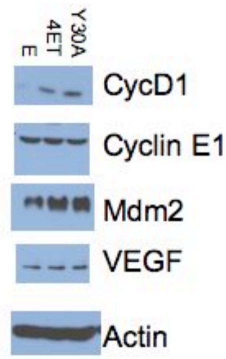


Figure 5.4. Western blot results for products of 4E-sensitive and 4E-SE containing transcripts.

Table 5.3. RNA elements in transcripts tested.

Gene	Cytoplasmic enrichment of RNA?	Elevated total protein?	translation -sensitive?	4E-SE	IRES[1]	ARE[2]	CPEB[3]
Cyclin D1	Yes	Yes	No	Yes	No	Yes	Yes
ODC	Yes	?	Yes	Yes	Yes	No	Yes
NONO.1	No	?	?	Yes	No	No	Yes
Mdm2	?	Yes	No	Yes	No	No	Yes
CGGbp	No	?	?	Yes	No	Yes	Yes
VEGF	No	No	Yes	No	Yes	Yes	Yes
Pim1	No	?	Yes	Yes	Yes	Yes	Yes
Nibrin	No	?	?	Yes	No	No	Yes
Cyclin E1	?	No	?	Yes	No	No	Yes

[1] results from database http://iresite.org;
[2] results from database http://rc.kfshrc.edu.sa/bssc/ARED_GENE;
[3] results from search for TTTTTAT, TTTTTG, TTTTTACU, TTTTAAT, TTTTACT, TTTTATT

Chapter 6. 4E-T overexpression effect on polysome profile.

Introduction

Having established that the 4ET and Y30A lines induce the formation of P-bodies (Chapter 2), I wanted to investigate the impact of P-body formation on polysome profile and the distribution of 4E-SE-containing, and 4E-sensitive transcripts on polysomes.

I reasoned that the induction of P-bodies could affect polysome profile because of the observation of an inverse relationship between translation and P-bodies (Teixeira, et al., 2005) and an apparent reciprocal relationship between polysomes and P-bodies (see (Brengues, et al., 2005).

Since 4E protein plays a role in the assembly of ribosomes to cap-bearing transcripts and it was observed that some 4E is relocalized to cytoplasmic puncta with 4ET overexpression (Chapter 4), I also wanted to investigate the distribution of 4E-SE-containing transcripts and 4E-sensitive transcripts.

Due to time constraints, I restrict my analyses to the 4ET and E cell lines. Fractions were collected for the Y30A line at the same time as data presented here. The Y30A data may be used at a later date.

Materials and Methods

Cell lines

Overexpression cell lines were those generated and described in Chapter 2 (4ET and E).

Polysome profile generation

Each cell line was seeded at (1 X 10^6 cells/plate) X4 and grown for 3 days at 37ºC 5% CO_2. Cells were treated with cyclohexamide (100 μg/ml) for 15 minutes at 37ºC, washed with PBS + cyclohexamide (100 μg/ml), scraped, centrifuged (800 X g, 5 minutes), and resuspended in 600 μl LSB (50 mM Tris pH 7.5, 250 mM KCl, 5 mM $MgCl_2$, 1mM DTT, 400 U/ml RNAseOUT, 100 ug/ml cyclohexamide and 1% TritonX100). Resuspensions were kept on ice for 30 minutes with occasional vortexing and then centrifuged at 3000 X g for 10 minutes at 4ºC. Supernatant was centrifuged at 12000 X g. for 10 minutes at 4ºC. 100 μl of this supernatant (ribosome preparation) was reserved for input RNA and 4 μl was resuspended in 400 μl H_20 to measure absorbance at 254 nm.

20-50% sucrose gradients were prepared (10 mM Tris pH 7.5, 250 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 20U/ml RNAseOUT, 100 μg/ml cyclohexamide) (Biocomp). Ribosome preparation was applied to gradient and centrifuged in ultracentrifuge at 38000 rpm for 2 hours at 4 ºC.

Fractions were collected (approximately 1 ml samples) and frozen at -80ºC until ready for RNA collection (by TrizolLS, Invitrogen). 1 μl of RNA from the input and each fraction was electrophorese on a 1% agarose gel to evaluate the quality of the RNA.

RT-qPCR

750 ng of RNA from each polysome and 1 μg of RNA from inputs was used to synthesize cDNA using the VILOkit (Invitrogen). cDNA was diluted 1 :9 for fractions and 1 :10 for input to be used in qPCR as described previously for tubulin, NONO.1, cyclin D1, CGGbp, Pim1, and VEGF.

Reporting of polysome RT-qPCR data

Ct data for inputs alone are presented. Distribution of mRNA in each polysome fraction is reported relative to input. The percentage of transcripts in each fraction is shown to demonstrate the distribution of transcripts in the profile.

Results

The polysome profile is very similar between the 4ET and E cell lines (see Figure 6.1A and 6.1B). The absorbance at 254 nm for 4ET was 0.378 and 0.343 for E. The profile of the RNA extracted for 4ET and E polysomes was also similar. Superposition of the polysome profiles for 4ET and E are almost identical (Figure 6.2).

The tubulin in the inputs and the distribution of tubulin in the polysome fractions for 4ET and E were very similar (Figure 6.3).

The inputs for transcripts tested all show no difference in raw Cts between 4ET and E (Figures 6.4-6.5).

The distribution for 4E-SE containing transcripts across polysomes was similar for 4ET and E (Figure 6.6). The peak of 4ET and E for NONO.1 occurred at fraction #9 and at fraction #4 for CGGbp (Figure 6.6A and C). The peak for cyclin D1 occurred at fraction #6 for 4ET and fraction #7 for E (Figure 6.6B).

The polysome profile for 4E-sensitive transcript VEGF was nearly identical for 4ET and E with a peak at fraction #5 (Figure 6.7A).

The distribution of 4E-SE containing 4E-sensitive transcript Pim1 was very similar for 4ET and E with a peak at fraction #6 (Figure 6.7B). The shape of the distribution of the 4E-SE containing 4E-sensitive transcript ODC was also similar for 4ET and E but the peak for 4ET was at fraction #4 and at fraction #5 for E (Figure 6.7C).

Discussion

Given that the shape of the polysome traces and the pattern of RNA seen in Figure 6.1 and 6.2, overexpression of 4ET does not have a large effect on global translation. Therefore, the induction of P-bodies in the 4ET cell lines did not result in changes in the polysome profile using the polysome preparation method used. The similarity in tubulin profile for 4ET and E and inputs also suggests that global loading of tubulin transcript was unaffected by 4ET-induced P-body formation.

The initial polysome preparation (input) contains the cytoplasmic fraction of cells. Since P-bodies and SGs are found within the cytoplasm, the input should also contain all cytosolic RNPs. Given that P-bodies contain several proteins (refer to Table 1.5), if they remain intact during the fractionation/extraction process, they will likely be "heavy" and sediment with the fraction with the most polysomes if at all. It would be possible to identify the fractions in which 4ET protein is found if samples were treated with 10% trichloroacetic acid and then subjected to Western blot (Wilusz, 2008). Therefore, I suggest that the transcript distribution on polysomes represents the activity of transcripts not associated with P-bodies.

The coincident peaks for 4ET and E for transcripts at the same fraction number of NONO.1, CGGbp, VEGF, and Pim1 suggest that the loading of ribosomes on these transcripts was not altered by the overexpression of 4ET (Figures 6.6 and 6.7). The shift of peak toward a lower polysome fraction for cyclin D1, and ODC in the 4ET line as compared to E suggests that overexpression of 4ET may have had a minor effect on the loading of ribosomes on these transcripts (Figures 6.6B and 6.7C). Since there was cytoplasmic enrichment for both cyclin D1 and ODC transcripts (Figure 5.2D and 5.3F), 4E-T induced P-bodies may have sequestered a proportion of these transcripts to impede their association with ribosomes. As ODC is a translationally-sensitive transcript while cyclin D1 is not (refer to Table 5.3), the dependence on 4E for ribosome assembly may be irrelevant with respect to the effect of 4ET overexpression on ribosome loading of these transcripts. Similarly, since cyclin D1 transcript does not possess an IRES while ODC transcript does (Table 5.3), the 4ET-induced reduction in the loading of ribosomes on these transcripts is also independent of the IRES motif. Western blot data for ODC would help solidify the hypothesis that 4ET overexpression affected the translation of ODC.

The overall modest effect of 4ET overexpression on distribution of transcripts tested suggests that overexpression of 4ET alone did not dramatically alter the loading of ribosomes onto these transcripts to increase or decrease their translation. Rather, polysome profiles suggest no effect on the translation of NONO.1, CGGbp, VEGF, and Pim1 and a decrease in translation of cyclin D1 and ODC. Again, perhaps co-overexpression of other P-body protein(s) might have produced a greater effect on the loading of polysomes onto transcripts.

Cyclin D1

Since the western blot data for cyclin D1 (Figure 5.4) is at odds with the polysome profile for 4ET as compared to E (Figure 6.6B), I suggest the following model: 4ET overexpression induces P-body formation in the cytoplasm. However, as the overexpression of 4ET is not accompanied by an increase in P-body associated mRNA degradation proteins, cyclin D1 transcript accumulates in P-bodies (as seen in its cytoplasmic/nuclear distribution; Figure 5.2D). This accumulation may be due to 4ET interacting with cyclin D1 transcript directly or with another protein, via an as yet to be determined RNA motif. P-body formation in 4ET does not alter the polysome profile (Figure 6.1) but may reduce the availability of cyclin D1 transcripts that can load ribosomes (as seen by a shift in the cyclin D1 transcript profile for 4ET relative to E; Figure 6.6B). The overexpressed 4ET protein interacts with cyclin D1 protein (confocal data of Figure 5.5) to increase its stability, which is seen as a relative increase as compared to E by Western blot (Figure 5.4).

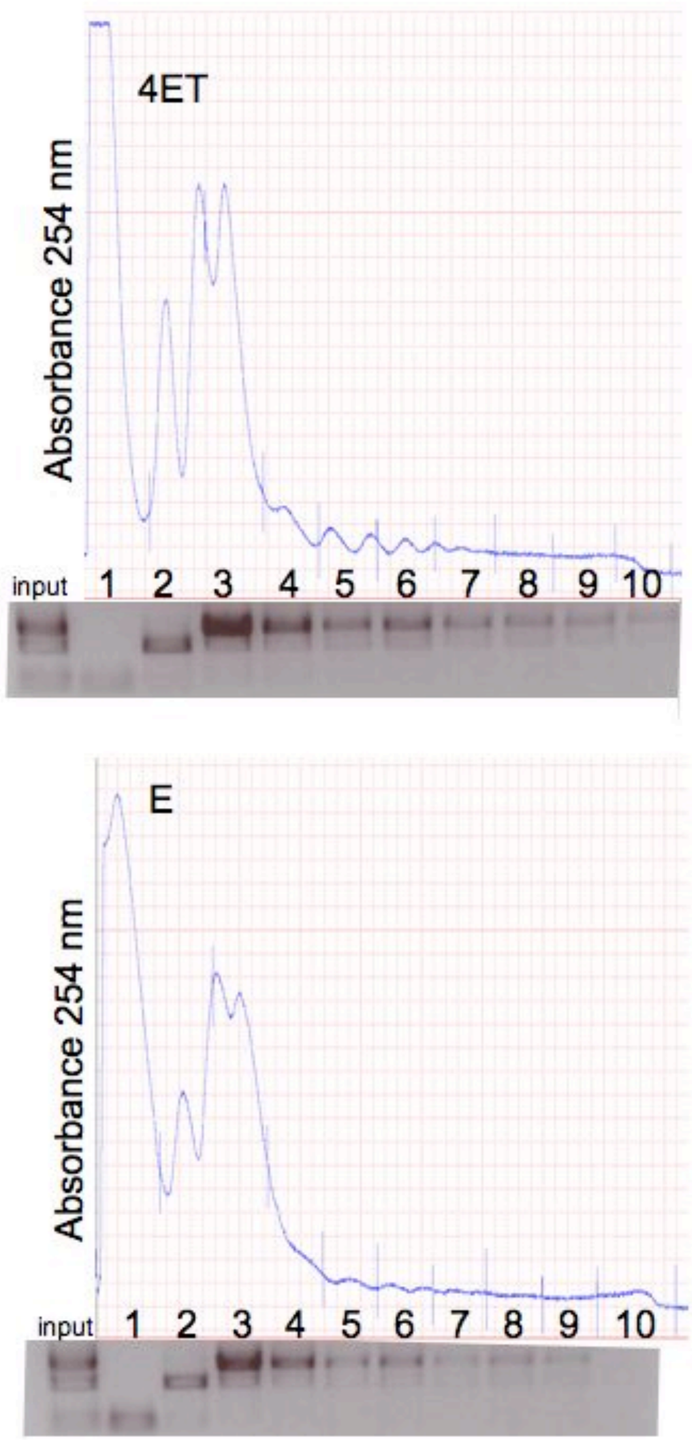


Figure 6.1. Polysome profiles and RNA of each faction. A) 4ET; B) E.

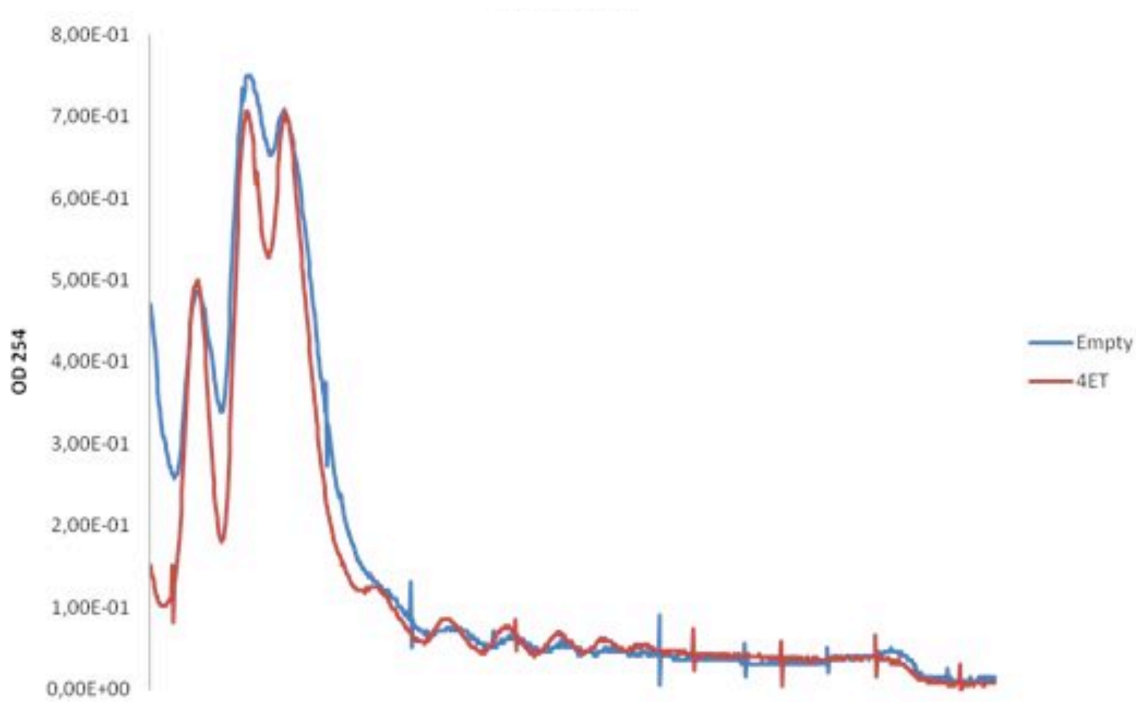


Figure 6.2. Superposition of polysome traces for 4ET and E.

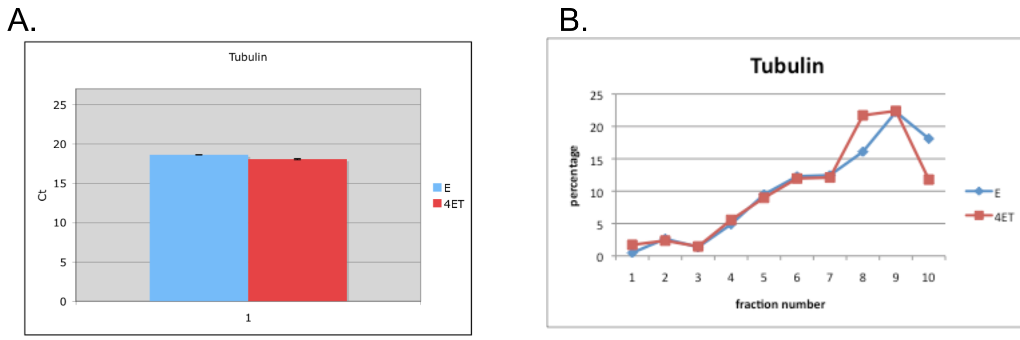


Figure 6.3. Tubulin data. A) Input Cts; B) polysome profile for tubulin.

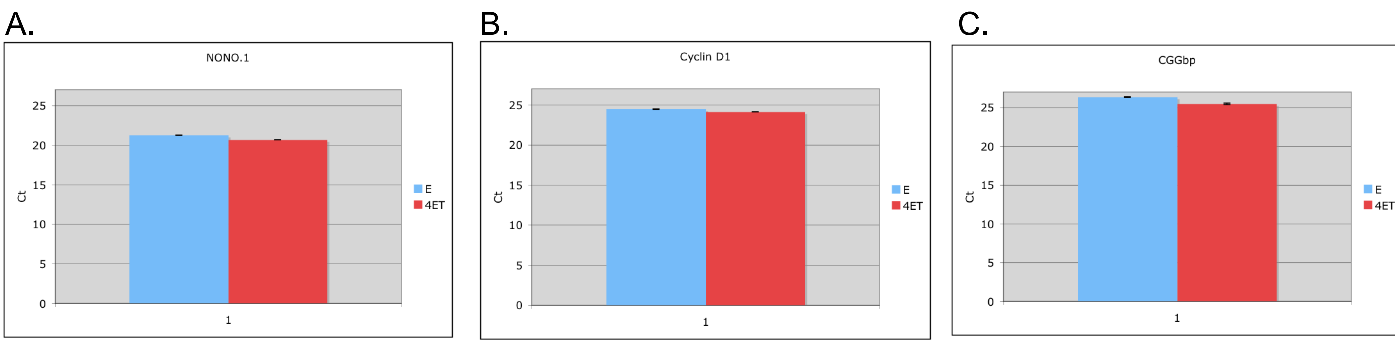


Figure 6.4. Raw Ct data for inputs of 4E-SE containing transcripts. A) NONO.1; B) Cyclin D1; C) CGGbp.

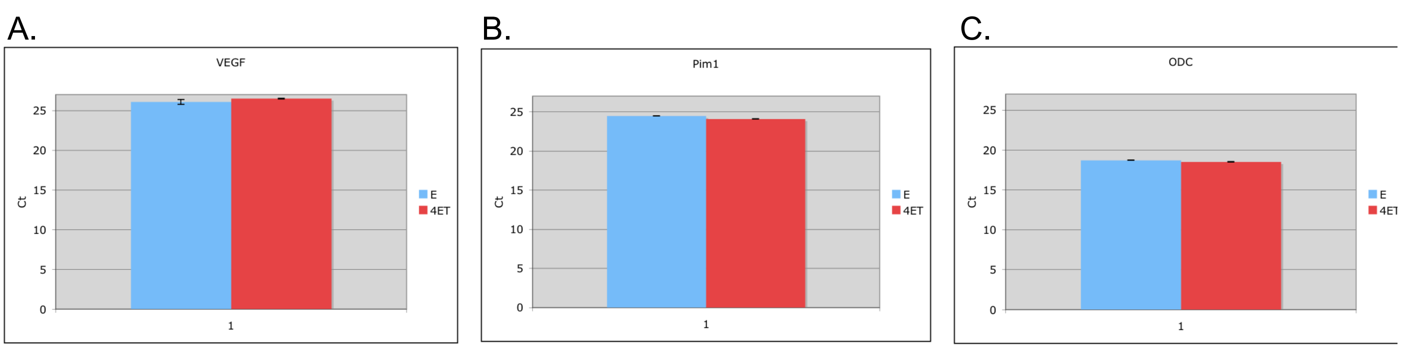
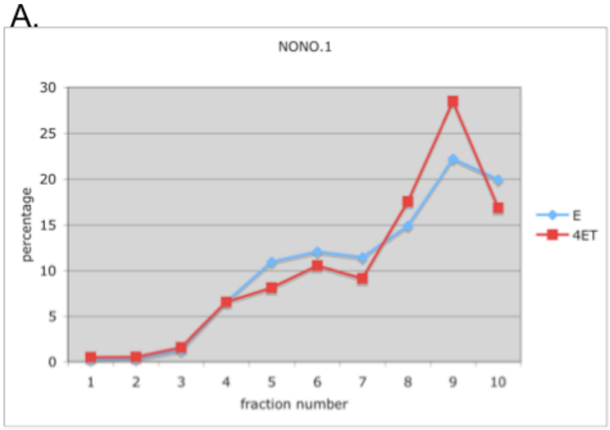
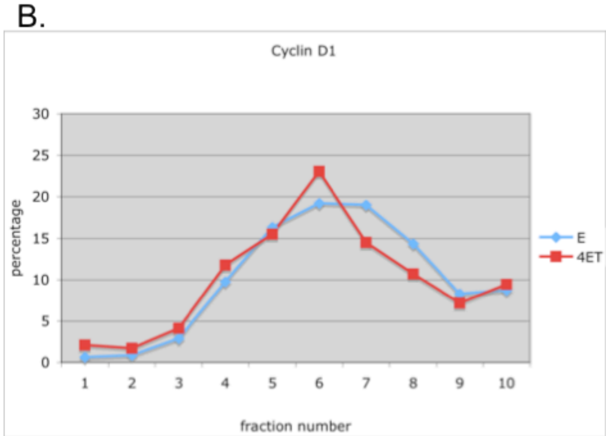


Figure 6.5. Raw Ct data for inputs of 4E-sensitive transcripts and 4E-SE containing 4E-sensitive transcripts. A) VEGF (4E-sensitive transcripts); 4E-SE containing 4E-sensitive transcripts B) Pim1; C) ODC.

A.



B.



C.

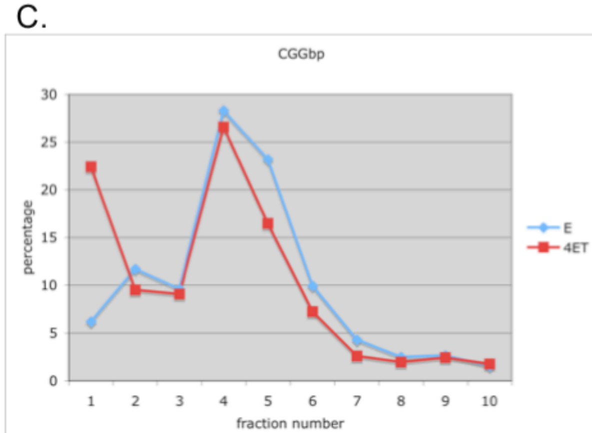
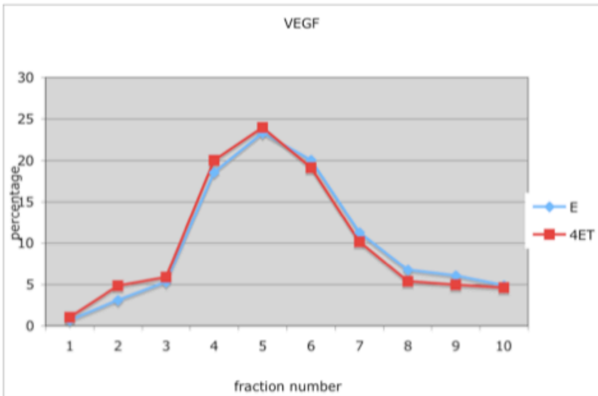
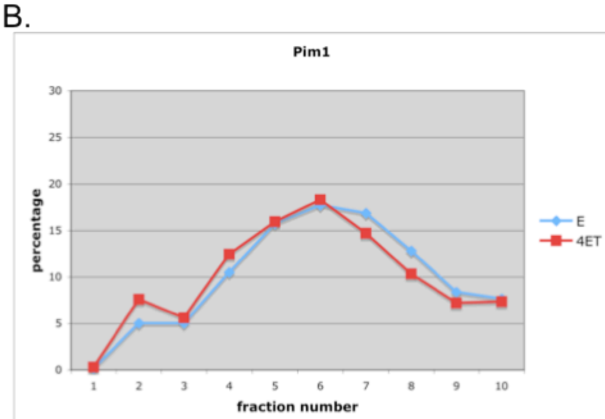


Figure 6.6. Transcript profiles for 4E-SE containing transcripts. A) NONO.1; B) Cyclin D1; C) CGGbp.

A.



B.



C.

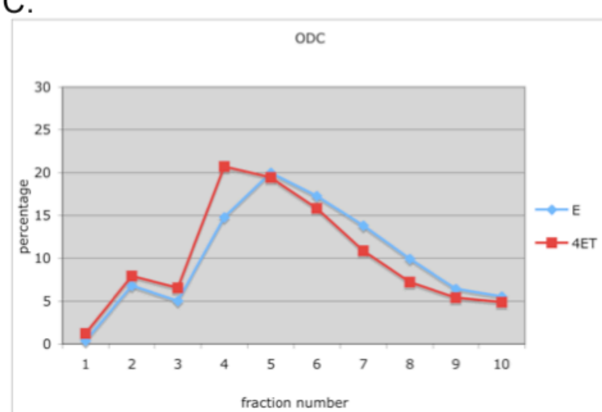


Figure 6.7. Transcript profiles for 4E-sensitive and 4E-SE containing 4E-sensitive transcripts. A) VEGF; B) Pim1; C) ODC.

Chapter 7. General discussion and Conclusion

The purpose of this memoire was to test the following hypothesis: 4E-T simultaneously modulates the activities of 4E in the nucleus (mRNA export), in the cytosol (translation), and in P-bodies (mRNA stability/sequestration) thereby reprogramming the proteome of the cell.

The data suggest that 4E-T overexpression alone does not affect 4E-mediated mRNA export or translation of most of the targets tested (Chapter 5). Cyclin D1 and ODC mRNAs may be sequestered in P-bodies and their sequestration appears to reduce the efficiency of ribosome loading onto these transcripts (Chapters 5 and 6). The interaction of 4ET with cyclin D1 and ODC transcripts (and perhaps others) may be direct or indirect (Figure 8.1). By Western blot, I have demonstrated that the translation of most of the targets tested was also unaltered (Chapter 5).

Therefore, overexpression of 4ET alone in U2Os cells does not appear to reduce the oncogenic potential of 4E in the cell system I generated. Perhaps overexpression of 4ET in a cell line that has endogenously high levels of 4E would have different effects. My attempts to overexpress 4ET in a cell line with high levels of 4E (the squamous cell carcinoma FaDu) were unsuccessful. I have also generated U2Os cell lines that co-overexpress 4E and 4ET but have not included them in this study.

Overexpression of 4ET in a different cell line might yield different results than those shown here not only due to 4E levels within the cell, but due to the activity of signaling pathways affecting post-translational modifications that affect 4ET function.

As suggested in previous chapters, the co-overexpression of other P-body components (see Table 1.6) may be important in more radically effecting P-body mediated changes in the proteome.

In all cases of co-overexpression, the balance between the level of 4ET overexpression and either 4E and/or P-body protein overexpression may be of critical importance. Controlling overepxression via an inducible promoter is possible but introduces another level of complexity in studying the relationship between 4E and 4ET.

Cyclin D1 and Mdm2

The observation that cyclin D1 protein and Mdm2 protein are elevated in Y30A and 4ET was unexpected and may reflect 4ET protein stabilizing these proteins.

Cyclin D1 protein levels change throughout the cell cycle. Cyclin D1 must be high in G1 to initiate DNA synthesis, then must be suppressed to low levels in S to allow synthesis. Cyclin D1 is degraded in S phase (via proteosomes in response to phophosphorylation at

Thr 286 by glycogen synthase kinase-3β (GSK-3β) (Diehl, Cheng, Roussel, & Sherr, 1998) and needs to be resynthesized to re-enter G2 (K. Yang, Hitomi, & Stacey, 2006). Cyclin D1 is reduced in the nucleus in many cell types during S phase. Overexpression of cyclin D1 alone is insufficient for oncogenic transformation, rather nuclear trafficking and proteolysis is critical for the manifestation of its oncogenicity (Kim & Diehl, 2009). The cyclin box of cyclin D1 is critical for its interaction with Cdk4 and the interaction is critical for nuclear transportation of the complex (Murakami, Horihata, Andojo, Yoneda-Kato, & Kato, 2009). Nuclear cyclin D1 overexpression is associated with cell proliferation (Itoi et al., 2000).

If cyclin D1 protein is retained in the cytoplasm by 4ET protein, then it could have no effect on proliferation despite accumulation. Sequestration of cyclin D1 in P-bodies may impede GSK-3β-cyclin D1 and/or cyclin D1-Cdk4 interactions. Confirmation of 4ET-cyclin D1 protein-protein interaction in the 4ET overexpression situation would need to be performed first before exploring this line of arguments.

Ectopic expression of cyclin D1 impairs proliferation and enhances apoptosis (Duquesne et al., 2001). A fluorescence-activated cell sorting (FACS) experiment could clarify if 4ET overexpression (and observed increase in cyclin D1 protein) affects cell susceptibility to apoptosis. My preliminary FACS data for Y30A and 4ET is inconclusive at present.

Mdm2 gene (murine double minute gene 2) encodes an E3 ubiquitin ligase that targets p53 for proteasome-mediated decay, thereby controlling the level of p53 and affecting transformation (Momand, Zambetti, Olson, George, & Levine, 1992). Mdm2 shuttles from nucleus to cytoplasm and appears to direct p53 to proteasomes (Kubbutat, Jones, & Vousden, 1997). Since Mdm2 protein is elevated in Y30A and 4ET, it too may be sequestered in P-bodies to prevent p53 degradation (analogous to cyclin D1 GSK-3β relationship). Again demonstration of 4ET and Mdm2 protein-protein interaction would need to be confirmed.

Future directions

As I have generated a cell line that overexpresses an HA-tagged version of 4ET, it offers the possibility to examine RNA and proteins that associate with overexpressed 4ET.

HA-RNAIP could be performed (Hassan, et al., 2010) to confirm the association of cyclin D1 and ODC transcripts with 4ET. The same type of experiment could be expanded to recover all transcripts (and possibly miRNA) that associate with 4ET. Once candidate transcripts have been identified, their presence in P-bodies could be confirmed by FISH combined with indirect immunofluorescence for 4ET and 4E. As suggested earlier, bioinformatics analysis could help identify sequence and/or secondary structure elements common to cyclin D1 and ODC. If direct interaction of 4ET protein with specific mRNA is found to be true, it may be analogous to the 4E-HP-bicoid-caudal mRNA relationship seen in *Drosophila* (Table 7.1).

Immunoprecipitation followed by mass-spectrometry of HA-tagged 4ET could be performed to identify other protein partners of 4ET. As discussed earlier, it may prove difficult to isolate enough material to perform such an experiment due to the transient nature of P-body components (Leung & Sharp, 2006). Other challenges in P-body isolation could arise due to their molecular mass, low abundance and/or incompletely understood physicochemical properties (e.g. high-density hydrophobic proteins such as GW182). Special precautions should be considered in the IP such as modified protocols described by (Moser, et al., 2009). Dependence of RNA for 4ET-other protein interactions can then be explored experimentally with RNAse.

Protein binding partners of 4ET could interact with specific mRNA elements (Figure 7.1B). If this 4E-4ET-protein X-mRNA relationship exists, it joins the list of other cases where 4E-binding proteins are recruited by specific proteins present only on a subset of mRNAs (Table 7.1).

Once the RNA motif of cyclin D1 and ODC is identified and the protein domain that binds the RNA motif is also identified, this information could be used in cancer therapy. For example, a protein domain that binds specific oncogenic RNA motifs could be fused to a P-body localization domain to create a chimeric protein that specifically degrades these transcripts. This engineered protein could potentially relieve the burden of excess oncogenic transcripts.

Specific overexpression of eIF41b combined with 4ET in cells that have high 4E could also be an interesting area of study. Since eIF41b has strong interaction with 4ET and translational repressor properties (Evsikov & Marin de Evsikova, 2009; Minshall, Reiter, Weil, & Standart, 2007) their co-overexpression could change the proteome in cells with high 4E. However, as was seen in the present study, it is possible that oncogenic protein could accumulate as a consequence (either direct or indirect) of 4ET overexpression.

All of these proposed therapeutic avenues involving 4ET require a thorough understanding of the activities of the protein and its interactions with RNA and with proteins. Therefore, further study of 4ET is warranted.

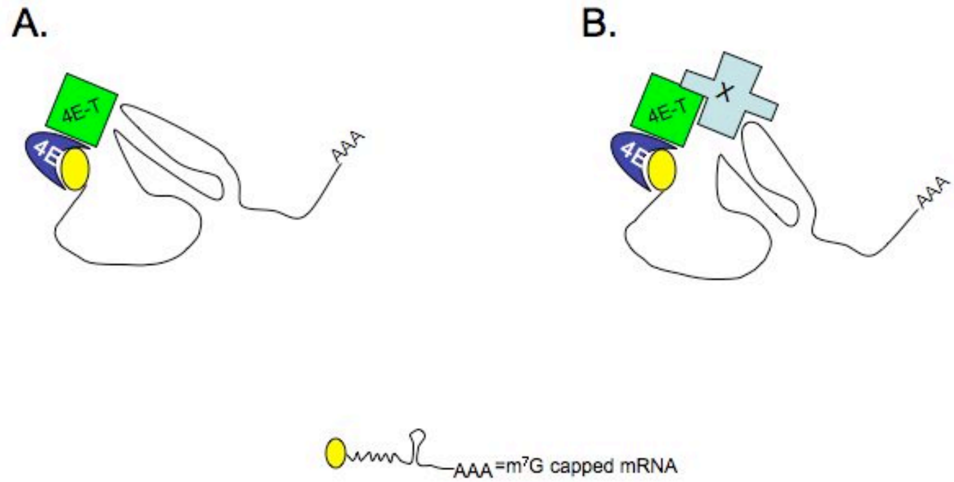


Figure 7.1. Models of potential interaction of 4ET and mRNA in P-bodies. A) direct 4ET interaction of an RNA element (sequence and/or secondary structure); B) indirect 4ET interaction (via a to be determined protein).

Table 8.1. Examples of 4E-4EBP-protein X-mRNA relationships. (adapted from (Cho et al., 2006; Jung, Lorenz, & Richter, 2006; Napoli et al., 2008; Nelson, Leidal, & Smibert, 2004; Standart & Minshall, 2008)

Class of 4E	4EBP	Protein X	mRNA element	mRNA
4E	eIF4G	PABP	polyA	Most mRNA
4E	Cup	Bruno	BRE	*Drosophila* oskar
4E	Cup	Smaug	SRE	*Drosophila* nanos
4E-HP	Bicoid	-	BBR	*Drosophila* caudal
4E-HP	Brat	Nanos/Pum	NRE	*Drosophila* hunchback
4E	Maskin	CPEB	CPE	*Xenopus* cyclin B1
eIF4E1b	4ET	CPEB	CPE	*Xenopus* cyclin
4E	CYFIP1	FMRP	unknown	Human *BC1, Map1b, αCaMKII, Arc, App* in brain
4E	neuroguidin	CPEB	CPE	unknown

Supplemental data

siRNA depletion of 4ET inhibits P-body formation in FaDu cells.

Introduction

As a demonstration that 4ET is required for P-body formation, I performed 4ET depletion (by siRNA) in FaDu cells (adherent human hypopharyngeal squamous cell carcinoma cell line) which has endogenously high 4E (as compared to Detroit 551, Borden lab unpublished).

Materials and Methods

FaDu cells (ATCC HTB-43) were grown to 50% confluence and then subjected to siRNA treatment by lipofectamine 2000(Invitrogen) as per manufacturer's instructions (Mock (no siRNA); si4ETC (sense 5'AUCCUGUAUUCAGACAAUGUACACUCC 3'; antisense 5' TAGGACAUAAGUCUGUUACAUGUGA 3'); siLuc (against firefly luciferase; 5'-CACGUACGCGGAAUACUUCGA-3').

Indirect immunflourescence was as performed in Chapter 2.

Results

siRNA for specific depletion of 4ET transcript reduced the number of 4ET positive cytoplasmic foci in FaDu cells (compare si4ETC to mock and siLuc).

Discussion

The absence of 4ET-positive cytoplasmic foci upon 4ET transcript depletion suggests that it is important for the formation of P-bodies. Depletion of 4ET protein is efficient by Western blot analysis (data not shown). Indirect immunofluorescence data demonstrating absence of Dcp1a-positive bodies upon 4ET depletion would be needed to more strongly support the notion that 4ET is important for P-body formation.

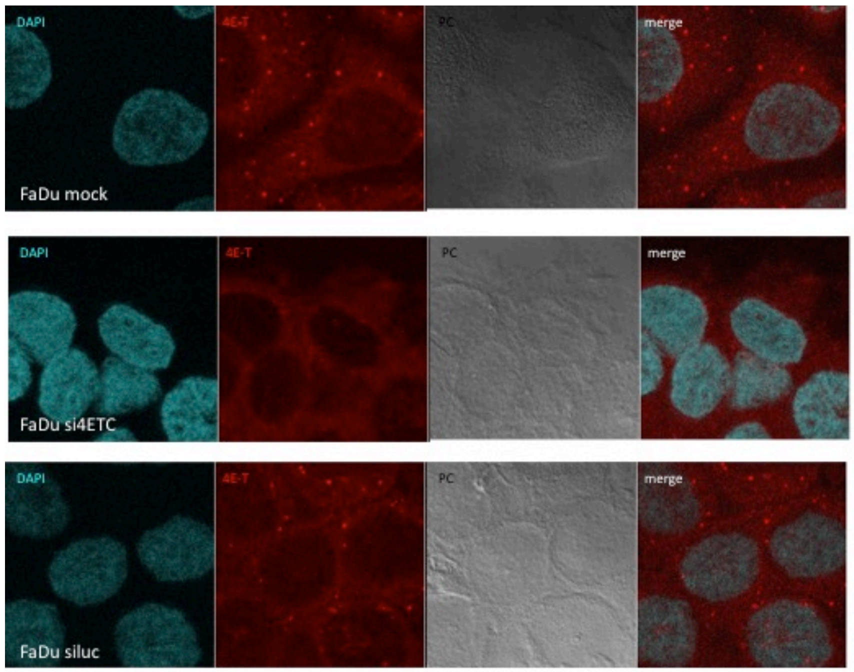


Figure S.1. FaDu cells transfected with si4ET do not have P-bodies.

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