The Cap-binding Inhibitor of Translation, d4EHP

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ABSTRACT

In eukaryotes, the initiation phase of protein synthesis or translation is a multi-step process that culminates in the positioning of the 80S ribosome at the initiation codon of a messenger RNA (mRNA). Recognition of the cap structure by eukaryotic initiation factor 4F (eIF4F; composed of three subunits: the cap-binding protein eIF4E, the RNA-helicase eIF4A and the scaffolding protein eIF4G) facilitates this process. The ability of eIF4F to bind to the cap, as a result of the Cap:eIF4E interaction is of particular importance, as it is the major target of translational regulatory mechanism.

Early embryogenesis requires the activity of various maternal determinants called morphogens, whose spatial and temporal expressions are tightly regulated at the level of translation. Positional information encoded within these factors is thus important for the establishment of body polarity. For instance, in *Drosophila*, when maternal Caudal (Cad) and Hunchback (Hb) proteins are allowed to accumulate inappropriately in an embryo, anterior and abdominal segmentations are blocked. Hence, the precision of Cad and Hb expression domains is critical for normal development.

An eIF4E-related protein called eIF4E-Homologous protein (4EHP) was first described in 1998. However, the function, if any, of 4EHP in translation has been elusive, since it does not interact with any known initiation factors. In order to elucidate its biological function, the power of *Drosophila* genetics was used. In this thesis, I show that the *Drosophila* homolog of 4EHP (d4EHP) interacts with Bicoid (Bcd) and Brain tumor (Brat) proteins to inhibit the translation of maternal *cad* and *hb* mRNAs. Simultaneous interaction of d4EHP with the cap and Bcd or Brat results in mRNA circularization, which renders *cad* and *hb* mRNAs translationally inactive. This example of cap-dependent translational control that is not mediated by eIF4E defines a new paradigm for translational inhibition involving tethering of the mRNA 5' and 3' ends.

Résumé

Chez les eucaryotes, la synthèse protéique est un processus énergétique très coûteux strictement contrôlé, principalement au niveau de l'initiation de la traduction. Ce contrôle se fait en partie grâce aux facteurs d'initiation appelés – eukaryotic Initiation Factors – ou eIFs, eIF4F étant le plus largement étudié. eIF4F permet le recrutement du complexe ribosomal 80S à l'ARN messager (ARNm). L'habilité de eIF4E d'interagir avec la coiffe – the cap structure – facilite ce processus.

Le développement embryogénique chez les eucaryotes nécessite l'activité de facteurs maternels appelés morphogènes. Leur expression spatiale et temporale est strictement contrôlée au niveau de la traduction. Les informations spatiales que contiennent ces protéines sont importantes pour l'établissement de la polarité corporelle. Par exemple, chez la *Drosophile*, lorsque les protéines maternelles Caudal (Cad) et Hunchback (Hb) s'accumulent dans des régions non-conventionelles, la segmentation antérieure et abdominale est bloquée. Or, la précision des domaines d'expression de Cad et Hb est critique pour un développement normal.

Une protéine reliée à l'eIF4E, appelée eIF4E-Homologous protéine (4EHP) a été clonée en 1998. Cependant, le rôle qu'elle joue dans la traduction n'a pu être déterminée, parce qu'elle n'interagit pas avec eIF4G ou 4E-BPs. Pour déterminer le rôle de 4EHP, on a utilisé le systéme génétique de la *Drosophile*. Dans cette thèse, nous démontrons pour la première fois que l'homologue de 4EHP chez la *Drosophile* (d4EHP) interagit avec Bicoid (Bcd) et Brain tumor (Brat) pour inhiber la traduction des ARNm *cad* et *hb*. L'inhibition est causée par une interaction simultanée de d4EHP avec la coiffe et Bcd ou Brat qui permet la formation d'ARNm circulaires. Cet exemple de régulation traductionnelle qui n'est pas contrôlée par eIF4E constitue un nouveau modèle d'inhibition de traduction.

PREFACE

This thesis is a compilation of one published and one submitted manuscripts for which I am the first author.

Chapter 2

Cho, P. F., Poulin, F., Cho-Park, Y. A., Cho-Park, I. B., Chicoine, J. D., Lasko, P., and Sonenberg, N. (2005). A New Paradigm for Translational Control: Inhibition via 5'-3' mRNA Tethering by Bicoid and the eIF4E Cognate 4EHP. Cell *121*, 411-423.

Chapter 3

Cho, P. F., Cho-Park, Y. A., Cho-Park, I. B., Lasko, P., and Sonenberg, N. (2005). Cap-Dependent Translational Inhibition Establishes Two Opposing Morphogen Gradients in *Drosophila* Embryo. Nature Cell Biology (*Submitted*).

I would like to acknowledge the work of my co-authors for each of the chapters, and thank them for their collaboration. In addition to the co-authors, I have received help from several people. Their contribution is acknowledged at the end of each chapter.

Chapter 2

Francis Poulin constructed the pcDNA3-FLAG-Bcd constructs, and co-supervised the initial part of the work. Andrew Yoon and Ian jointly constructed pcDNA3-*rLuc*-BBR^{sense}, pcDNA3-*rLuc*-BBR$^{anti\text{-}sense}$, pProEX-His-d4EHP and pGEX-d4EHP constructs, and designed the experiments presented in Figure 2.11. Jarred Chicoine and Paul Lasko taught me *Drosophila* genetics and provided expert advice. I have performed all other experiments and wrote the entire manuscript.

Chapter 3

Andrew Yoon and Ian jointly constructed pcDNA3-FLAG-Brat, pProEX-His-Brat, pProEX-His-Nos and pGEX-Pum constructs. Paul Lasko provided expert advice and helped characterize various mutant phenotypes presented in this chapter. I have performed all other experiments and wrote the entire manuscript.

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To begin with, I would like to thank Nahum for giving me the opportunity to work in his lab. Under his tutelage, I have learned great many things about science. Thank you! I would also like to take this opportunity to thank Paul for all his support and expert advices.

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Lastly, I would like to extend my heartfelt gratitude to my family for their unwavering love and support. To mother and father, thank you for everything! To In and Yoon, may the future bring you much success and happiness! Despite all the difficulties I have encountered during the course of my study, because of you it was all worth it in the end! Remember, this is only the beginning...

"Do not lead the truth, but let the truth lead you"

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CHAPTER 1 – General Introduction

1.1 Prologue

With the advent of technology, we have now deciphered our genome in its entirety; a monumental achievement that could potentially yield great benefits to mankind. However, the genome by itself is only a code, a complex biological riddle or blueprint that needs deciphering. Produced in a process called translation - the mechanism by which organisms decode the genomic code encrypted within macromolecules called messenger RNA (mRNA) - proteins are biology's chief architect, worker and construction material of choice. Therefore, if one wishes to qualify DNA as the "book" of life, proteins are its very fabric. Only through their activity can life be sustained. Due to their paramount importance, defects that interfere with the process of protein synthesis often result in fatality (Abbott and Proud, 2004; Ainsworth, 2005; Calkhoven et al., 2002; Meric and Hunt, 2002; Nader et al., 2002; Pandolfi, 2004; Rosenwald, 2004). Understanding how proteins are synthesized and work *in vivo* will, thus, have a profound consequence in our quest to understand life itself.

1.2 Importance of Translational Control

It has long been established that an overwhelming majority of regulatory events controlling gene expression occur at the level of mRNA biosynthesis, otherwise known as transcription (Levy and Darnell, 2002; Mathews et al., 2000). Typically, transcriptional control operates by modulating the overall rate of mRNA synthesis and their subsequent transport from nuclei to cytoplasm; all of which are time-consuming processes. While important, due to these unique characteristics, the concept of transcriptional regulation can never be applied in biological systems such as early embryogenesis, whose first hours are devoid of all detectable transcriptional activity, or in enucleated mature reticulocytes (Mathews et al., 2000). Coincidentally, it was in

these systems that researchers observed the earliest known cases of translational control (Mathews et al., 2000). Regulation of gene expression at the level of translation, therefore, by bypassing the need to invoke the nuclear pathway for mRNA synthesis and transport, allows an organism to respond rapidly to external stimuli and provides the theoretical groundwork for the idea of post-transcriptional gene regulation. It is precisely for these reasons that translational regulation plays such a critical role in development, differentiation, cell cycle progression, growth, and apoptosis (Mathews et al., 2000). At the physiological level, defects that are known to interdict translational control are manifested by the onset of various pathology, most of which are known to be fatal (Abbott and Proud, 2004; Ainsworth, 2005; Calkhoven et al., 2002; Meric and Hunt, 2002; Nader et al., 2002; Pandolfi, 2004; Rosenwald, 2004).

1.3 Brief Overview of Translation

In eukaryotes, translation can be broken into four stages: initiation, elongation (the decoding step), termination and recycling. Translation begins when a ribosome (discussed in section 1.5) is recruited to the 5' end of an mRNA in a process called translation initiation (discussed in section 1.6). Successful recognition of an initiator AUG by a ribosome activates translation elongation. It is during this phase that aminoacyl transfer RNAs (tRNAs) are recruited to a ribosome and catalyze the formation of peptide bonds. Finally, translation is terminated when a processing ribosome encounters a stop codon and releases the completed polypeptide. In the ensuing recycling phase, ribosomal subunits get dissociated, releasing the mRNA and the deacylated tRNAs, to be used in another round of initiation.

1.4 *Anatomy of mRNA*

In eukaryotes, in addition to the coding sequence, an mRNA contains non-coding sequence elements that modulate its translation efficiency and protect it from degradation. These elements will be discussed in further detail in the following section.

1.4.1 *The Cap Structure*

Present at the 5' end of all nuclear transcribed eukaryotic mRNAs, the cap structure (m7GpppN, where N is any nucleotide; Figure 1.1)(Shatkin, 1976) facilitates translation (Muthukrishnan et al., 1975) by promoting ribosome binding to an mRNA via an interaction with the initiation factor 4E (eIF4E; section 1.7.1)(Sonenberg et al., 1978; Sonenberg et al., 1980). In addition to its role in translation, the cap is critically required for pre-mRNA splicing, nucleocytoplasmic transport of mRNAs and mRNA stability (Varani, 1997). For viruses that are known to produce mRNAs that lack the cap structure (i.e.: poliovirus, hepatitis C virus and encephalomyocarditis virus), an alternative translation initiation mechanism, which requires a unique RNA secondary structure called the internal ribosome entry site (IRES), has been developed to facilitate the hijacking of the host translational machinery (section 1.6.3)(Hellen and Sarnow, 2001).

1.4.2 *The Poly(A) tail*

With the notable exception of histone mRNAs, all eukaryotic mRNAs contain a poly(A) tail (ranging 50-200 bases) in the 3' end that is added post-transcriptionally in the nucleus (Hall, 2002). Poly(A) tail plays an important role in the initiation of translation and stability of mRNAs (Hall, 2002; Munroe and Jacobson, 1990; Sachs, 2000; Wilusz et al., 2001). Indeed, through the activity of the poly(A)-binding protein

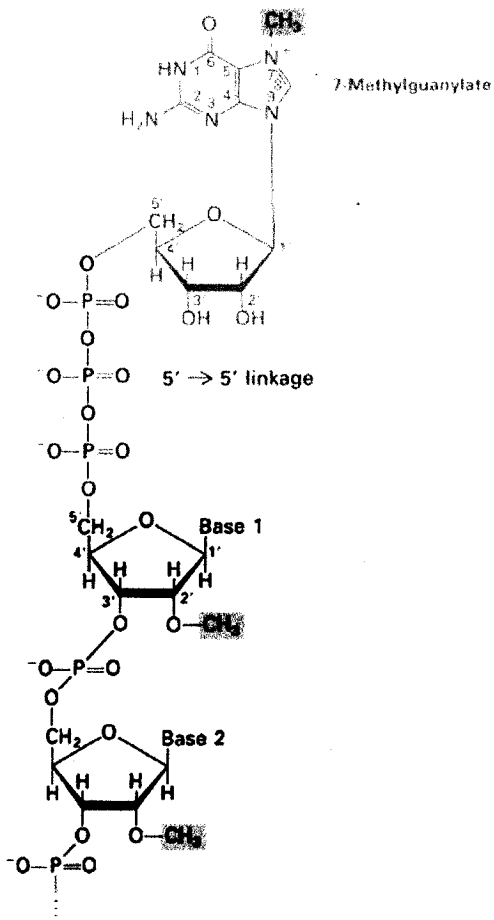


Figure 1.1 The Structure of the 5' methylated cap of eukaryotic mRNA.
The distinguishing chemical features are the 5'-to-5' linkage of 7-methylguanylate to the initial nucleotide of the mRNA molecule and the methyl group at the 2' hydroxyl of the ribose of the first nucleotide (Base 1). Both these features occur in all animal cells and in cells of higher plants; yeast lack the methyl group on Base 1. The ribose of the second nucleotide (Base 2) also is methylated in vertebrates.

(PABP), poly(A) tail synergizes with the cap structure to stimulate translation (Gallie, 1991; Kahvejian et al., 2005). Specifically, by interacting simultaneously with both eIF4G and poly(A) tail, PABP induces mRNA circularization (section 1.8) and allows the 5'-3' translational synergy to take place (Kahvejian et al., 2001; Wells et al., 1998).

1.4.3 *cis-Acting Elements*

In addition to the cap structure and the poly(A) tail, eukaryotic mRNAs contain a number of *cis*-acting elements that influence the translational efficiency of a given transcript. Such elements include sequences that surround the initiator AUG (Kozak, 1991), secondary structures (Jang et al., 1989; Koromilas et al., 1992; Pelletier and Sonenberg, 1988), upstream open reading frames (uORF)(Geballe and Sachs, 2000) and oligopyrimidine tracts located at the extreme 5' end of an mRNA (5' TOP)(Hornstein et al., 2001). Furthermore, 5' and 3' untranslated regions (UTR) of an mRNA contain translational control elements that are known to recruit regulatory proteins to modulate the expression of specific mRNAs (Kuersten and Goodwin, 2003). Trancripts that require such an elaborate translational control include, amongst others, those involved in iron metabolism (Hentze and Kuhn, 1996; Hentze et al., 2004), early erythrocyte development (Ostareck et al., 2001; Ostareck et al., 1997) and in early embryo development (discussed in section 1.9.2)(Gebauer and Hentze, 2004; Johnstone and Lasko, 2001; Kuersten and Goodwin, 2003; St Johnston and Nusslein-Volhard, 1992).

1.5 *The Ribosome*

Found at the heart of the protein synthesis machinery of all known living organisms, ribosome is a large ribonucleoprotein particle (i.e.: 70S prokaryotic ribosome is

composed of ribosomal RNAs (rRNAs) of 4530 nucleotides in length and contains more than 50 proteins), and a ribozyme, that consists of two subunits (Figure 1.2)(Nissen et al., 2000; Noller, 2005; Ogle and Ramakrishnan, 2005; Ramakrishnan, 2002; Yusupov et al., 2001). Whereas in prokaryotes, these subunits are designated 30S and 50S, and together make up the 70S ribosome, in eukaryotes they are referred to as 40S and 60S ribosomal subunits, which join together to form the 80S ribosome. Each subunit has three binding sites for tRNA, designated as A (aminoacyl), which accepts the incoming aminoacylated tRNA; P (peptidyl), which holds the tRNA with the nascent peptide chain; and E (exit), which holds the deacylated tRNA before it leaves the ribosome (Ogle and Ramakrishnan, 2005; Ramakrishnan, 2002)(Figure 1.3). In bacteria, it has been demonstrated that the small 30S ribosomal subunit binds mRNA and the anticodon stem-loops of tRNA, and contributes to the fidelity of translation by monitoring base pairing between the mRNA codon and the tRNA anticodon in the decoding process. The 50S subunit, on the other hand, binds to the acceptor arms of tRNAs and catalyzes peptide bond formation between an incoming amino acid on the A-site tRNA and the nascent peptide chain attached to the P-site tRNA (Ramakrishnan, 2002). At any given time, depending on cellular needs, an mRNA may be bound by a single or multiple ribosomes. Therefore, the number of ribosomes recruited to an mRNA is directly proportional to the rate of translation.

1.6 *Eukaryotic Mechanism of Ribosome Recruitment: Translation Initiation*

Requiring a large number of proteins, RNA factors and ribosome, eukaryotic translation initiation is a complex energy-dependent process that culminates in the positioning of the 80S ribosome at the initiator AUG of an mRNA. Conceptually, the initiation phase of translation can be divided into four distinct steps: 1) Binding of the Met-tRNA$_i$-

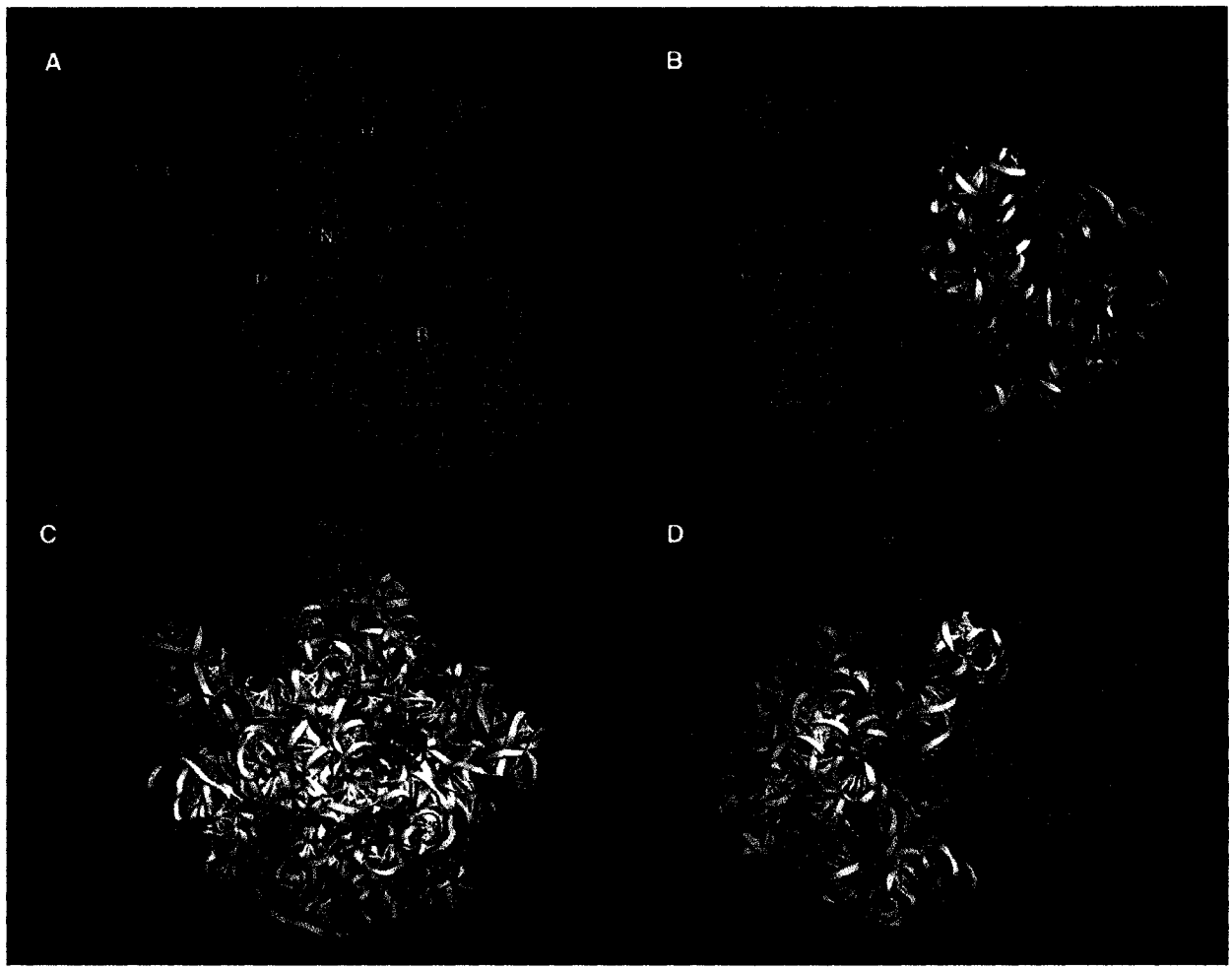


Figure 1.2 Structure of the T. thermophilus 70S ribosome.
A. View from the back of the 30S subunit. H, head; P, platform; N, neck; B, body. B. View from the right-hand side, showing the subunit interface cavity, with the 30S subunit on the left and the 50S on the right. The anticodon arm of the A-tRNA (gold) is visible in the interface cavity. C. View from the back of the 50S subunit. D. View from the left-hand side, with the 50S subunit on the left and the 30S on the right. The anticodon arm of the E-tRNA (red) is partly visible. The different molecular components are colored for identification: cyan, 16S rRNA; gray, 23S rRNA; light blue, 5S rRNA (5S); dark blue, 30S proteins; magenta, 50S proteins. Proteins fitted to the electron density are numbered in orange; 50S proteins whose electron density has been identified but not fitted are numbered in magenta. The A-, P-, and E-site tRNAs are colored gold, orange, and red, respectively (Figure adapted from Yusupov et al., 2001).

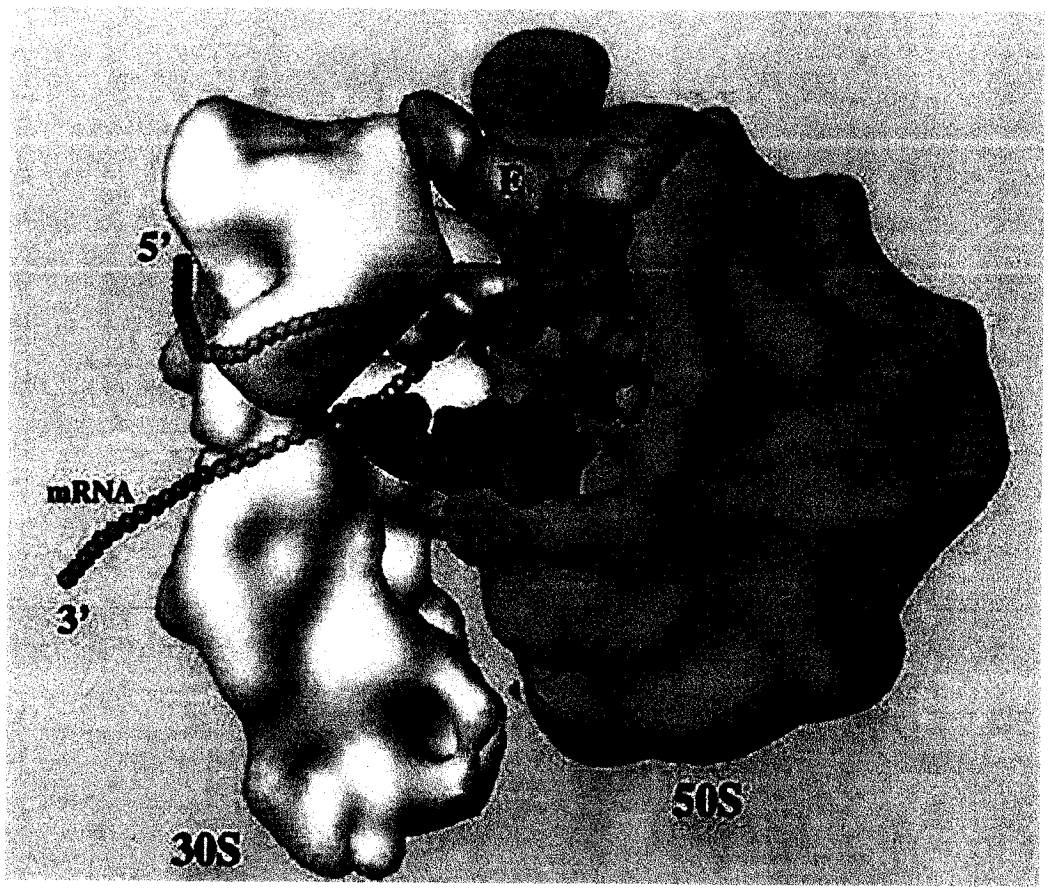


Figure 1.3 The A-, P-, and E- site tRNAs bound to 70S ribosome.
Drawing of a 70S ribosome bound to an mRNA and A-, P-, and E-site tRNAs.

GTP-eIF2 ternary complex with the 40S ribosomal subunit. 2) Recruitment of the small ribosomal subunit to the 5' cap of an mRNA. 3) Scanning of the mRNA 5'UTR by the small ribosomal subunit to reach the initiator AUG. 4) Joining of the large ribosomal subunit to generate a translationally competent ribosome. Several modes of ribosome recruitment have been proposed for eukaryotic and viral protein synthesis: the Scanning model and the Ribosome shunting, both requiring the 5' cap structure, and a cap-independent translation initiation mechanism that is mediated via IRES. Although each model will be given its due consideration, the main focus of this thesis will be the scanning model for cap-dependent initiation of translation.

1.6.1 *The Scanning Model*

In eukaryotes, a new round of translation begins when an 80S ribosome dissociates into 40S and 60S ribosomal subunits (Figure 1.4, step 1). At the same time, nearby, a large protein complex that is composed of eIF1, eIF3 and eIF5 recruits a ternary complex composed of eIF2-GTP and the initiator methionyl-tRNA (Met-tRNA$_i$-GTP-eIF2 ternary complex; step 2). This complex together with eIF1A, binds to a 40S ribosomal subunit to form the 43S pre-initiation complex (step 3). While the 43S is being formed, there is another group of specialized initiation factors, the eIF4 family, that join together to form the eIF4F cap-binding complex. It is through the activity of eIF4F, more specifically via the eIF4E:Cap interaction, that the 43S pre-initiation complex gets recruited to an mRNA (step 4). Together, the eIF4F and 43S pre-initiation complex make up the 48S ribosomal complex. Individual proteins and regulatory mechanisms that govern step 4 will be described in further detail in section 1.7. Following the events of step 4, the 48S ribosomal subunit bound to the initiation factors scans the mRNA in a 5'->3' direction until an initiation codon in a favorable context is found (step 5). Once

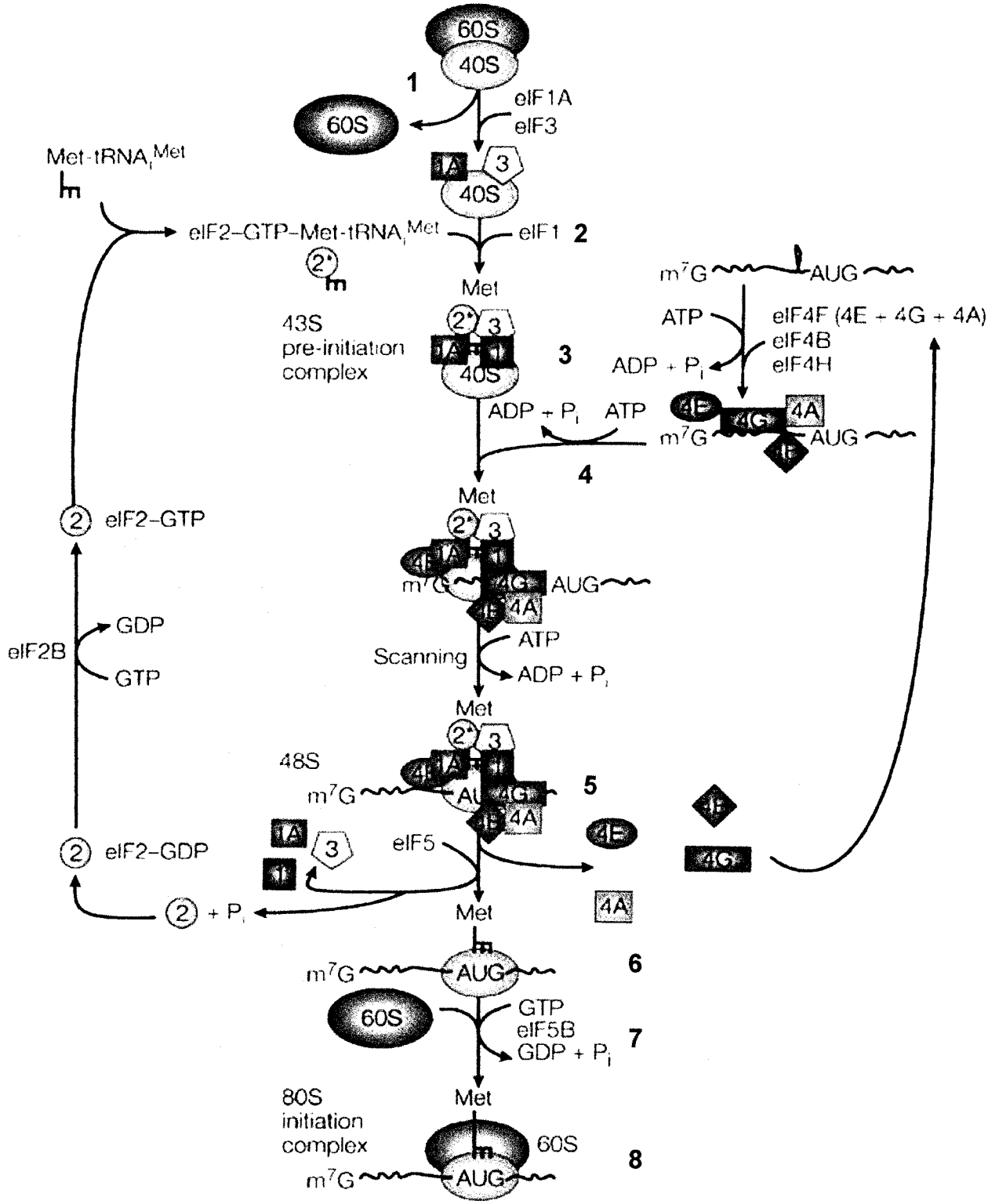


Figure 1.4 Initiation of Translation.
The translation of eukaryotic mRNAs involves the recognition and recruitment of mRNAs by the translation-initiation machinery, and the assembly of the 80S ribosome on the mRNA. This process is mediated by proteins that are known as eukaryotic initiation factors (eIFs). Important steps in the initiation phase of translation are denoted by the numbers colored red. See text for details (Figure adapted from Holcik and Sonenberg, 2005).

the AUG start codon has been identified via base pairing with the anticodon of the Met-$tRNA_i$, eIF5-dependent eIF2-GTP hydrolysis occurs. As a result of GTP hydrolysis to GDP, all associated initiation factors get dissociated from the 48S subunit, leaving the Met-$tRNA_i$ in the P-site base-paired to the AUG start codon on the mRNA; this is in stark contrast with other aminoacyl tRNAs that are recruited to the A site (step 6)(Dever, 2002). Subsequent binding of eIF5B and eIF1A to the 48S subunit allows another round of GTP hydrolysis, which promotes the recruitment of the 60S ribosomal subunit to the initiator AUG. (step 7). Finally, with the dissociation of the remaining two initiation factors, the 80S ribosome can now begin polypeptide elongation (step 8).

1.6.2 *Ribosome Shunting*

Initially proposed by Futterer and colleagues in 1993, the idea of ribosome shunting explains how the 35S mRNA of the cauliflower mosaic virus, which contains a 600 nucleotides leader with several small ORFs that by themselves inhibit translation, gets translated (Futterer et al., 1993). Ribosome shunting, also known as ribosome jumping, discontinuous scanning or repositioning, therefore, describes a process through which a particular segment of the 5'UTR is bypassed by a ribosome. Although examples of ribosome shunting have been described in other virus systems (i.e.: Sendai virus Y protein, adenovirus late mRNAs and papillomavirus E1 mRNA)(Curran and Kolakofsky, 1988; Latorre et al., 1998; Remm et al., 1999; Yueh and Schneider, 1996), this mechanism of translation initiation still remains poorly understood.

1.6.3 *Internal Ribosome Entry Site - Cap-independent Mode of Translation Initiation*

An alternative, cap-independent, mode of translation initiation has been proposed for both viral and eukaryotic mRNA translation. This process is called internal entry of

ribosome and is mediated by specific mRNA sequences termed IRES (Hellen and Sarnow, 2001). Studies on picornavirus (*i.e.*: Poliovirus and Encephalomyocarditis virus) mRNA translation were essential for the discovery of this unusual translational initiation mechanism. Unlike their cellular counterparts, picornavirus mRNAs are naturally uncapped at their 5' ends (Nomoto et al., 1976) and have an unusually long structured 5' UTRs; features that have traditionally been linked to translation repression. Unexpectedly, however, it was later demonstrated that instead of being a translation repressor, the long picornavirus mRNA 5'UTR served as an enhancer of translation (Pelletier et al., 1988a; Pelletier et al., 1988b; Trono et al., 1988). These early observations led to the discovery of the cap-independent translation initiation via IRES, in 1988, by two independent groups who have provided the first tangible evidence that the picornavirus 5'UTR serves as a "ribosome landing pad" to promote the recruitment of ribosomes to the viral mRNA (Jang et al., 1988; Pelletier and Sonenberg, 1988).

How does a stretch of RNA sequence, without the 5' mRNA Cap:eIF4E interaction recruit a ribosome? This question triggered a frantic search for an IRES-binding protein that would be also capable of recruiting a ribosome. eIF4G, the scaffolding protein essential for the cap-dependent mode of translation initiation, emerged as the prime candidate. Indeed, it was demonstrated that not only does eIF4G possesses a binding site for eIF3 (the initiation factor that physically binds to the ribosome for its recruitment to an mRNA), but also contain an RNA-binding domain that was shown to bind to IRES (Imataka and Sonenberg, 1997; Lamphear et al., 1995; Marcotrigiano et al., 2001; Ohlmann et al., 1996; Pestova et al., 1996; Thoma et al., 2004). Therefore, by interacting with both eIF4E and IRES, eIF4G plays a central role in both cap-dependent and cap-independent mechanisms of translation initiation. The role of eIF4G in translation initiation will be further discussed in the following section.

1.7 eIF4 family of initiation factors

1.7.1 *eIF4E*

eIF4E is an evolutionarily conserved cap-binding protein, that interacts with eIF4G to form the eIF4F cap-binding complex. The NMR and crystal structures of yeast and mouse eIF4E bound to m^7GDP demonstrates the conservation of the cap-binding mechanism among the eIF4E of higher eukaryotes (Marcotrigiano et al., 1997; Matsuo et al., 1997)(Figure 1.5). eIF4E is the central component of the cap-dependent translation initiation machinery. When eIF4E, and associated proteins, are depleted from cell-free extracts, translation of capped mRNA is dramatically reduced (Svitkin et al., 1996). Two forms of regulatory mechanisms have been discovered to modulate eIF4E activity in translation initiation. First, a small group of proteins called eIF4E binding proteins (4E-BPs) were shown to inhibit the cap-dependent translation by sequestering eIF4E away from the eIF4F complex (Gingras et al., 1999). The inhibitory activity of 4E-BPs is mediated by their ability to compete directly with eIF4G via a conserved eIF4E-binding motif found in eIF4G and 4E-BPs (YxxxxLΦ, Φ denotes any hydrophobic amino acid and x any amino acid)(Mader et al., 1995). Second, the activity of eIF4E in translation may also be regulated via post-transcriptional modification. It has been shown that eIF4E is a phosphoprotein that gets phosphorylated on a single site, serine 209 (Ser209), by MAP kinase activated kinase Mnk1 (Pyronnet et al., 1999; Raught et al., 2000; Waskiewicz et al., 1997). Although the role of eIF4E phosphorylation is not completely understood, several lines of evidence suggest that it plays an important role in the regulation of eIF4E activity. For instance, phosphorylation of eIF4E was shown to decrease its affinity for the mRNA 5' cap-structure (Scheper et al., 2002; Zuberek et al., 2003). In addition, when the human

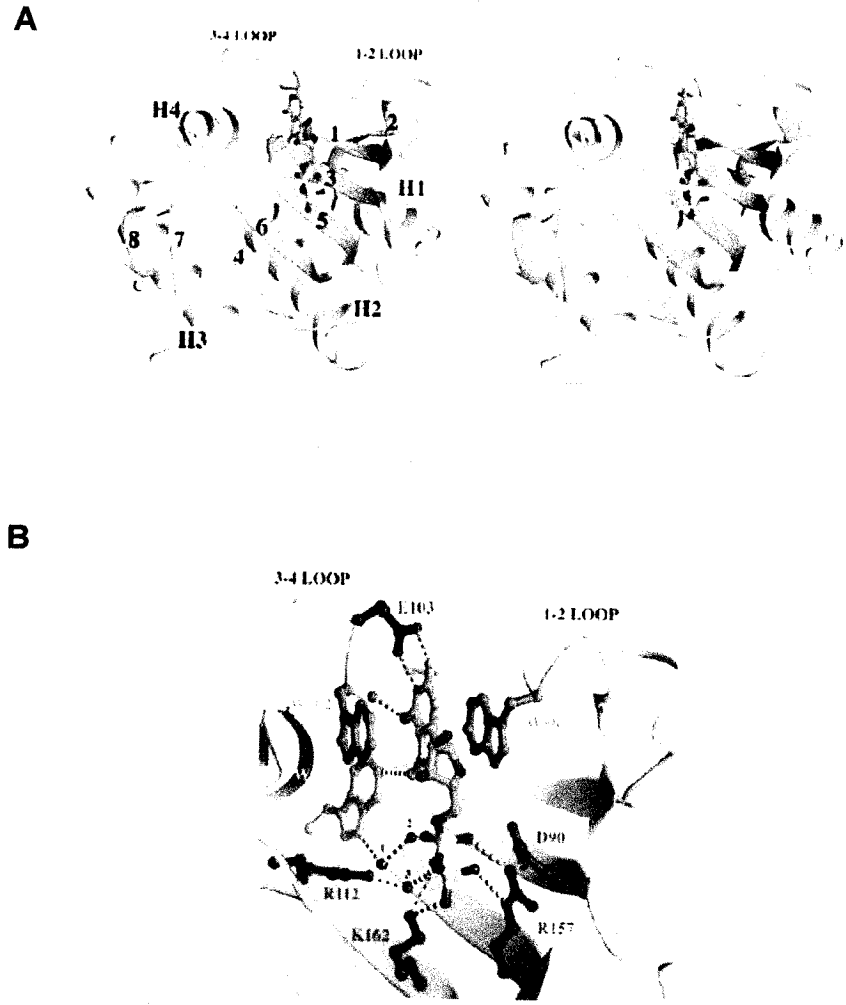


Figure 1.5 Structure of the murine eIF4E-7-methyl-GDP complex.
A. RIBBONS stereodrawing showing the concave cap-binding surface of eIF4E(28-217). 7-methyl-GDP, included as an atomic stick figure, is located in the cap-binding slot. α helices are labeled H1-H4 and β strands are labeled 1-8, with the N and C termini labeled with N and C, respectively. B. RIBBONS drawing of 7-methyl-GDP in the cap-binding slot of eIF4E, showing selected residues involved in cap-analog recognition. Hydrogen bonds, van der Waals interactions, and salt-bridges are indicated with dotted lines. The three bridging water molecules are shown as black spheres, labeled 1, 2, and 3 (Figure adapted from Marcotrigiano et al., 1997).

Ser209 equivalent residue (Ser251) was mutated to alanine in *Drosophila* eIF4E, growth and viability is severely affected; escapers developed more slowly than control siblings and were smaller in size (Lachance et al., 2002).

Besides its role in translation initiation, eIF4E is also involved in cellular transformation (Mamane et al., 2004). Since eIF4E is present in limited amount in cells, any changes that affect its concentration relative to other factors will be detrimental. Indeed, when Lazaris-Karatzas et al. overexpressed eIF4E in mouse fibroblasts, they observed a significant increase in tumor formation (Lazaris-Karatzas et al., 1990). In addition, high levels of eIF4E have been detected in various forms of human cancers (Mamane et al., 2004). It was later demonstrated that the ability of eIF4E to bind to the cap structure is critical for cellular transformation, since a mutant of eIF4E that could no longer bind to the cap structure failed to evoke a robust transformational response (Cohen et al., 2001).

1.7.2 *eIF4G*

Formerly known as p220, eIF4G is the scaffolding component of the eIF4F cap-binding complex. Because of its ability to bind to eIF4E and IRES, eIF4G is important for both cap-dependent and cap-independent initiation of translation. Two forms of eIF4G (eIF4GI and eIF4GII) with identical role in translation initiation have been identified in mammals and yeast (Goyer et al., 1993; Gradi et al., 1998; Imataka et al., 1998; Yan et al., 1992). As determined by viral protease cleavage patterns, human eIF4GI can be divided into three distinct functional domains of approximately 500 amino acids (amino-terminal, middle and carboxy-terminal domains; Figure 1.6)(Lamphear et al., 1995). Found within each of these domains are motifs that allow eIF4G to bind to other initiation factors to facilitate ribosome recruitment.

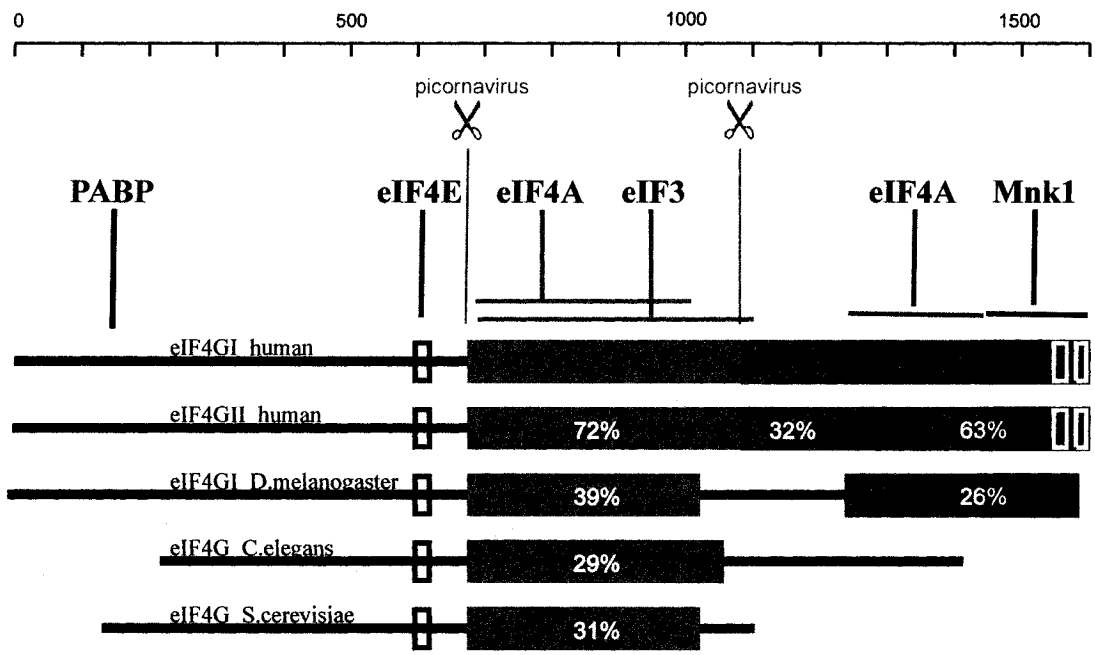


Figure 1.6 Domain/Motif organization of eIF4G.
Comparison of eIF4G domain/motif organization in different species. Colored blocks identify homologous regions with % sequence identity to human eIF4GI (4G/M, blue; phosphoregion, orange; 4G/C1, green; 4G/C2, red). The small purple box denotes the eIF4E-binding site (Figure adapted from Bellsolell et al., 2005).

The amino-terminal third of eIF4G contains the binding site for eIF4E (Mader et al., 1995), and is therefore crucial for cap-dependent translation initiation (Imataka et al., 1998; Lamphear et al., 1995; Mader et al., 1995; Morino et al., 2000). Interestingly, because the PABP-binding domain of eIF4G is also found in this domain, it is essential for mRNA circularization and 5'-3' translational synergy (Imataka et al., 1998; Kahvejian et al., 2005). In steady state, the N-terminal domain of eIF4G is found largely unstructured. However, upon binding to eIF4E, it assumes the correct folding and becomes translationally active (Gross et al., 2003). In addition, the binding of eIF4E to the eIF4G N-terminal domain dramatically enhances its cap-binding ability (Gross et al., 2003; Haghighat and Sonenberg, 1997; Ptushkina et al., 1998).

The middle domain of eIF4G interacts with eIF3 and eIF4A; critical components of the translational initiation machinery (Gingras et al., 1999). Interestingly, it is mainly composed of conserved HEAT repeats (Marcotrigiano et al., 2001), which derive their name from the first four proteins in which this characteristic sequence pattern was identified: ***H***untingtin, ***E****longation* Factor 3, PR65/***A*** subunit of PP2A and ***T***or1 (Andrade and Bork, 1995). Protein domains composed of HEAT repeats are known to be involved in protein-protein interactions (Andrade and Bork, 1995). Besides the HEAT domain, the eIF4G middle domain contains an RNA recognition motif (RRM)-like domain that is critically required for the IRES-mediated cap-independent translation initiation (Imataka and Sonenberg, 1997; Lamphear et al., 1995; Marcotrigiano et al., 2001; Ohlmann et al., 1996; Pestova et al., 1996; Thoma et al., 2004).

The carboxy-terminal domain of eIF4G contains a second, independent, eIF4A-binding site (Imataka and Sonenberg, 1997; Morino et al., 2000) and a binding site for Mnk1, the protein kinase that phosphorylates eIF4E (Pyronnet et al., 1999).

Similar to the eIF4G middle domain, the C-terminus of eIF4G is also composed of two HEAT repeats and contains two Aromatic/Acidic-boxes (AA-boxes) that mediate specific binding to proteins containing one or more segments of positively charged residues such as Mnk1 (Bellsolell et al., 2005). However, while the C-terminal domain of eIF4G mediates such important interactions, it does not appear to be important for translation. Indeed, the middle domain, including the region responsible for the eIF4E binding site, is sufficient in mediating ribosome binding and translation of capped mRNAs (Morino et al., 2000). Nevertheless, because the binding of eIF4A to the C-terminal third region of eIF4G is required for a robust translation, it was proposed that the eIF4G C-terminal domain functions as a translational modulator (Morino et al., 2000).

1.7.3 *eIF4A*

As the founding member of the DEAD-box RNA helicase family, named after one of the conserved sequence motifs found in this family, eIF4A is a bidirectional RNA helicase, an RNA-dependent ATPase, and a crucial component of the eIF4F cap-binding complex (Benz et al., 1999; Gingras et al., 1999; Oberer et al., 2005; Pause et al., 1994b; Pause and Sonenberg, 1992; Rogers et al., 2002). The primary role of eIF4A, as a component of the eIF4F complex is to melt secondary RNA structures found in the 5' UTR of an mRNA that would otherwise impede proper ribosomal "scanning" (Gingras et al., 1999; Kozak, 2002). Although eIF4A can, by itself, unwind RNA secondary structures, its helicase activity can be further enhanced when eIF4A is found as part of the eIF4F-complex and via its interaction with eIF4B and eIF4H (Oberer et al., 2005; Richter et al., 1999; Rogers et al., 2001; Rogers et al., 1999; Rozen et al., 1990). In mammals, three forms of eIF4A have been identified (eIF4AI, eIF4AII and

eIF4AIII)(Li et al., 1999). While eIF4AI and eIF4AII are known to be involved in translation initiation, and are functionally identical (Li et al., 1999; Nielsen and Trachsel, 1988; Weinstein et al., 1997; Yoder-Hill et al., 1993), the role of eIF4AIII in translation remained ambiguous (Li et al., 1999; Weinstein et al., 1997). However, it was recently demonstrated that eIF4AIII plays an important role in non-sense mediated decay of mRNA (NMD), the process by which the cellular machinery degrades prematurely terminated mRNA species to prevent the synthesis of, presumably, dominant negative proteins that might be deleterious for cell growth and development (Chan et al., 2004; Conti and Izaurralde, 2005; Ferraiuolo et al., 2004; Lejeune and Maquat, 2005; Palacios et al., 2004; Shibuya et al., 2004).

1.7.3 *eIF4B*

The function of eIF4B in the initiation of translation is not well understood. eIF4B stimulates the helicase activity of eIF4A and facilitates the binding of the 40S ribosomal subunit to the mRNA (Gingras et al., 1999; Methot et al., 1996a). Unlike other members of the eIF4 family, however, and since the 48S ribosomal initiation complex can still be formed in its absence, eIF4B is not a critical component of the translation initiation machinery (Altmann et al., 1993; Coppolecchia et al., 1993; Pestova et al., 1996). eIF4B functions in translation initiation via multiple protein-protein interactions. In mammals, eIF4B protein was shown to form a homodimer and interacts with the p170 subunit of eIF3 (Methot et al., 1997; Methot et al., 1996b). In addition, eIF4B was recently shown to interact with PABP (Bushell et al., 2001). Although it was proposed that the eIF4B:PABP interaction, in conjunction with the binding of eIF4G to PABP, facilitates the functional association of the 5' and 3' ends of mRNA (Bushell et al., 2001), it is still not clear whether this interaction is functionally

significant. Recently, an eIF4B-related protein (termed eIF4H) was discovered based on its stimulatory activity in a reconstituted *in vitro* translation assay (Richter-Cook et al., 1998). Similar to eIF4B, eIF4H enhances translation efficiency by stimulating the helicase activity of eIF4A (Richter et al., 1999; Rogers et al., 2001; Rogers et al., 1999). Further analysis will be required to elucidate the exact role of eIF4B and eIF4H in translation initiation.

1.8 *mRNA Circularization - Role in Translation Initiation*

The idea of mRNA circularization and the 5'-3' translational synergy was first proposed by Jacobson and Favreau in 1983 (Jacobson and Favreau, 1983). However, proof of direct interaction between mRNA 5' and 3' ends proved elusive until the discovery of the eIF4G:PABP interaction some 13 years later (Le et al., 1997; Tarun and Sachs, 1996). Ensuing atomic microscopy experiments using recombinant eIF4G, eIF4E and PABP finally allowed scientists to witness the existence of a circularized RNA in yeast (Wells et al., 1998).

What is the biological significance of an mRNA circularization? It is postulated that circularization of an intact mRNA induces a selective increase in its translation efficiency. Such a mechanism would prevent the expression of nicked mRNAs that could potentially be harmful (Kahvejian et al., 2001). Developmentally regulated trans-acting factors that bind to the 3' end of specific transcripts also influence translation at the level of initiation by affecting the ability of the mRNA to circularize (Kuersten and Goodwin, 2003; Richter and Sonenberg, 2005). Indeed, when the eIF4G:PABP interaction, and by inference the mRNA circularization, is abrogated in *Xenopus* by means of mutagenesis, translation of polyadenylated mRNAs and progesterone induced oocyte maturation is severely affected (Wakiyama et al., 2000). Furthermore, since the

mRNA degradation pathway requires, as a prerequisite, the removal of the 5' cap structure (decapping) and the 3' poly(A) tail (deadenylation)(Coller and Parker, 2004), by shielding these two mRNA elements from the cellular degradation machinery, mRNA circularization may potentially stabilize an mRNA. Interestingly, however, the function of mRNA circularization does not seem to be isolated to enhancement of translation alone. As a matter of fact, various translational repression mechanisms are found to adopt such a mechanism to repress the expression of specific transcripts in time- and location-dependent manner (Kuersten and Goodwin, 2003; Richter and Sonenberg, 2005; Wickens et al., 2000). Some of these mechanisms will be discussed in detail in the following sections.

1.9 *Translational Regulatory Mechanisms*

In a complex and multi-step pathway such as protein synthesis, regulation can be exerted at many levels. However, as confirmed by a large body of evidence, translational rate is primarily regulated at the initiation phase (Mathews et al., 2000). Furthermore, depending on which step of the initiation stage a regulatory pressure is applied, a general or specific translational control can be evoked. The following sections will explore the various translational regulatory mechanisms that have been discovered to date.

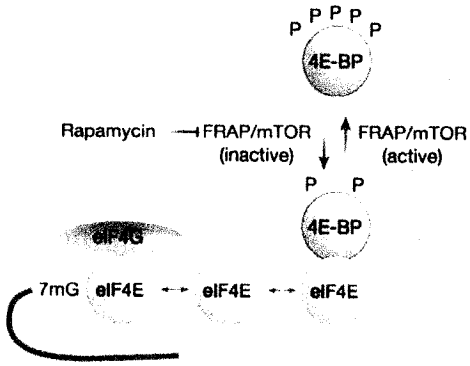
1.9.1 *General Translational Repression*

There are three well known mechanisms in the translation initiation phase, through which organisms control general translation. The first of such mechanisms requires the activity of 4E-BPs. Initially cloned in 1994 (Lin et al., 1994; Pause et al., 1994a), 4E-BPs are general translational repressors that compete with eIF4G for eIF4E binding via

the conserved eIF4E-recognition motif and inhibit the eIF4F formation (Mader et al., 1995; Miron et al., 2001; Pause et al., 1994a; Poulin et al., 1998). The translational repressor activity of 4E-BPs is primarily regulated via phosphorylation (Gingras et al., 1999; Raught et al., 2000). Upon mitogen and nutrient stimulation, 4E-BPs undergoes rapid hierarchical phosphorylation by the mTOR (mammalian target of rapamycin) kinase, which function as the nutrient-sensing checkpoint of a cell (Gingras et al., 2001; Hay and Sonenberg, 2004; Raught et al., 2000). While the hyperphosphorylated form of 4E-BPs increases translation by releasing eIF4E and allowing the formation of the eIF4F complex, the hypophosphorylated form of 4E-BPs inhibits it (Figure 1.7A)(Gingras et al., 1999; Raught et al., 2000). In mammals, there are three forms of 4E-BPs (4E-BP1, 4E-BP2 and 4E-BP3) that are functionally identical, yet display tissue specific expression patterns: whereas 4E-BP1 is expressed at high levels in fat and muscles, the highest concentration of 4E-BP2 and 4E-BP3 proteins are found in brain and colon, respectively (Tsukiyama-Kohara et al., 2001).

The second mechanism of translational control implicates eIF2B, the guanine-nucleotide exchange factor for eIF2 (Dever, 2002; Gebauer and Hentze, 2004). Typically, following a single round of initiation, eIF2 is found in complex with GDP, and therefore functionally inactive. This is partly due to the fact that eIF2 exhibits higher affinity for GDP than GTP. As such, eIF2 requires the guanine-nucleotide exchange activity of eIF2B to replace GDP with GTP to regain its activity for subsequent rounds of translation initiation. Similar to 4E-BPs, eIF2 activity is controlled via phosphorylation (Figure 1.7B). When eIF2, which consists of three subunits (α, β and γ), becomes phosphorylated on the residue Ser51 of the α subunit, it switches from a substrate to an inhibitor of eIF2B and stops protein synthesis (Dever, 2002). Four eIF2 kinases (HRI, PERK, PKR and GCN2) have been found to date.

A



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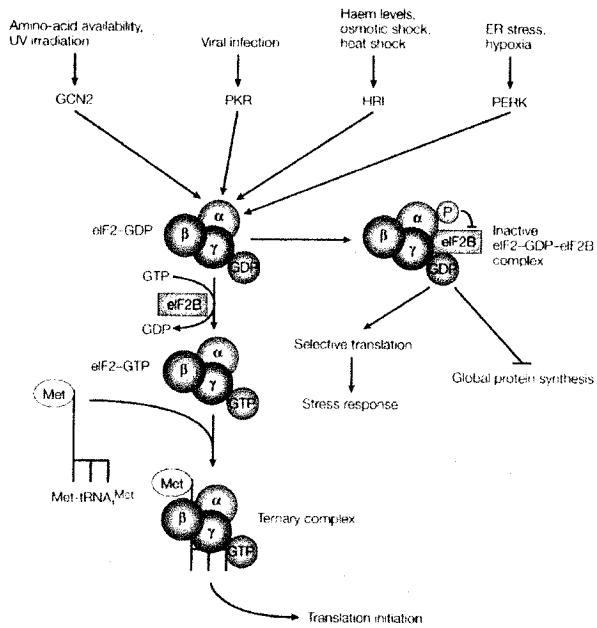

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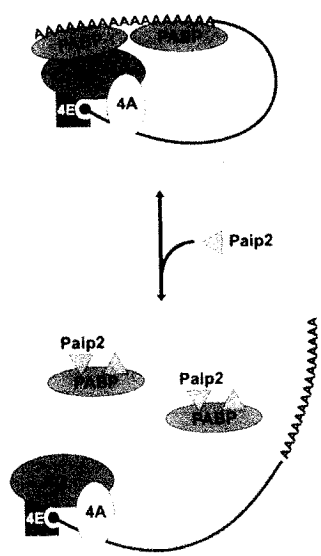

Figure 1.7 General Translational Regulation.

A. Translation regulation and 4E-BP phosphorylation. The kinase FRAP/mTOR hyperphosphorylates 4E-BP on several sites; this causes the liberation of eIF4E from 4E-BP, and the association of eIF4E with both capped mRNA and eIF4G. The inhibition of FRAP/mTOR by rapamycin leads to the hypophosphorylation of 4E-BP and enhanced binding to eIF4E (Figure adapted from Richter and Sonenberg, 2005).

B. Integration of stress responses by the phosphorylation of eukaryotic initiation factor-2 . Many stress conditions result in the phosphorylation of eukaryotic initiation factor-2 (eIF2), which is accomplished by four distinct protein kinases: GCN2, PKR, HRI and PERK. See text for details (Figure adapted from Holcik and Sonenberg, 2005).

C. Model for the inhibition of translation by Paip2. Paip2 inhibits PABP binding to the mRNA and may interfere with the PABP/eIF4G interaction on the mRNA, resulting in the disruption of the circular conformation (Figure adapted from Kahvejian et al., 2001).

These kinases, which become active when cells are treated with heat shock, osmotic, oxidative and endoplasmic reticulum (ER) stresses, interferon, UV-irradiation and amino-acid starvation, all inhibit general translation by phosphorylating eIF2α (Dever, 2002; Holcik and Sonenberg, 2005).

The third and last major translational control mechanism at the initiation step implicates a small polypeptide that was shown to strongly interdict the interaction of PABP with the poly(A)-tail. Typically, PABP interacts with eIF4G to induce 5'-3' translational synergy by mRNA circularization. The observed translation activation is further enhanced when PABP interacts with a protein called PABP-interacting protein 1 (Paip1)(Craig et al., 1998). Recently, a second form of Paip (Paip2) was cloned (Khaleghpour et al., 2001). Unlike Paip1, however, Paip2 was shown to compete not only with Paip1, but also with poly(A)-tail for PABP binding (Khaleghpour et al., 2001; Roy et al., 2004). Therefore, by sequestering PABP, and the mRNA 3', away from the 5' translation initiation machinery, Paip2 represses general translation with high efficiency (Figure 1.7C)(Kahvejian et al., 2001; Khaleghpour et al., 2001; Roy et al., 2004).

1.9.2 *Gene Specific Translational Repression*

It is becoming increasingly clear that through evolution organisms have developed different translational control mechanisms to regulate gene expression (Kim, 2005; Kuersten and Goodwin, 2003; Richter and Sonenberg, 2005; Wickens et al., 2000; Zamore and Haley, 2005). Whereas a general mechanism of translational control may be invoked, occasionally, to regulate expression of genes in situations where an overall shift in cellular output is demanded (i.e.: metabolism and cellular stress conditions), gene specific translational control mechanisms have been developed to provide an

organism with the ability to compartmentalize the influence of a gene in a time- and location-dependent manner. This is especially important during embryogenesis and in early differentiating cells, where slight changes in the level of developmentally important protein factors may result in the alteration of fate map (Kuersten and Goodwin, 2003; St Johnston and Nusslein-Volhard, 1992; Wickens et al., 2000).

To date, all gene specific translational controls have been shown to involve a sophisticated series of *trans*-acting factors to regulate gene expression with pinpoint accuracy. The 3' UTR has emerged as a particularly common site for such regulatory interactions. Indeed, for the past decade or so, we have witnessed a tremendous surge in interest by the scientific community for the role of the 3'UTR in gene expression. It is during this period of renaissance that the translational control field witnessed the identification of vast number of 3'UTR translational control elements in several different species (Kim, 2005; Kuersten and Goodwin, 2003; Richter and Sonenberg, 2005; Wickens et al., 2000; Zamore and Haley, 2005). For some of these 3'UTR elements, subsequent analysis has yielded a significant amount of data that allowed scientists to better understand the underlying mechanism of translational control (Figure 1.8)(Kuersten and Goodwin, 2003; Nakamura et al., 2004; Nelson et al., 2004; Stebbins-Boaz et al., 1999; Wickens et al., 2000; Wilhelm et al., 2003).

In order to develop into mature erythrocytes, early erythroid precursor cells must undergo systematic organelle degradation process that rids them of their organelles and nucleus. 15-lipooxygenase (LOX), which mediates organelle degradation, is only expressed in early erythroid cells just before they become mature erythrocytes (Ostareck et al., 2001; Rapoport and Schewe, 1986; van Leyen et al., 1998). Translational control was found to be responsible for this temporal restriction of LOX synthesis (Hohne et al., 1988). More specifically, the expression of l*ox* mRNA is

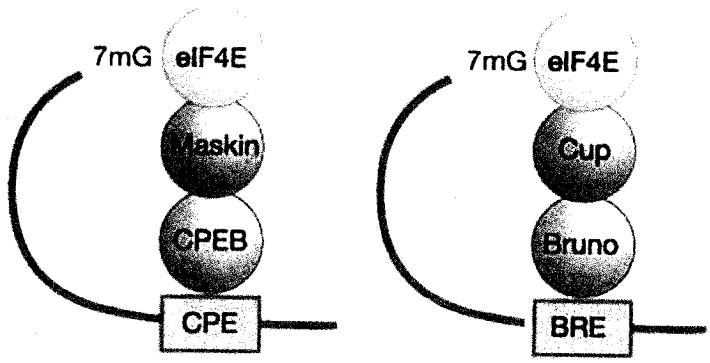

Figure 1.8 Gene Specific Translational Regulation
Through its association with CPEB, Maskin interacts with eIF4E only on RNAs that contain a CPE; disruption of the eIF4E:eIF4G complex by this protein is therefore mRNA-specific. In a similar manner, Cup, through its association with Bruno, binds and displaces the eIF4G from eIF4E only on mRNAs that contain a Bruno response element (BRE)(Figure adapted from Richter and Sonenberg, 2005).

silenced in early erythroid precursor cells by a specific mRNA-protein complex between a 3'UTR control element called DICE and hnRNPs K and E1 (Ostareck et al., 2001), which prevents the recruitment of the 60S ribosomal subunit to the mRNA (Ostareck et al., 2001).

In *Xenopus*, oocyte maturation is preceded by the activation of *cyclin B1* and *c-mos* mRNA translation. Cytoplasmic polyadenylation is thought to mediate this activation (Mendez and Richter, 2001). Indeed, only when the short poly(A) tails of early *cyclin B1* and *c-mos* mRNAs are extended to a significant length (~ 150 nucleotides long), is their translation allowed to be activated (Mendez and Richter, 2001). Therefore, prior to their polyadenylation, translation of *c-mos* and *cyclin B1* mRNAs is mostly inhibited in oocytes to prevent premature development. Identified in 1999, Maskin is an eIF4E-binding protein that was found to inhibit translation in *Xenopus* oocyte (Cao and Richter, 2002; Stebbins-Boaz et al., 1999). Similar to 4E-BPs, Maskin represses translation by competing with eIF4G for eIF4E binding via a putative eIF4E-binding motif (Cao and Richter, 2002; Stebbins-Boaz et al., 1999). Surprisingly, Maskin also interacts with the cytoplasmic polyadenylation element (CPE) binding protein (CPEB), which is known to bind to the CPE found in the 3' UTR of *cyclin B1* and *c-mos* mRNAs (Cao and Richter, 2002; Stebbins-Boaz et al., 1999). Therefore, by interacting with both eIF4E and CPEB, Maskin induces the circularization of *cyclin B1* and *c-mos* mRNAs, and inhibits their expression (Figure 1.8)(Cao and Richter, 2002; Richter and Sonenberg, 2005; Stebbins-Boaz et al., 1999).

1.10 *Drosophila Development and Translational Control*

During early embryo development, immediately after fertilization, embryos undergo dramatic changes that essentially determine the fate of the organism. It is precisely

during this time of development, when the embryo is devoid of any transcriptional activity, that translational control plays a vital role. In *Drosophila,* translational control during early embryogenesis is crucial for the initial establishment of the major spatial axes (Johnstone and Lasko, 2001; Kuersten and Goodwin, 2003; St Johnston and Nusslein-Volhard, 1992; Wickens et al., 2000).

1.10.1 *Translation Activation and Drosophila Embryogenesis*

In *Drosophila,* upon fertilization, various maternally contributed transcripts become translationally active; producing factors that are critically needed for the initial phase of embryogenesis. For some of these transcripts, similar to *Xenopus cyclin B1* and *c-mos* mRNAs, cytoplasmic polyadenylation seems to be at the heart of their translation control (Johnstone and Lasko, 2001; Wickens et al., 2000). For example, in the case of *bicoid* (*bcd*) mRNA, which is localized exclusively at the anterior end of an embryo, it was shown that the lengthening and shortening of its poly(A) tail correlate with its translational status (Salles et al., 1994). Similar to *bcd,* shortly after fertilization, translation of *nanos* (*nos*) mRNA at the posterior end of an embryo also gets activated (Johnstone and Lasko, 2001). Since it does not localize to the posterior if the pole plasm has not been assembled, it is likely that genes that are needed for the pole plasm assembly are directly or indirectly required for *nos* translation activation (Johnstone and Lasko, 2001). Further highlighting the importance of translation activation during early *Drosophila* embryogenesis, mutant alleles that fail to activate the translation of both *bcd* and *nos* mRNAs develop severe abdominal segmentation defects (Johnstone and Lasko, 2001; Kuersten and Goodwin, 2003; St Johnston and Nusslein-Volhard, 1992; Wickens et al., 2000).

1.10.2 *Translational repression and Drosophila Embryogenesis*

It is clear that translation activation plays a vital role during early *Drosophila* embryogenesis. However, activation is not the only mechanism of translational control at work during early embryo development. Repression of protein-synthesis also plays a central role during this phase of *Drosophila* life-cycle to ensure survival.

Proteins encoded by several maternally-contributed mRNAs, including *oskar* (*osk*), *nos*, *caudal* (*cad*), and *hunchback* (*hb*), are essential for anterior-posterior patterning. In order to carry out their functions in development, Osk, Nos, and Cad proteins must be restricted in space to the future posterior of the embryo, while Hb must be restricted to the anterior (Johnstone and Lasko, 2001; Kuersten and Goodwin, 2003; St Johnston and Nusslein-Volhard, 1992; Wickens et al., 2000). To achieve asymmetric distribution of the proteins, translation of all these mRNAs is repressed in regions of the embryo where the relevant protein needs to be excluded. Translational repression of *osk, nos* and *cad* mRNAs, and to some degree *hb* mRNA occurs at the mRNA 5' cap structure recognition step (see below for further details)(Chagnovich and Lehmann, 2001; Dubnau and Struhl, 1996; Nakamura et al., 2004; Nelson et al., 2004; Rivera-Pomar et al., 1996; Wilhelm et al., 2003).

1.10.2.1 *Translational Regulation of osk and nos mRNAs*

In the case of *osk* and *nos* mRNAs, their cap-dependent translation repression is mediated via an eIF4E-binding regulator protein called Cup. Cup competitively inhibits the interaction between eIF4E and eIF4G, an essential step of cap-dependent translation initiation, to block ribosome recruitment (Figure 1.8)(Nakamura et al., 2004; Nelson et al., 2004; Wilhelm et al., 2003). However, significant amounts of *osk* and *nos* mRNAs were also found to be associated with polysome fractions under conditions where their

protein products do not accumulate; providing evidence that suggests the involvement of a transient repression mechanism that operates post-initiation (Braat et al., 2004; Clark et al., 2000; Cook et al., 2004). Further complicating the story, RNA interference (RNAi) also contributes to the translational repression of *osk*. Indeed, mutations in any of four genes affecting assembly or transport of the RNA-induced silencing complex (*armitage*, *spindle-E*, *aubergine*, and *maelstrom*) do not affect *osk* mRNA level but result in premature Osk expression (Cook et al., 2004; Tomari et al., 2004). In addition to translational control, mechanisms also exist to localize and enrich the concentrations of these transcripts at the posterior pole where the proteins they encode are required (Johnstone and Lasko, 2001).

1.10.2.2 *Cap-dependent Translational Inhibition of cad and hb mRNAs*

In sharp contrast to *osk* and *nos* mRNAs, *cad* and *hb* are uniformly distributed across the anterior-posterior axis of an embryo and, thus, require a sophisticated localized translational repression mechanism that would not interfere with their translation in other parts of an embryo (Kuersten and Goodwin, 2003; Wickens et al., 2000). In addition, while the inhibitory mechanism that acts upon *cad* and *hb* mRNA also implicates the 5' cap structure and requires such RNA-binding regulatory proteins as Bicoid (Bcd) and Brain Tumor (Brat), unlike *osk* and *nos*, their localized repressions are meant to be permanent (Chagnovich and Lehmann, 2001; Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996; Tautz, 1988; Tautz et al., 1987). Although much is known about the *cad* and *hb* mRNA translational inhibition, the exact mechanism by which these mRNAs are permanently inhibited in early embryo still remains unknown.

1.11 *Rationale*

The human eIF4E-homologous protein (h4EHP) was first identified based on its homology to eIF4E (30% identity and 60% similarity)(Kim, 1998; Rom et al., 1998). Similar to eIF4E, h4EHP is an evolutionarily conserved cap-binding protein (Hernandez et al., 2005; Keiper et al., 2000; Kim, 1998; Rom et al., 1998; Ruud et al., 1998). Surprisingly, despite its homology to eIF4E, h4EHP does not interact with either eIF4G or 4E-BPs (Rom et al., 1998). In addition, when h4EHP is over-expressed or depleted in HeLa cells, the overall translational rate remained unaffected (Kim, 1998). Therefore, the function, if any, of 4EHP in translation remained unknown. Due to the difficulties we had encountered in assaying for the effects of 4EHP in translation, we soon began to entertain the idea that 4EHP, instead of affecting general translation, may be modulating the translational rate of specific transcripts. Since a large number of examples of translational control mechanisms have been discovered in *Drosophila*, and because it was recently shown that the *Drosophila* homolog of h4EHP (d4EHP) is expressed at high levels throughout the life-cycle of this model organism (Arbeitman et al., 2002), we decided to use it to test our hypothesis. In summary, to understand the function of 4EHP in translational control, we created a mutant of d4EHP and studied its effect in *Drosophila* embryogenesis.

CHAPTER 2 - A new paradigm for translational control: inhibition via 5'-3' mRNA tethering by Bicoid and the eIF4E cognate 4EHP

2.1 Abstract

Translational control is a key genetic regulatory mechanism implicated in regulation of cell and organismal growth, and early embryonic development. Initiation at the mRNA 5' cap structure recognition step is frequently targeted by translational control mechanisms. In the *Drosophila* embryo, cap-dependent translation of the uniformly distributed *caudal* (*cad*) mRNA is inhibited in the anterior by Bicoid (Bcd) to create an asymmetric distribution of Cad protein. Here, we show that d4EHP, an eIF4E-related cap-binding protein, specifically interacts with Bcd to suppress *cad* translation. Translational inhibition depends on the Bcd binding region (BBR) present in the *cad* 3' untranslated region. Thus, a simultaneous interaction of d4EHP with the cap structure, and Bcd interaction with BBR, renders *cad* mRNA translationally inactive. This example of cap-dependent translational control that is not mediated by canonical eIF4E defines a new paradigm for translational inhibition involving tethering of the mRNA 5' and 3' ends.

2.2 Introduction

In the absence of transcription during early embryogenesis, many genes are regulated at the level of translation (Wickens et al., 2000). Translation rates are often controlled at the initiation phase, a multi-step process involving the recruitment of the 40S small ribosomal subunit to the 5' end of an mRNA, which culminates in the positioning of the ribosome at the initiation codon (Hershey and Merrick, 2000; Poulin and Sonenberg, 2003). The mRNA 5' cap structure (m^7GpppN, where N is any nucleotide)(Shatkin, 1976), facilitates ribosome binding to the mRNA via an interaction with the cap-binding complex, eukaryotic initiation factor (eIF) 4F.

eIF4F is composed of three subunits: eIF4E, eIF4A, and eIF4G. Simultaneous interaction of eIF4G with eIF4E and poly(A)-binding protein (PABP) brings about mRNA circularization and promotes the recruitment of the 40S ribosomal subunit (Gebauer and Hentze, 2004; Kahvejian et al., 2005; Sachs, 2000). Because of their key roles, eIF4E and PABP have emerged as major targets of translational regulatory mechanisms. Several mechanisms of modulating their activity have now been described. eIF4E-binding proteins (4E-BPs) inhibit general cap-dependent translation by sequestering eIF4E from the eIF4F complex (Gingras et al., 1999; Raught et al., 2000). An mRNA-specific mechanism of cap-dependent inhibition involves proteins such as Cup (Nakamura et al., 2004; Nelson et al., 2004; Wilhelm et al., 2003) and Maskin (Stebbins-Boaz et al., 1999), which interact simultaneously with eIF4E and, either directly or indirectly, with the 3' end of an mRNA (Richter and Sonenberg, 2005). Another mechanism involves PABP-interacting protein 2 (Paip2), which binds to PABP and displaces it from the poly(A) tail, effectively inhibiting translation by interdicting mRNA circularization (Kahvejian et al., 2005; Khaleghpour et al., 2001).

Embryonic pattern is established in *Drosophila* by several proteins that are

targeted to defined regions of the cytoplasm, and translational regulation plays a central role in their localization (Johnstone and Lasko, 2001; Kuersten and Goodwin, 2003; St Johnston and Nusslein-Volhard, 1992; Wickens et al., 2000). For example, a posterior-to-anterior gradient of Caudal (Cad) protein is established in early embryogenesis from uniformly-distributed maternal *cad* mRNA, and this gradient is essential for posterior patterning. Establishment of the Cad gradient requires Bicoid (Bcd), which mediates cap-dependent translational repression of *cad* mRNA dependent on the Bcd-binding region (BBR), an element in its 3' untranslated region (UTR) (Chan and Struhl, 1997; Dubnau and Struhl, 1996; Niessing et al., 1999; Rivera-Pomar et al., 1996). It has been proposed that Bcd blocks *cad* mRNA translation by interacting with eIF4E to prevent eIF4F complex formation (Niessing et al., 2002).

An eIF4E-related protein called human eIF4E-Homologous protein (h4EHP) was previously described (Rom et al., 1998). However, the function, if any, of 4EHP in translation has been elusive, since it does not interact with eIF4G (Hernandez et al., 2005; Rom et al., 1998) and thus cannot function in ribosome recruitment. Here, we show that the *Drosophila* 4EHP homolog (d4EHP) interacts with Bcd to inhibit the anterior translation of maternal *cad* mRNA. Translational regulation of *cad* mRNA thus involves a unique translational inhibitory mechanism.

2.3 Results

2.3.1 d4EHP is a cap-binding protein

4EHP is evolutionarily conserved in metazoans and plants (Figure 2.1A). The *d4EHP* gene (Genbank: NM_176552; Gadfly: CG33100) encodes a 223 amino acid protein with a predicted molecular mass of 26 kDa. Most amino acids implicated in eIF4E binding to the cap structure (Marcotrigiano et al., 1997; Matsuo et al., 1997), are

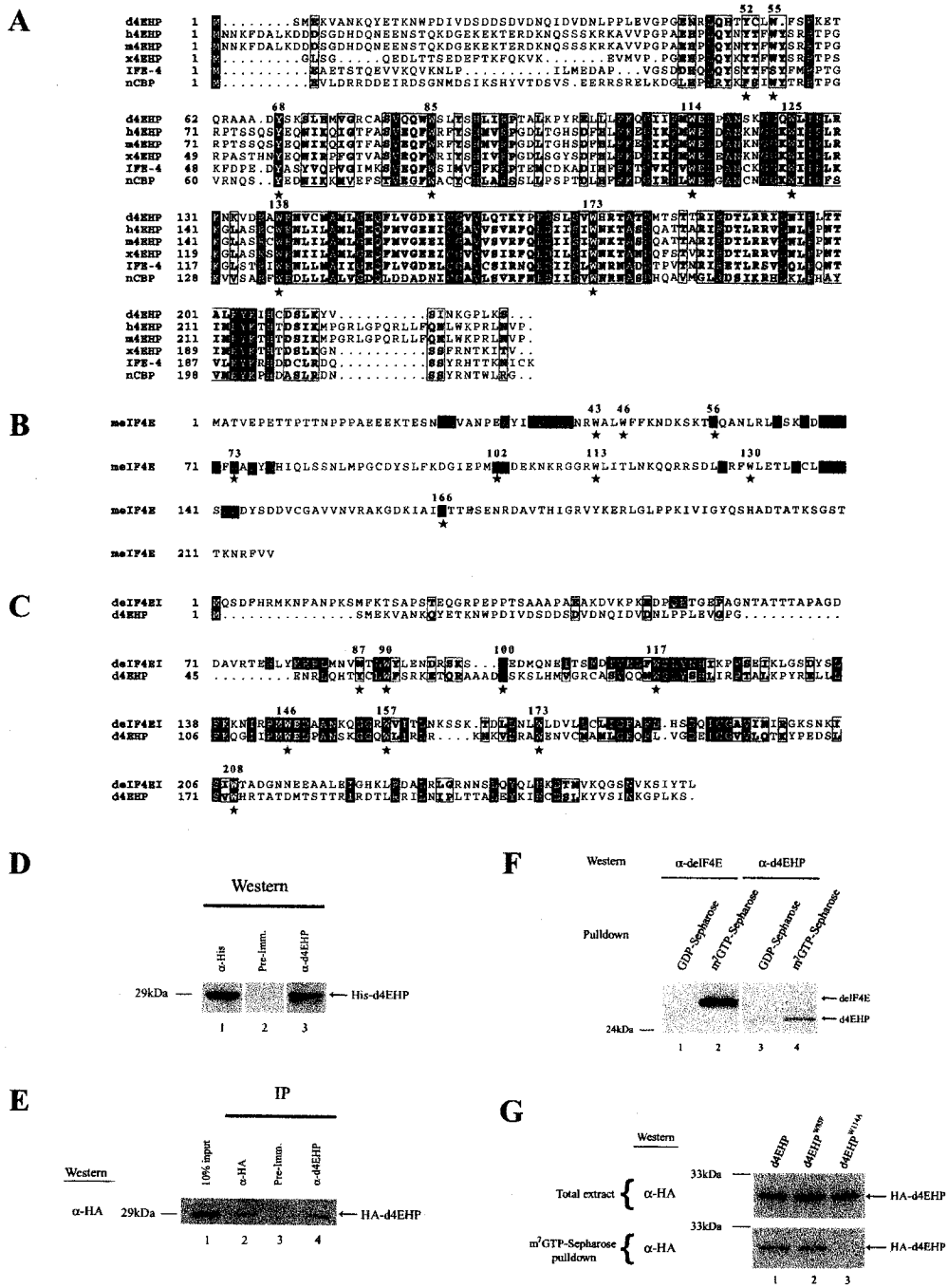

Figure 2.1 4EHP is an evolutionarily conserved cap-binding protein

A. Sequence alignment of 4EHP from D.melanogaster (d4EHP), human (h4EHP), mouse (m4EHP), X.laevis (x4EHP), C.elegans (IFE-4), and A.thaliana (nCBP). B. Amino acid sequence of mouse eIF4E (meIF4E). C. d4EHP is similar to deIF4E. Sequence alignment of D.melanogaster eIF4EI (deIF4EI) and d4EHP. A-C. Identical amino acids are highlighted in red and conserved ones in yellow. Residues that function in cap-binding (Marcotrigiano et al., 1997; Matsuo et al., 1997) are highlighted in green. Conserved residues which form contacts between eIF4E and eIF4G (Gross et al., 2003; Marcotrigiano et al., 1999) are highlighted in blue. Stars indicate the position of the eight tryptophan residues conserved in eIF4E through evolution; Trp43 and Trp56 are replaced by tyrosines in 4EHP (black stars). The eIF4E residue Trp73, critical for eIF4G and 4E-BP interaction in the mouse and by inference in flies, is indicated by a red star. D. d4EHP antiserum detects recombinant d4EHP. Recombinant His-tagged d4EHP (lane 1) is detected in a Western blot with a d4EHP antiserum (lane 3), but not with pre-immune serum (lane 2). E. d4EHP antiserum immunoprecipitates d4EHP from cell extracts. HA-tagged d4EHP was transfected in 293 cells (lane 1) and immunoprecipitated with an anti-HA antibody (lane 2), pre-immune serum (lane 3), or d4EHP antiserum (lane 4). F. d4EHP is a cap-binding protein. Cap-binding proteins were affinity purified from Drosophila S2 cell extracts using m7GTP-Sepharose (lanes 2 and 4). The eluates were analyzed by Western blotting for the presence of deIF4E or d4EHP. GDP-Sepharose affinity purification was used as a negative control (lanes 1 and 3). G. Trp114 is critical for the d4EHP:Cap interaction. 293 cell extracts (top panel) containing transfected HA-tagged d4EHP (lane 1), d4EHPW85F (lane 2) and d4EHPW114A (lane 3), were incubated with m7GTP-Sepharose, and the eluate was analyzed by Western blotting (bottom panel).

conserved in d4EHP (Figures 2.1A-C), although two of eight conserved tryptophan residues in eIF4E are replaced by tyrosines in 4EHP (Figures 2.1A-C). While one of these residues, Trp56 in eIF4E (Tyr68 in d4EHP; Figure 2.1A-B), directly interacts with the ring structure of the m^7G cap (Marcotrigiano et al., 1997; Matsuo et al., 1997), it is replaced with other aromatic amino acids in disparate cap-binding proteins, such as VP39 and CBP20, indicating that the aromatic ring is the important chemical moiety for cap interaction (Calero et al., 2002; Hodel et al., 1997; Mazza et al., 2001).

An antiserum against GST-d4EHP fusion protein, which recognizes recombinant His-d4EHP by immunoblotting, was raised (Figure 2.1D). The specificity of the antiserum was established by immunoprecipitation of HA-tagged d4EHP from transfected 293 cells (Figure 2.1E).

Like deIF4E, d4EHP binds to m^7GTP-Sepharose, but not to GDP-Sepharose (Figure 2.1F). eIF4E and d4EHP share a common cap-binding mechanism, since mutation of the d4EHP equivalent of murine eIF4E Trp102 (Trp114 in d4EHP), significantly reduced the ability of d4EHP to bind to the cap structure (Figure 2.1G). Mutation of d4EHP Trp85, a residue to be discussed later in this report, does not affect cap-binding (Figure 2.1G).

2.3.2 d4EHP genetically interacts with *cad*

d4EHP is uniformly distributed in early *Drosophila* embryos (Figure 2.2A). To investigate its biological function, we produced mutants by imprecise excision of a P-element inserted within the first exon of *d4EHP* (BG017013, Figure 2.3A)(Bellen et al., 2004). One of several deletion lines we obtained, which is referred to as $d4EHP^{CP53}$, carries an excision of ~2.1 kb that deletes all of exon I, including the translation start site, and part of intron I (Figure 2.3A). The resulting mutant is hypomorphic, as

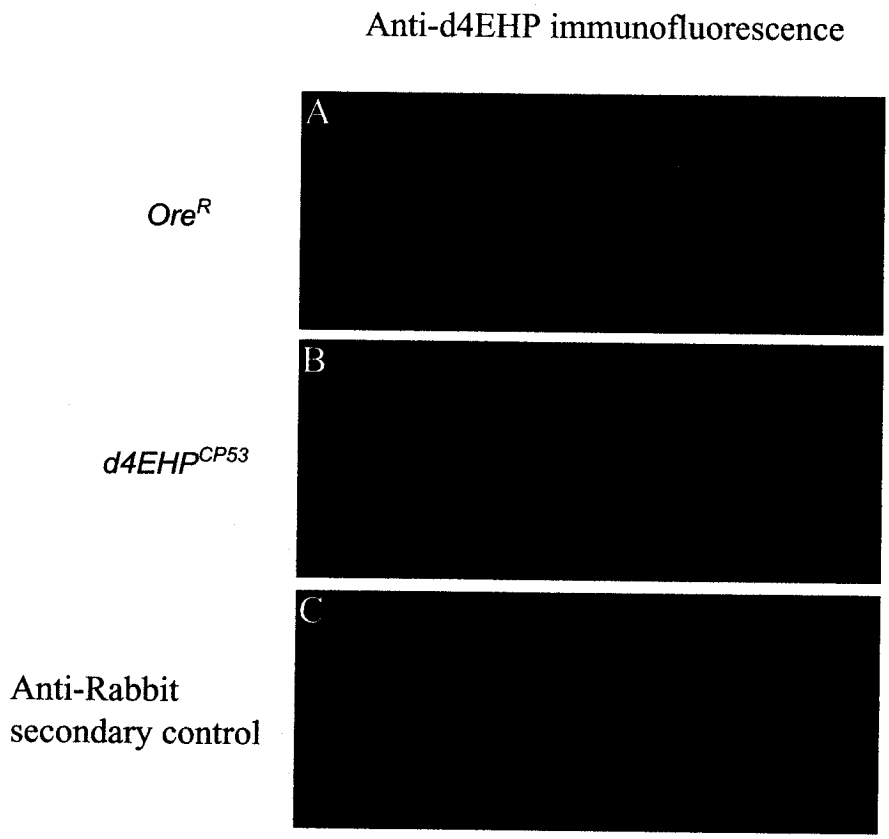

Figure 2.2 d4EHP expression pattern in Drosophila embryos
A and B. Anti-d4EHP immunofluorescence was performed on wild-type (A) and d4EHPCP53 mutant (B) embryos. Anti-d4EHP was used at 1:500 dilution. C. Control immunofluorescence was performed using anti-Rabbit secondary antibody.

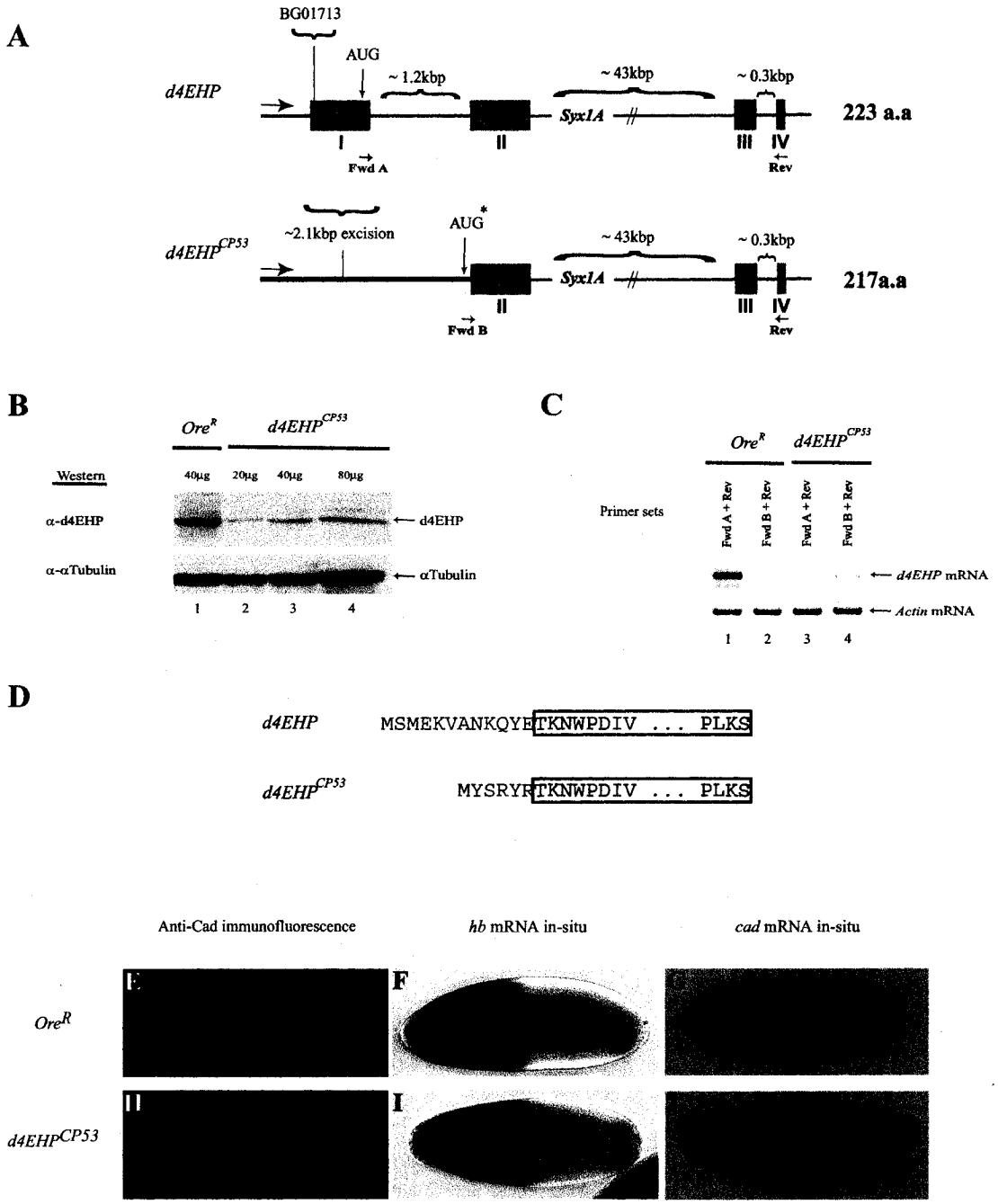

Figure 2.3 Characterization of d4EHPCP53 mutant

A. Schematic representation of the d4EHP gene. The d4EHP gene spans ~ 45Kbp, and comprises four exons (boxes) and three introns (solid lines). Syntaxin 1A (Syx1A) is nested in the second intron of d4EHP (yellow box), and is transcribed in the same orientation. P-element (BG01713) is inserted in exon I of the gene (d4EHP panel). AUG* in d4EHPCP53 is an alternative translation start site that becomes active upon excision of exon I and part of intron I (d4EHPCP53 panel). Location of RT-PCR primers are indicated by blue arrows. The number of amino acids encoded by the mRNA is indicated on the right. B. Reduced d4EHP expression in the d4EHPCP53 mutant. Wild-type (OreR) and d4EHPCP53 embryo extracts were analyzed by Western blotting using anti-d4EHP and anti-αtubulin as a loading control. C. RT-PCR analysis of total RNA using primers specific for the wild-type d4EHP (Fwd A in exon I) or d4EHPCP53 mutant (Fwd B in intron I). Actin mRNA is used as a loading control. D. Translation from the wild-type and mutant genes is predicted to produce different N-terminal ends. E-G. OreR embryo displays wild-type Cad gradient (E), zygotic hb transcription (F), and cad distribution (G). H-J. 0-2h d4EHPCP53 mutant embryo show ectopic Cad expression at the anterior end (H), yet has normal zygotic hb activation (I) and cad localization (J). To maximize signal-to-background ratio, sagittal sections of embryos are used to display Cad gradients; hence the reason for the absence of surface nuclei that are evident in embryo images presented in Figures 2.8 and 2.10. Orientation of embryos is anterior left and dorsal up.

immunoblotting (Figure 2.3B) and immunostaining (Figure 2.2B) detected the presence of small amounts of d4EHP in mutant embryo extracts (7%, relative to wild-type; Figure 2.3B). This is most probably due to the presence of an in-frame AUG (AUG*) at the end of the first intron (Figure 2.3A) that remains present in the $d4EHP^{CP53}$ deletion. Consistent with this, a transcript that contains part of intron I and the AUG* is detected, albeit at reduced levels (~5%), in the $d4EHP^{CP53}$ mutant line (Figure 2.3C, compare lanes 1 and 4). The predicted mutant d4EHP lacks the first 12 amino acids of the wild-type protein, which are replaced by six new amino acids (Figure 2.3D). Expression of *syntaxin 1A* (*syx1A*), located in the second intron of *d4EHP* (Figure 2.3A), is not affected by the $d4EHP^{CP53}$ mutation (Figure 2.4).

The $d4EHP^{CP53}$ mutant is homozygous viable, and does not display any obvious zygotic phenotype. However, embryos produced by homozygous $d4EHP^{CP53}$ females have a substantially reduced hatching frequency (52%) compared to wild-type flies (93%). Flies that do hatch have no conspicuous phenotypic defects, even when genetically homozygous themselves. The embryos that do not hatch exhibit patterning defects mostly affecting anterior segmentation (Figure 2.5).

Because of these patterning defects, we investigated whether d4EHP activity is involved in translational regulation of maternal *cad* mRNA. Remarkably, in contrast to wild-type embryos (Figure 2.3E), those from mothers homozygous for $d4EHP^{CP53}$ (subsequently termed $d4EHP^{CP53}$ mutant embryos) show Cad ectopically expressed at the anterior end (Figure 2.3H; note however that the anterior expression of Cad is weaker than in the posterior, most likely because of the residual d4EHP). The expression of Cad in the anterior is not due to an alteration in the Bcd gradient, since Bcd-dependent zygotic *hunchback* (*hb*) mRNA expression (Tautz, 1988) is unaffected

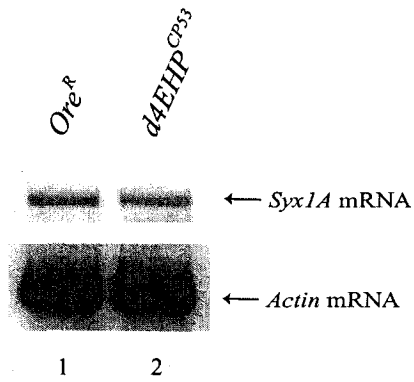

Figure 2.4 Syx1A expression is unaffected in d4EHPCP53 embryos
RT-PCR analysis of total RNA from wild-type (OreR) and d4EHPCP53 embryos using primers specific for Syx1A and Actin mRNAs.

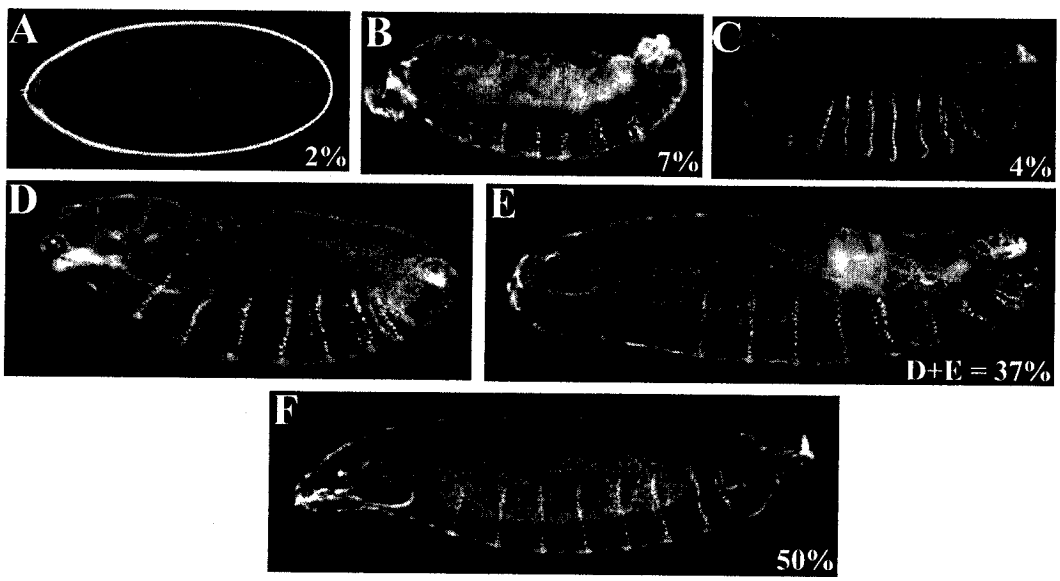

Figure 2.5 Cuticle preparations of embryos that fail to hatch from d4EHPCP53 females Percentages indicate the proportion of embryos exhibiting each phenotype. A. No apparent cuticle; these probably represent unfertilized eggs. B. All head and thoracic segments are deleted, as are one or two anterior abdominal segments. An open hole in the anterior cuticle allows internal tissue and yolk to escape. C. All eight abdominal segments are present, but head segments and denticle belts corresponding to thoracic segments are not observed. Open anterior holes are not apparent. D and E. Head structures are present but clearly reduced, and one or more thoracic denticle belts may also be lacking. F. Only relatively minor head defects are apparent. Note that posterior segmentation remains intact in these mutant embryos, as is the case for the progeny of bcd females.

in *d4EHP*^{CP53} mutant embryos (Figure 2.3I). In addition, *cad* mRNA expression levels (Figure 2.6A) and localization (Figure 2.3J) are unaltered in *d4EHP*^{CP53} mutant embryos. These results demonstrate that *d4EHP* activity is required to repress Cad expression at the anterior of the embryo.

2.3.3 d4EHP interacts biochemically with the anterior determinant Bcd

Next, we investigated whether d4EHP and Bcd interact *in vivo*. Extracts prepared from 0-2h wild-type embryos were treated with RNAse and used to examine the interaction between Bcd and deIF4E or d4EHP (Figure 2.7A). Pre-immune sera failed to precipitate deIF4E or d4EHP. Anti-deIF4E immunoprecipitated deIF4E, but not Bcd. In contrast, anti-d4EHP readily co-immunoprecipitated endogenous Bcd, thus demonstrating that Bcd exhibits much stronger affinity for d4EHP than for deIF4E. These results are at variance with a paper published by Niessing et al. (2002), which concluded that an interaction between deIF4E and Bcd exists. This discrepancy will be addressed in the Discussion.

Three alternatively spliced forms of *bcd* mRNA produce different variants of Bcd protein (Driever and Nusslein-Volhard, 1988b). Only two of these variants, Bcd$^{1-489}$ and Bcd$^{1-494}$, contain the homeobox domain that is critical for inhibiting anterior *cad* translation (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). To determine whether Bcd$^{1-489}$ and Bcd$^{1-494}$ differ in their ability to interact with d4EHP, HA-tagged deIF4EI (as a negative control) or d4EHP were transfected into 293 cells with each of the two FLAG-tagged Bcd variants. Immunoprecipitation with an anti-FLAG antibody demonstrates that neither one of the Bcd spliced variants interacts detectably with deIF4EI, while both exhibit a comparable interaction with d4EHP (Figure 2.7B). We therefore used the Bcd$^{1-494}$ isoform for all subsequent experiments, and will refer to it

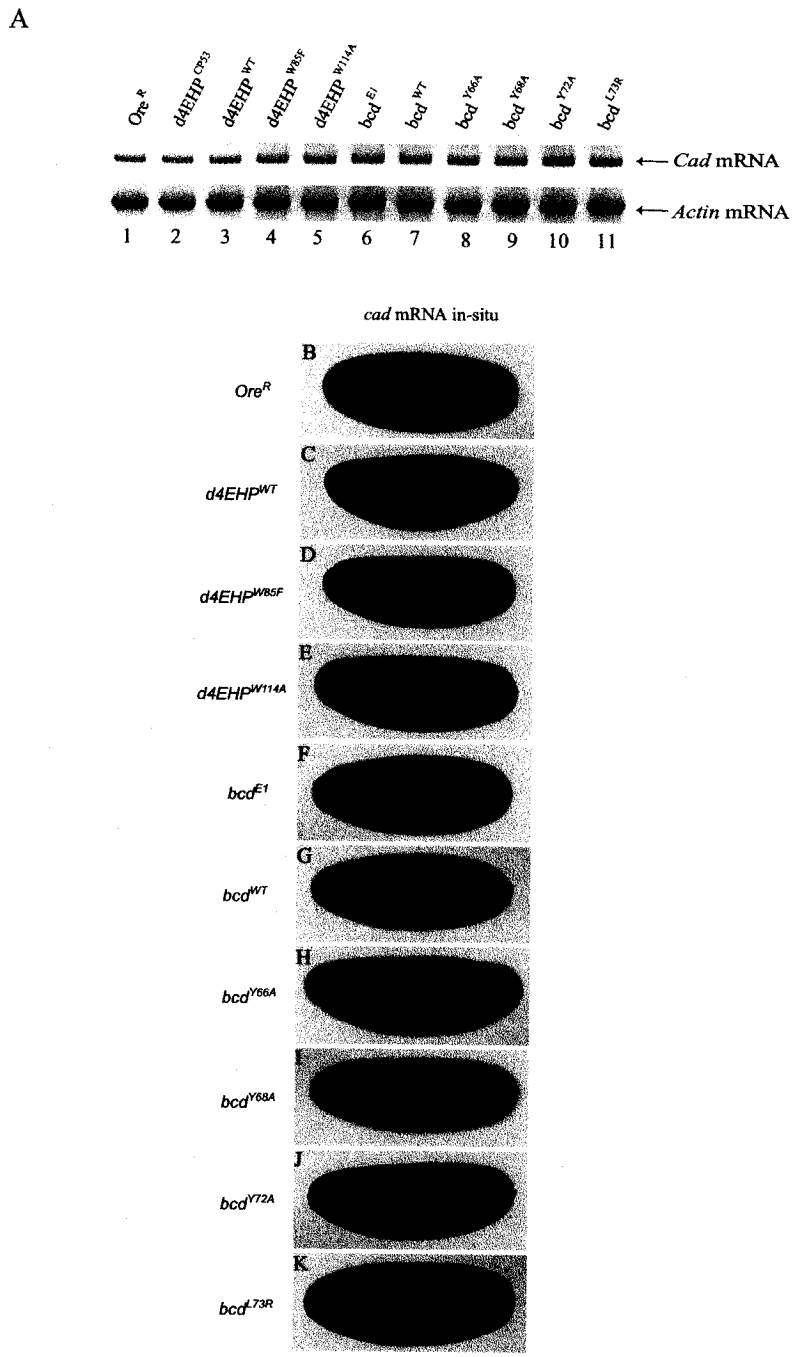

Figure 2.6 Cad mRNA expression and localization in mutant Drosophila embryos
A. RT-PCR analysis of total RNA from wild-type (lane 1), and d4EHP (lanes 2-5) or bcd (lanes 6-11) mutant embryos using primers specific for cad and Actin mRNAs. B-K. cad mRNA in situ hybridization in wild-type (B), and d4EHP (C-E) or bcd (F-K) mutant embryos.

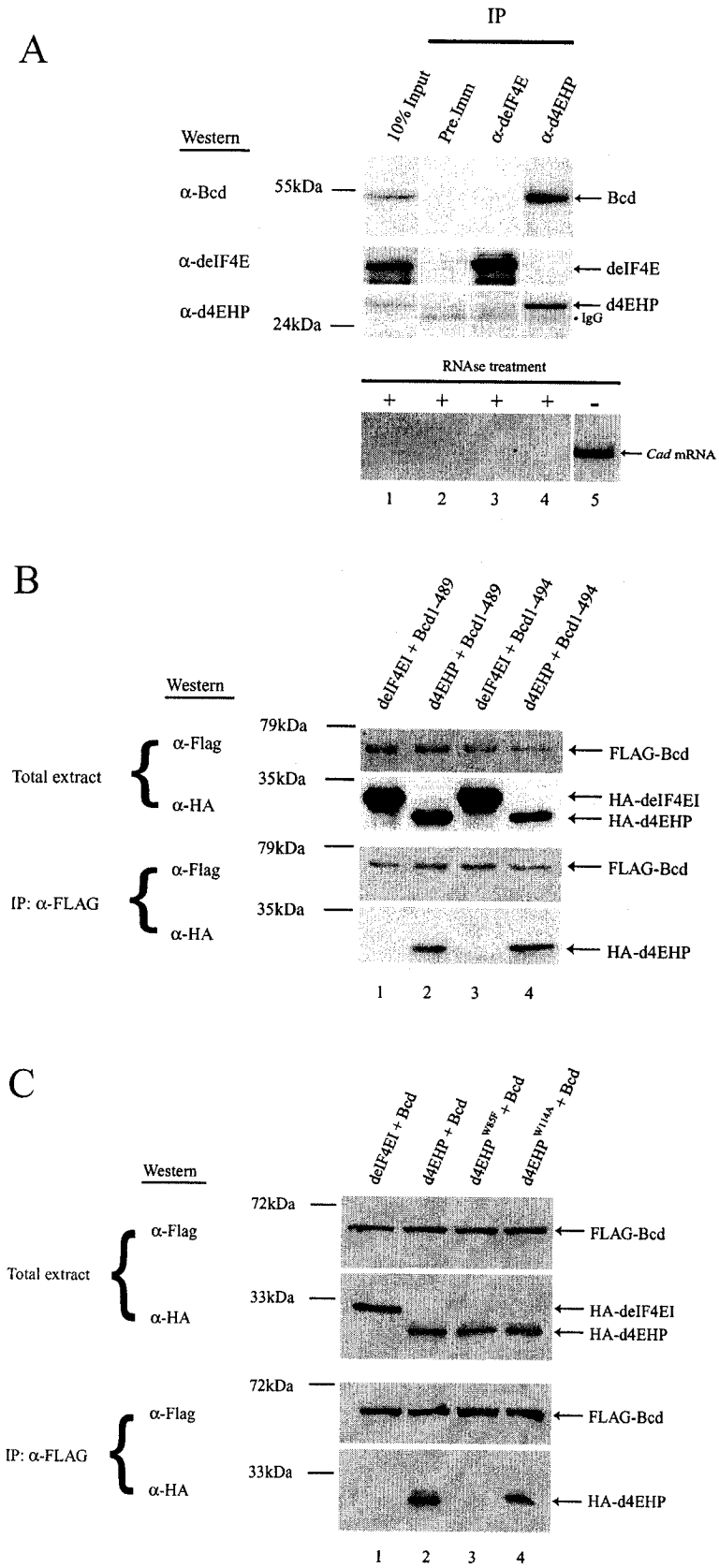

Figure 2.7 d4EHP interacts with Bcd in vivo and in vitro

A. d4EHP interaction with endogenous Bcd. 0-2h OreR embryo extract (lane 1) was immunoprecipitated (IP) using pre-immune (lane 2), anti-deIF4E (lane 3), or anti-d4EHP (lane 4). Immunoprecipitated proteins were analyzed by Western blotting for the presence of Bcd (top panel), deIF4E (second panel) and d4EHP (third panel). The presence of endogenous cad mRNA in the extracts was analyzed by RT-PCR (bottom panel). B. d4EHP interacts with two alternatively spliced variants of Bcd. FLAG-tagged Bcd1-489 and Bcd1-494 were transfected in 293 cells together with HA-tagged deIF4EI or d4EHP. Extracts were immunoprecipitated (IP) with anti-FLAG and analyzed by Western blotting. C. Bcd interaction requires a conserved tryptophan residue (W85) in d4EHP. FLAG-tagged Bcd was transfected in 293 cells with HA-tagged deIF4EI (lane 1), d4EHP (lane 2), d4EHPW85F (lane 3), and d4EHPW114A (lane 4). Extracts (top panels) were immunoprecipitated (IP) with anti-FLAG and analyzed by Western blotting (bottom panels).

as Bcd for simplicity.

The similarity between 4EHP and eIF4E is not limited to the amino acids that participate in binding the 5' cap structure. Also highly conserved between the two proteins are several residues that play a role in the interaction between eIF4E and eIF4G or 4E-BP (Figure 2.1B-C)(Gross et al., 2003; Marcotrigiano et al., 1999). In heIF4E, Trp73 directly contacts eIF4G and 4E-BP (Marcotrigiano et al., 1997; Marcotrigiano et al., 1999; Pyronnet et al., 1999). Despite the fact that the equivalent of Trp73 is conserved in all 4EHPs (Trp85 in d4EHP), 4EHP does not interact with eIF4G in mammals (Rom et al., 1998; Tee et al., 2004) and in *Drosophila* (data not shown, Hernandez et al., 2005). It was therefore pertinent to determine whether Trp85 is required for d4EHP interaction with Bcd. To this end, HA-tagged deIF4EI, d4EHP, d4EHP^{W85F} and d4EHP^{W114A} mutants were transfected in 293 cells along with FLAG-tagged Bcd. FLAG-Bcd co-immunoprecipitated wild-type HA-d4EHP, but not HA-deIF4EI (Figure 2.7C). Mutation of Trp85 in d4EHP to Phe abrogated Bcd binding, while the W114A mutation, which affects cap binding, did not (Figure 2.7C). Taken together, these results demonstrate that the interaction between d4EHP and Bcd occurs on the convex dorsal surface of d4EHP, as determined by the predicted position of Trp85 on a homology model (data not shown, Rom et al., 1998). This emulates the mechanism used by eIF4Gs/4E-BPs for their interaction with eIF4E (Gross et al., 2003; Marcotrigiano et al., 1999).

2.3.4 d4EHP interacts with both Bcd and *cad* mRNA 5' Cap structure to inhibit Cad expression

To show that the Bcd- and cap-binding abilities of d4EHP are required for the translational inhibition of *cad* mRNA, we generated transgenic fly lines that

overexpress wild-type or mutant forms of d4EHP (Figure 2.8A), and assessed their ability to rescue the *d4EHP*^{CP53} mutation. Three independent insertion lines were examined for each construct. For simplicity, embryos will be referred to by their maternal genotype. In contrast to wild-type embryos (Figure 2.8B), *d4EHP*^{CP53} embryos show Cad expression domains extending further towards the anterior (Figure 2.8C). While transgene-derived expression of wild-type *d4EHP* (*d4EHP*^{WT}) rescued this effect (Figure 2.8D), the Bcd- and cap-binding mutants of d4EHP (*d4EHP*^{W85F} and *d4EHP*^{W114A}, respectively) failed to establish a wild-type Cad gradient (Figure 2.8E-F), even though *cad* mRNA expression levels (Figure 2.6A) and distribution (Figure 2.6C-E) are indistinguishable from wild-type. Thus, the ability of d4EHP to bind to both Bcd and the cap structure is critical for the efficient inhibition of anterior *cad* mRNA translation.

2.3.5 Delineation of the Bcd d4EHP-binding motif

A Bcd mutant (Y68A/L73R) that fails to inhibit anterior *cad* mRNA translation was previously described (Niessing et al., 2002). The two residues that were changed simultaneously in this mutant affect the canonical YxxxxLΦ eIF4E-binding motif (Mader et al., 1995; Figure 2.9A), and are critical for binding to eIF4E in all eIF4E-binding proteins. We investigated whether the canonical eIF4E binding site of Bcd was required for binding to d4EHP. Point mutations were engineered to replace four amino acids that are near to, or fall within, the eIF4E-binding site. As noted above, two of the single point mutations, Y68A and L73R, replace amino acids that are critical for the interaction between eIF4E and its partners. Two other mutations, Y66A and Y72A, change residues at position -2 and +4, relative to the conserved Tyr68 (Figure 2.9B).

Transgenic fly lines carrying targeted *bcd* mutations were crossed into a *bcd-*

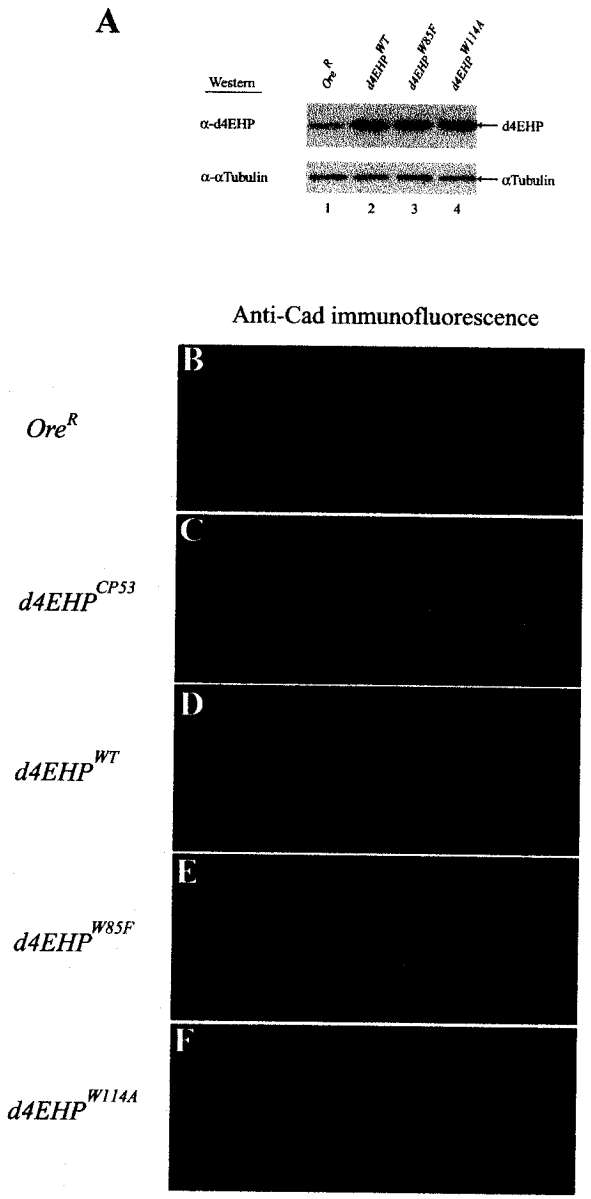

Figure 2.8 d4EHP interaction with Bcd and the cap structure is crucial for cad mRNA translation inhibition

A. Western blot analysis of d4EHP expression in transgenic embryos (Top panel). Anti-α-tubulin was used as a loading control (Bottom panel). B. OreR embryos display wild-type Cad gradient. C. d4EHPCP53 mutant embryo display ectopic Cad expression at the anterior end. D. Expression of a d4EHPWT transgene rescues the d4EHPCP53 mutant phenotype. E and F. Embryos from transgenic females expressing d4EHPW85F (E) and d4EHPW114A (F) in the d4EHPCP53 mutant background show ectopic expression of Cad at the anterior end. Orientation of embryos is anterior left and dorsal up.

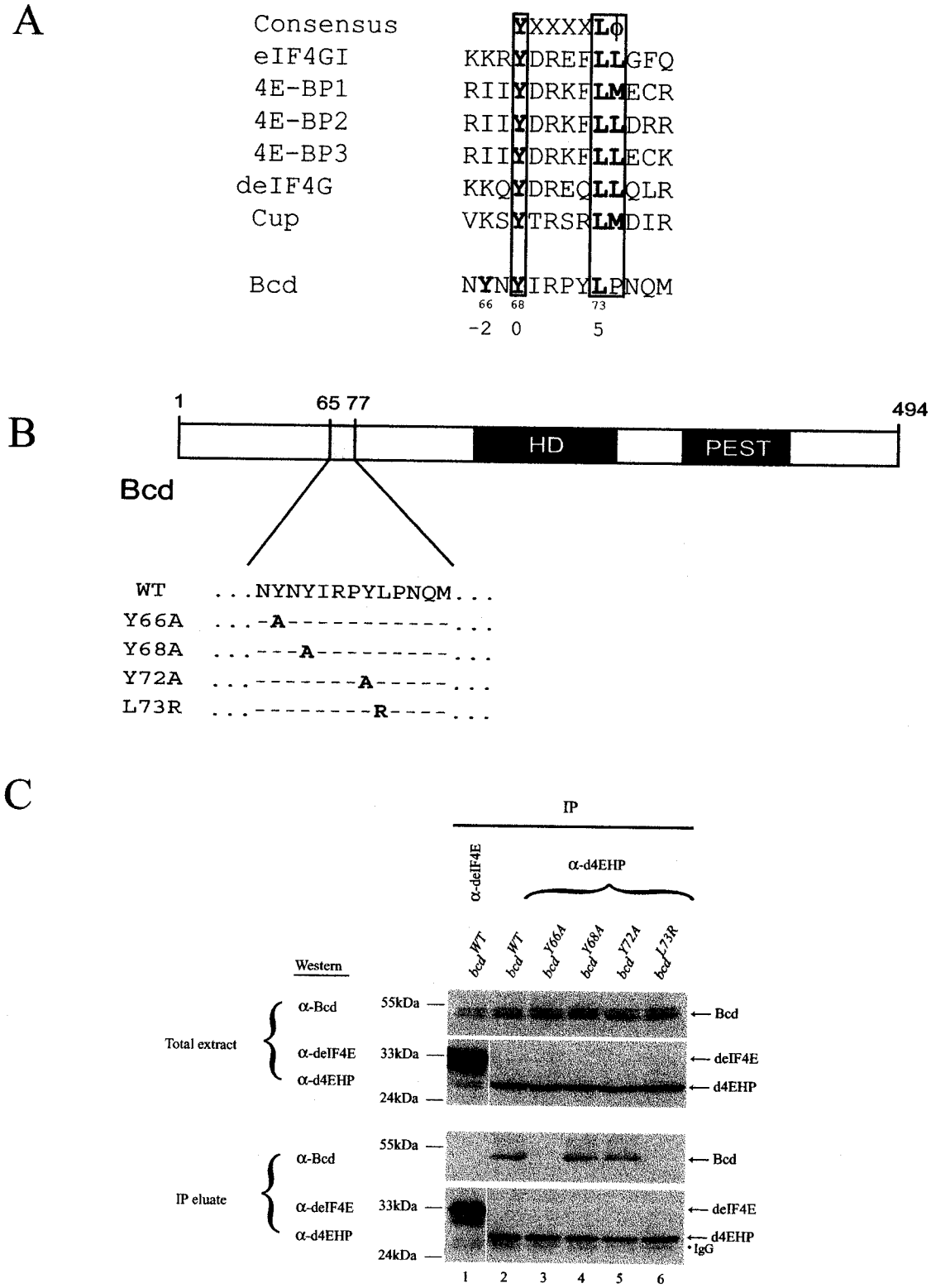

Figure 2.9 Bcd contains a d4EHP binding motif

A. Alignment of eIF4E binding motifs from mammalian eIF4GI and 4E-BPs, Drosophila eIF4G (deIF4G) and Cup, with Bcd amino acids 65 to 77. ϕ denotes any hydrophobic amino acid and X any amino acid. B. Schematic depiction of Bcd showing mutations in the putative d4EHP binding motif. C. In vivo interaction of Bcd mutants with d4EHP. 0-2h embryo extracts from females homozygous for the listed genotypes were immunoprecipitated (IP) with anti-deIF4E (lane 1), or anti-d4EHP (lane 2-6). Eluted proteins were analyzed for the presence of Bcd, deIF4E and d4EHP by Western blotting.

null background (bcd^{E1}), and embryos from females expressing Bcd only from the transgenes were obtained. RNAse-treated embryo extracts were then immunoprecipitated with deIF4E or d4EHP-specific antibodies, and the immunoprecipitates were analyzed by immunoblotting for the presence of Bcd (Figure 2.9C). Consistent with our earlier results, endogenous Bcd co-immunoprecipitates with d4EHP, but not with deIF4E. Surprisingly, however, the Y68A mutation failed to affect the interaction of Bcd with d4EHP, whereas the Y66A mutation, which changes a residue outside of the canonical consensus eIF4E-binding motif, abrogated the interaction. d4EHP:Bcd interaction was also abolished by the L73R mutation, but was not affected by the Y72A mutation. Consequently, we conclude that Bcd interaction with d4EHP, unlike that of eIF4G or 4E-BP binding to eIF4E, requires a sequence motif that is distinct from the canonical YxxxxLΦ eIF4E-recognition motif.

2.3.6 The d4EHP:Bcd interaction is required for embryonic patterning and development

We investigated the effects of these targeted *bcd* mutants on embryonic development. As previously described, Ore^{R} embryos (0-2h) show an anterior-to-posterior Cad gradient (Figure 2.10A) and normal cuticle segmentation pattern (Figure 2.10B), while in bcd^{E1} embryos, Cad is evenly distributed throughout the embryo (Figure 2.10C) and a *bcd* mutant cuticle pattern develops (Figure 2.10D)(Driever and Nusslein-Volhard, 1988a). Transgene-derived expression of wild-type *bcd* (bcd^{WT}) rescued all mutant phenotypes associated with bcd^{E1} (Figure 2.10E, F). Embryos expressing forms of Bcd unaffected for d4EHP binding (bcd^{Y68A} or bcd^{Y72A}) exhibited both a normal Cad gradient (Figure 2.10I, K) and normal cuticle pattern (Figures 2.10J, L). In contrast, bcd^{Y66A} and bcd^{L73R} mutant embryos exhibit defects in anterior patterning (Figure 2.10H,

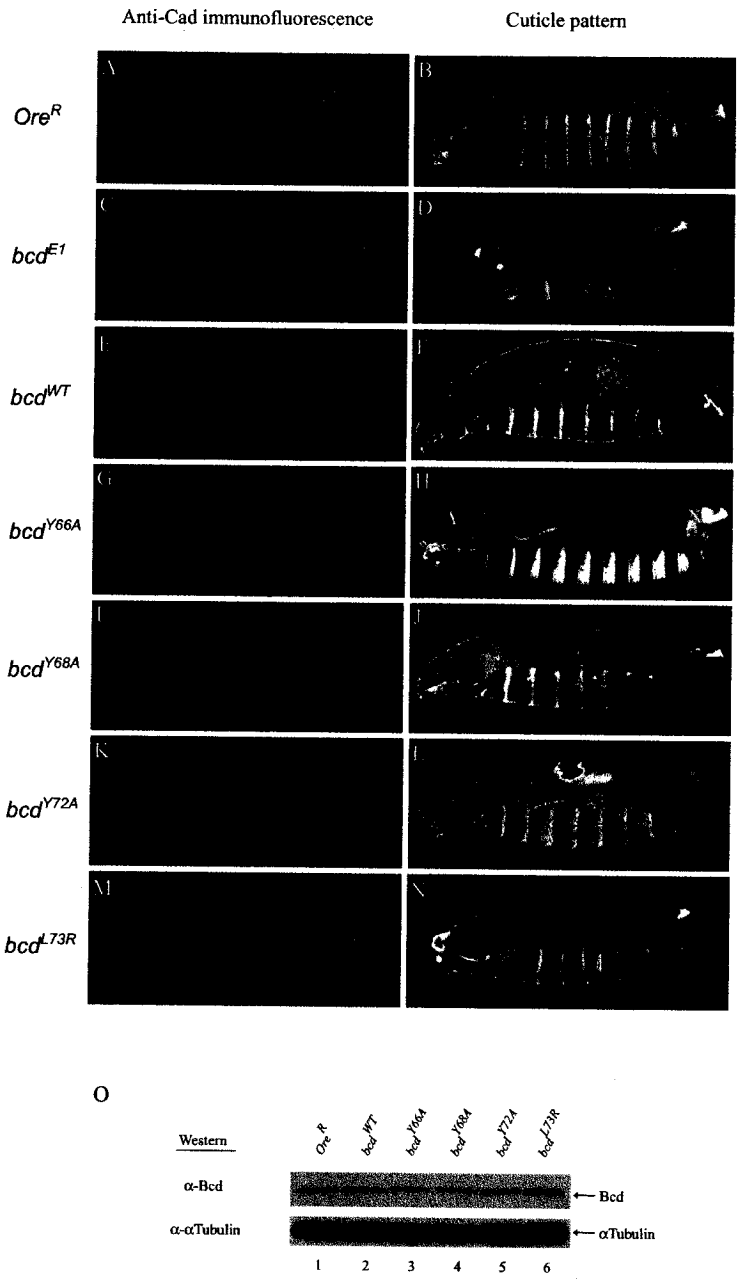

Figure 2.10 Functional analysis of mutant Bcd in transgenic Drosophila embryos
A and B. OreR embryos display wild-type Cad gradient (A) and cuticle pattern (B). C and D. Embryos derived from homozygous bcdE1 females fail to repress anterior cad mRNA translation (C) and show a bcd mutant cuticle phenotype (D). E and F. Transgenic embryos derived from females expressing bcdWT rescues the mutant Cad gradient (E) and cuticle pattern (F). G and H. Embryos derived from females expressing the mutant bcdY66A gene fail to repress cad mRNA translation (G) and develop seemingly normal larval segmentation with improperly assembled head elements (H). I and J. Embryos derived from females expressing the mutant bcdY68A gene demonstrate wild-type cad mRNA translation (I) and have normal cuticle pattern (J). K and L. Embryos derived from females expressing the mutant bcdY72A gene demonstrate wild-type Cad gradient (K) and have normal cuticle pattern (L). M and N. Embryos derived from females expressing the mutant bcdL73R gene fail to repress cad mRNA translation (M) and develop seemingly normal larval segmentation with improperly assembled head elements (N). Orientation of embryos is anterior left and dorsal up. O. Western blot analysis of embryo extracts using monoclonal anti-Bcd, or anti-α-tubulin as a loading control.

N), and do not establish a Cad gradient (Figure 2.10G, M). Bcd-dependent zygotic *hb* expression is normal in all mutant transgenic lines (data not shown, Niessing et al., 2002), demonstrating that the mutations we examined specifically affect the d4EHP interaction. Also, *cad* mRNA expression levels (Figure 2.6A) and localization are normal in all the mutant embryos (Figures 2.6F to 2.6K). Transgene-dependent Bcd expression levels were similar in all mutant transgenic lines (Figure 2.10O).

Next, we examined whether disruption of the Cad gradient through abrogation of the d4EHP:Bcd interaction affects hatching and development of *bcd* mutant embryos. Ore^{R} control embryos showed a 94% hatching frequency, and all negative-control bcd^{E1} mutant embryos failed to hatch (Table 2.1). Expression of bcd^{WT}, bcd^{Y68A} and bcd^{Y72A} rescued the ability of bcd^{E1} embryos to hatch (Table 2.1), and to give rise to adult flies. However, the two mutations that abrogate the Bcd:d4EHP interaction, bcd^{Y66A} and bcd^{L73R}, caused a substantial decrease in the number of hatching embryos (Table 2.1). Moreover, at 25°C, most of the hatched bcd^{Y66A} and bcd^{L73R} mutant larvae failed to develop into adults. Taken together, our data demonstrate a critical requirement of the d4EHP:Bcd interaction in *Drosophila* development.

2.3.7 The interaction of Bcd and d4EHP is required for translation inhibition

To demonstrate that the d4EHP:Bcd interaction is required for the BBR-mediated inhibition of *cad* translation, capped reporter mRNAs containing BBR sequences in their 3' UTR were used as a template for in vitro translation reactions. The BBR was inserted either in the sense or anti-sense orientation in the 3' UTR of the *Renilla reniformis* Luciferase (*rLuc*) reporter mRNA (Figure 2.11A). Mouse Krebs-2 cell-free translation extracts were used for the assay because they are more cap-dependent than the reticulocyte lysate system (Svitkin et al., 2001) and because they do not contain

Table 2.1 Hatching Frequency

Females*	Males	Total Eggs	Hatched	%Hatching
Ore^R	Ore^R	175	165	94
bcd^{E1}	Ore^R	137	0	0
bcd^{WT}	Ore^R	169	155	92
bcd^{Y66A}	Ore^R	628	198	32
bcd^{Y68A}	Ore^R	437	368	84
bcd^{Y72A}	Ore^R	198	180	91
bcd^{L73R}	Ore^R	540	158	29

Females and male flies of the listed genotypes were introduced into cages and were allowed to lay eggs for a 24-h period. Eggs were scored 30 h later for their ability to hatch. *All females are homozygous for the listed genotypes.

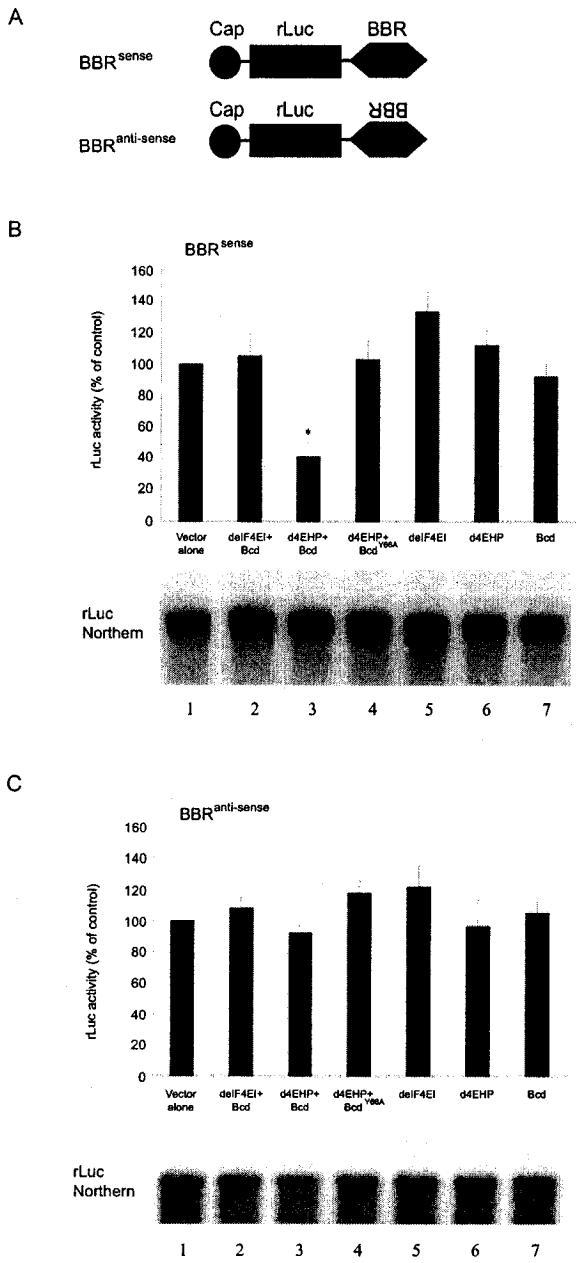

Figure 2.11 Bcd specifically represses translation in the presence of d4EHP
A. The Bcd binding region (BBR) from the 3'UTR of cad mRNA was inserted into the 3'UTR of Renilla reniformis luciferase (rLuc) mRNA, either in the sense (BBRsense) or the anti-sense (BBRanti-sense) orientation. B and C. In vitro translation of BBRsense and BBRanti-sense reporters. In vitro translation of the BBRsense (B) and the BBRanti-sense (C) reporter mRNAs (lanes 1) was performed in the presence of deIF4EI and wild-type Bcd (lanes 2), d4EHP and wild-type Bcd (lanes 3), d4EHP and Bcd Y66A (lanes 4), deIF4EI (lanes 5), d4EHP (lanes 6), and wild-type Bcd (lanes 7). Stability of the reporter mRNAs were determined by Northern blotting (bottom panels). Data are presented as mean ± standard deviation from three independent experiments. The value obtained for vector alone was set as 100%. *, p=0.0004. Statistical analysis was performed with a two-tailed, paired, t-test.

endogenous Bcd. deIF4EI, d4EHP and Bcd were synthesized *in vitro* by incubating the translation extract with their corresponding mRNAs for a period of one hour. Following this pre-incubation period, the extract was programmed with the reporter mRNA and incubated for an additional hour, and rLuc activity was then measured (Figure 2.11B, C). We first determined whether Bcd or d4EHP individually affect the translation of the BBR^{sense} and the $BBR^{anti\text{-}sense}$ reporter. No significant effect of d4EHP or Bcd was detected. However, addition of Bcd and d4EHP in combination caused a reduction of ~60% in translation of the *rLuc* mRNA containing the BBR^{sense} sequence. In contrast, the combination of Bcd and deIF4EI failed to inhibit translation. The effect of adding Bcd and d4EHP was dependent on their ability to interact, since the Bcd^{Y66A} mutant failed to inhibit translation. The inhibition by d4EHP and Bcd was also dependent on the interaction of Bcd with mRNA, because a reporter mRNA with an inverted BBR sequence was not regulated by d4EHP:Bcd-complex. Northern blotting analysis shows that the stability of the reporter mRNA in the translation extract was not affected by the expression of d4EHP or Bcd (Figure 2.11B, C, bottom panels).

2.4 Discussion

2.4.1 *cad* translation is repressed by a novel d4EHP-dependent mechanism, not by sequestering eIF4E

We describe here a new mode of mRNA-specific translational inhibition, which acts by tethering the mRNA 5' and 3' end via d4EHP, an eIF4E-related protein, and Bcd. d4EHP binds to the *cad* mRNA 5' cap structure, while Bcd binds to BBR in its 3' UTR. The interaction between d4EHP and Bcd is mediated through a sequence motif in Bcd that resembles, but is distinct from, the consensus eIF4E binding domain present in classical eIF4E-binding proteins such as 4E-BPs and eIF4G. Inhibition of *cad* mRNA translation by the d4EHP:Bcd complex demonstrates for the first time the involvement of a cellular cap-binding protein other than eIF4E in cap-dependent translational control. Furthermore, it provides a new molecular mechanism governing the formation of morphogenetic gradients during early *Drosophila* embryo development.

It was previously reported that Bcd inhibits anterior Cad synthesis through a direct interaction with eIF4E (Niessing et al., 2002). This conclusion was based largely on an *in vitro* demonstration that Bcd could be recovered from *Drosophila* extracts using a cap-affinity resin, which was pre-bound to an excess amount of recombinant eIF4E. However, under these conditions, only a small fraction of Bcd was recovered from the extracts (Niessing et al., 2002). It is therefore a distinct possibility that Bcd actually bound to the cap-affinity resin through endogenous d4EHP that was also present in the extracts. This possibility is consistent with both the previous data and our present study. Further supporting this conclusion, endogenous deIF4E and Bcd were not shown to interact in the previous study. Our data also indicate that the L73R mutation alone is sufficient to explain the previously reported $bcd^{Y68A/L73R}$ double mutant phenotype (Niessing et al., 2002).

2.4.2 Proposed d4EHP mode of action

The role of 4E-BPs in regulating cap-dependent translation is well documented (Gingras et al., 1999). 4E-BPs inhibit translation by competing with eIF4G for binding to eIF4E, and are therefore general inhibitors of cap-dependent translation, although the degree of inhibition varies among different mRNAs (Figure 2.12A). Cup and Maskin are eIF4E binding proteins that regulate translation during oogenesis and embryonic development (Nakamura et al., 2004; Nelson et al., 2004; Stebbins-Boaz et al., 1999; Wilhelm et al., 2003). They inhibit the translation of specific mRNAs by a simultaneous interaction with eIF4E at the mRNA 5' end and proteins bound to sequence elements in the 3' UTR (Richter and Sonenberg, 2005). Thus, Cup and Maskin have to compete with eIF4G for binding to eIF4E. While the exact binding affinities of these proteins for eIF4E have not been determined (Nakamura et al., 2004; Nelson et al., 2004; Stebbins-Boaz et al., 1999; Wilhelm et al., 2003), it is known that Maskin interacts rather weakly with eIF4E (Stebbins-Boaz et al., 1999). As a comparison, the 4E-BPs' affinity for eIF4E (Kd = 15 ± 3 nM) is comparable to that of eIF4G (Kd = 27 ± 6 nM)(Marcotrigiano et al., 1999).

In contrast to 4E-BP, Cup and Maskin, Bcd does not need to compete with eIF4G to interact with d4EHP. Rather, it is d4EHP that competes with eIF4E for cap-binding, which results in translation being inhibited at the level of cap recognition (Figure 2.12B). As a result of bypassing the need to disrupt the very stable eIF4E:eIF4G interaction, d4EHP should interdict translation more efficiently than 4E-BPs or other eIF4E-binding proteins. 4EHP-mediated translational regulation may have a particularly important role in germ line development, based on our results and on a recent report that a mutant allele of *C.elegans 4EHP* (*ife-4*) shows a severe egg

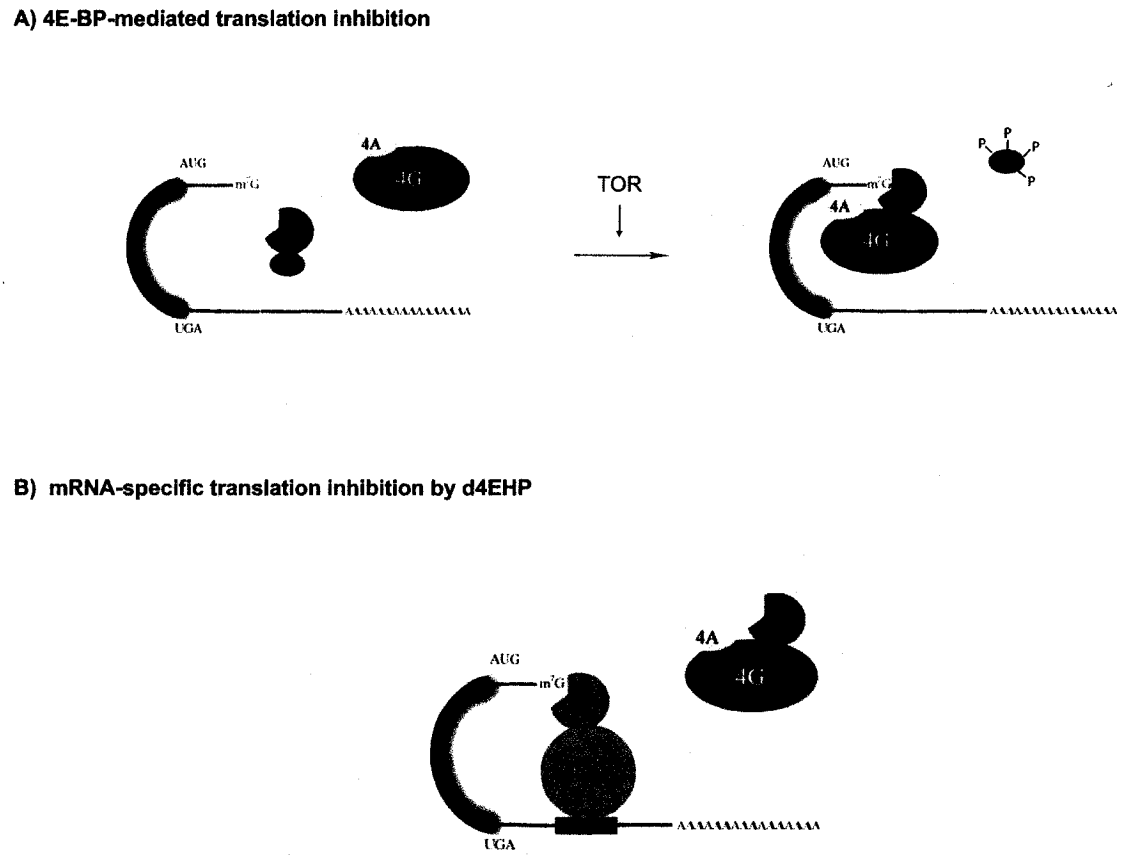

Figure 2.12 d4EHP:Bcd Translation Repression Model

A. By mimicking the eIF4G canonical YxxxxLϕ eIF4E binding motif, 4E-BP sequesters eIF4E from the initiation complex to inhibit cap-dependent translation. Phosphorylation of 4E-BP by TOR (Target of Rapamycin, Hay and Sonenberg, 2004) releases it from eIF4E. B. In contrast to 4E-BP, Cup and Maskin, d4EHP binds directly to the cap structure to inhibit translation of a specific mRNA. The simultaneous interaction of d4EHP with the mRNA 5' cap structure and Bcd with the BBR, renders cad mRNA translationally inactive.

laying defect (Dinkova et al., 2005)

The delineation of a d4EHP-recognition sequence in Bcd (YxxxxxxL) that interacts with d4EHP via its Trp85 residue highlights the similarities between the d4EHP:Bcd interaction and that of eIF4G with eIF4E (YxxxxLΦ in eIF4G; Trp73 in eIF4E)(Mader et al., 1995; Marcotrigiano et al., 1999). Despite these parallels, the inability of Bcd to bind to eIF4E must be explained by structural differences. The presence of two proline residues at position +3 and +6 of the Bcd d4EHP-binding motif (Figure 2.9A) is predicted to significantly alter the α-helical structure assumed by the YxxxxLΦ peptide upon binding to eIF4E (Marcotrigiano et al., 1999), and thus prevent Bcd association with deIF4E. Furthermore, the eIF4E interaction surface of eIF4G is not limited to the YxxxxLΦ motif, but extends over a larger interface; the N-terminal domain of eIF4E is also required for folding and tight binding to eIF4G (Gross et al., 2003). Indeed, the ability of d4EHP to bind specifically to Bcd, and not to deIF4G and d4E-BP (Hernandez et al., 2005), can be explained by the importance of the N-terminal KHPL sequence of eIF4E in the interaction with eIF4G and 4E-BP (Gross et al., 2003; Marcotrigiano et al., 1999), since this sequence is not conserved in d4EHP (Figure 2.1C).

2.4.3 Many different mechanisms repress translation of specific mRNAs in the *Drosophila* embryo

Our demonstration that *cad* translation is repressed through a d4EHP- and Bcd-dependent tethering mechanism adds to the diversity of translational control mechanisms operating in the early *Drosophila* embryo. Why are so many translational repression pathways necessary? If an individual mechanism alone can reduce translation of a specific mRNA, but not completely abrogate it, a combination of

inhibitory interactions may be needed in order to accomplish strict translational control. This can be advantageous if the diversity of factors, like Bcd, that can confer mRNA specificity for a given mechanism, is relatively limited. Multiple mRNAs also have to be translationally repressed in overlapping spatial and temporal domains. Controlling these mRNAs through mechanisms that target different components of the general translational machinery, rather than through a common mechanism, might allow more precise regulation of their individual expression patterns.

It is noteworthy that although 4EHP is conserved through evolution, Bcd exists only in higher dipterans (Lynch and Desplan, 2003). Thus, in other organisms, 4EHP must function during development through proteins that are analogous to Bcd. In summary, we describe here a novel mode of translational control in *Drosophila* development. Because cap-dependent translation regulation plays such an important role in gene expression, and since 4EHP is also expressed in somatic cells, we predict that examples of d4EHP-mediated translational repression other than *cad* are most likely to exist.

2.5 Experimental Procedures

2.5.1 Plasmids

A cDNA coding for d4EHP (SD07020; Research Genetics) was obtained from the Berkeley *Drosophila* Genome Project (Rubin et al., 2000). Subcloning and mutagenesis of d4EHP, deIF4EI and Bcd were performed using the polymerase chain reaction (PCR). The PCR-amplified open reading frames, flanked by an *EcoR*I site in the 5' and an *Xho*I site in the 3', were subcloned into the pcDNA3-3HA vector (d4EHP and deIF4EI) and the pcDNA3-C-term-FLAG vector (Bcd). For recombinant protein expression, d4EHP was subcloned into pProEx-His and pGEX6p-1 vectors using *BamHI*/*EcoR*I and *EcoRI*/*Xho*I sites, respectively. To create pUASP-***d4EHP construct*** rescue vectors, d4EHP constructs were inserted into the pUASP vector using *KpnI*/*BamHI* restriction sites. The Bcd binding region (BBR) from *cad* mRNA was introduced into the 3'UTR of pcDNA3-rLuc-Δ*Apa*I reporter vector, either in the sense (BBR^{sense}) or the anti-sense ($BBR^{anti\text{-}sense}$) orientation, using PCR with oligonucleotides containing *Xba*I sites. To create pCaSpeR4-*nos* promoter-***Bcd construct***-Bcd 3' UTR rescue vectors, Bcd constructs were inserted into the pKS-*nos* promoter-**X**-Bcd 3'UTR vector (**X** denotes a multiple cloning site) using *NdeI*/*BamHI* restriction sites. Subsequently, a *KpnI*/*Not*I cassette from the pKS-*nos* promoter-***Bcd construct***-Bcd 3'UTR vectors were transferred into the pCaSpeR4 vector. All inserts were fully sequenced.

2.5.2 Recombinant Protein Purification

For the purification of GST-d4EHP and His-d4EHP fusion proteins, *E.coli* BL21 was transformed with the pGEX6p-d4EHP and the pProEx-His-d4EHP construct. Following a 2h induction at 37°C with 0.1 mM IPTG, the fusion proteins were purified on a

Glutathione Sepharose™ 4B resin (Amersham Pharmacia) and TALON™ Metal Affinity resin (BD Bioscience), respectively, according to the manufacturer's instructions.

2.5.3 Anti-d4EHP Antiserum and Western Blotting Analysis

An anti-d4EHP antiserum (#3444) was raised in a New Zealand white rabbit injected with GST-d4EHP. For Western blotting, proteins were resolved by SDS-PAGE and transferred onto a 0.22μm nitrocellulose membrane. Membranes were blocked overnight at 4°C with 5% milk in phosphate buffered saline (PBS) and 0.5% Tween-20 (PBST). Membranes were incubated for 90 min with one of the following antibodies: mouse monoclonal anti-HA (Babco; 1:5000); mouse monoclonal anti-FLAG (Sigma; 1:5000); mouse monoclonal anti-His (Qiagen; 1:1000); rabbit polyclonal anti-deIF4E (1:5000 Sigrist et al., 2000); rabbit polyclonal anti-d4EHP (1:5000); mouse monoclonal anti-Bcd (Bcd mab23 ATCC; 1:50). This was followed by a 1h incubation with horseradish peroxidase-coupled sheep anti-mouse or anti-rabbit (Amersham Pharmacia; 1:5000), or goat anti-rabbit Fc-specific IgG (Jackson ImmunoResearch; 1:3000). Detection was performed with Western Lightning™ (PerkinElmer).

2.5.4 Cell Culture

Cationic lipid reagent (20μl of Lipofectamine and 30μl of Plus™; Invitrogen) was diluted in serum free media (Opti-MEM; Invitrogen) for transfection in Human Embryonic Kidney 293 cells (100mm dish). Following a 3h incubation, the medium was replaced with Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS). Transfected cells were harvested in PBS 36h following the addition of serum containing media. The cells were then lysed

by repeated freeze/thaw cycles in 600µl of lysis buffer (20mM Hepes-KOH, pH 7.6, 200mM KCl, 0.5mM EDTA, 10% glycerol, 1% Triton X-100 and Protease Inhibitor Cocktail (Complete™; Roche)) that contains RNAse A (50µg/ml). Cell debris was pelleted by centrifugation, and the protein concentration in the supernatant was determined using the Bio-Rad assay. S2 cells were grown at 25°C in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% FBS. S2 cell extract and 0-2h embryo extract were prepared as described above.

2.5.5 Cap-affinity Assay

S2 cell extract (200µl; 8$\mu g/\mu l$) was brought up to 1ml with cap-binding buffer (50mM Tris-HCl, pH 7.5, 300mM KCl, 1mM DTT, 1mM EDTA and Protease Inhibitor Cocktail (Complete™, Roche)), and pre-cleared for 1h at 4°C with 25µl of Protein A Sepharose. The supernatant was incubated for 2h at 4°C with 25µl of GDP-sepharose (Sigma) or m^7GTP-Sepharose™ 4B resin (Amersham Pharmacia). The resin was washed three times with 1ml of the cap-binding buffer and the bound proteins were eluted in 2X Laemmli sample buffer.

2.5.6 Co-Immunoprecipitation

For co-immunoprecipitation, 293 cell extract (200µl; 6-10$\mu g/\mu l$) was brought up to 1ml with the lysis buffer and pre-cleared for 1h at 4°C with 25µl of Protein A Sepharose. The supernatant was immunoprecipitated for 1h at 4°C with 25µl of anti-FLAG®M2-Affinity Gel (Sigma). The resin was washed twice with lysis buffer and once with lysis buffer containing 300mM KCl. Immunoprecipitates were eluted in 2X Laemmli sample buffer. For anti-HA, anti-deIF4E and anti-d4EHP immunoprecipitations, 25μl of Protein A Sepharose were pre-incubated for 2h with anti-HA (3μl), anti-deIF4E (5μl)

and anti-d4EHP (5μl). The resin was washed three times with the lysis buffer prior to immunoprecipitation as described above. Embryo extract was used at a concentration of 12μg/μl.

2.5.7 P-element Excision and Transgenic Rescue Experiment

The excision experiment was performed as previously described (Thomson and Lasko, 2004). Transgenic flies were generated by P-element mediated germline transformation of *yw* recipients using pCaSpeR4-*nos* promoter-***Bcd construct***-Bcd 3'UTR or pUASP-***d4EHP construct*** vectors. To express d4EHP, the UAS transformant lines were crossed to *Act-GAL4* driver line. Transformed *bcd* and *d4EHP* lines were crossed to bcd^{E1} and $d4EHP^{CP53}$ mutants, respectively, and tested for the rescue of mutant phenotypes. Antibody staining and *in situ* hybridization were carried out as described (Kobayashi et al., 1999). d4EHP and Cad immunostainings were visualized using AlexaFluor® 488 goat anti-rabbit IgG (1:500; Molecular Probes) under confocal laser scanning microscope.

2.5.8 RT-PCR

Total RNA was isolated from embryos using the RNAeasy kit (Qiagen) and then used to analyze various mRNAs by RT-PCR using the One Step RT-PCR kit (Qiagen) according to the manufacturer's instructions.

2.5.9 In-vitro Transcription and Translation Assay

Plasmids were linearized with *ApaI* and transcribed using T7 RNA polymerase (MBI). Capped mRNA synthesis was performed using the RiboMAX system (Promega). Krebs-2 cell extract (12.5μl) was incubated for 1h at 30°C with 300ng of capped-

mRNA encoding for individual proteins assayed herein. The extracts were subsequently programmed with 15ng of the reporter mRNA (capped-*rLuc-BBR*^{sense} or capped-*rLuc-BBR*$^{anti\text{-}sense}$) and incubated for an additional hour. Aliquots (2μl) were assayed for luciferase activity using the Dual-Luciferase® reporter assay system (Promega) in a Lumat LB 9507 bioluminometer (Berthold Technologies). ^{35}S-methionine labelling was performed as previously described (Brasey et al., 2003).

2.6 Acknowledgments

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Connecting text

In order to better understand the role of d4EHP in translation during early *Drosophila* embryo development, we used the information we have obtained in our study of the d4EHP:Bcd interaction to identify additional interacting proteins and mRNAs that undergo d4EHP-dependent translational inhibition. The results of these studies are presented in Chapter 3.

CHAPTER 3 - Cap-Dependent Translational Inhibition Establishes Two Opposing Morphogen Gradients in Drosophila Embryo

3.1 Abstract

In the early *Drosophila* embryo, asymmetric distribution of transcription factors, established as a consequence of translational control of their maternally-derived mRNAs, initiates pattern formation (Gebauer and Hentze, 2004; Johnstone and Lasko, 2001; St Johnston and Nusslein-Volhard, 1992; Wickens et al., 2000). For instance, translation of the uniformly distributed maternal *hunchback* (*hb*) mRNA is inhibited at the posterior to form an anterior-to-posterior protein concentration gradient along the longitudinal axis (Tautz, 1988; Tautz et al., 1987). Inhibition of *hb* mRNA translation requires an mRNP complex (the NRE-complex) that consists of Nanos (Nos), Pumilio (Pum) and Brain tumor (Brat) proteins, and the Nos responsive element (NRE) present in the 3' UTR of *hb* mRNA (Chagnovich and Lehmann, 2001; Sonoda and Wharton, 2001; Wharton and Struhl, 1991). The identity of the mRNA 5' effector protein that is responsible for this translational inhibition remained elusive. Here we show that d4EHP, a cap-binding protein which represses *caudal* (*cad*) mRNA translation, also inhibits *hb* mRNA translation by interacting simultaneously with the mRNA 5' cap structure (m^7GpppN, where N is any nucleotide)(Shatkin, 1976) and Brat. Thus, by regulating Cad and Hb expression, d4EHP plays a key role in establishing anterior-posterior axis polarity in the *Drosophila* embryo.

3.2 Introduction/Results

In the absence of transcription during early embryonic development, many genes are regulated at the level of translation (Wickens et al., 2000). Translation rates are often controlled at the initiation phase, a multi-step process involving the recruitment of the 40S ribosomal subunit to the 5' end of an mRNA and culminating in the positioning of the 80S ribosome at the initiation codon (Hershey and Merrick, 2000; Poulin and Sonenberg, 2003). Recognition of the cap structure by eIF4F (composed of three subunits: eIF4E, eIF4A and eIF4G) facilitates this process. Simultaneous interaction of eIF4G with eIF4E and the poly(A)-binding protein (PABP) results in mRNA circularization and promotes the recruitment of the 40S ribosomal subunit (Gebauer and Hentze, 2004; Kahvejian et al., 2005; Sachs, 2000). Consistent with their importance, eIF4E and PABP have emerged as major targets of translational regulatory mechanisms mediated by such modulator proteins as 4E-BPs and Paip2 (Kahvejian et al., 2005; Khaleghpour et al., 2001; Richter and Sonenberg, 2005).

Embryonic development in many metazoans requires the activity of various maternal determinants called morphogens, whose spatial and temporal expressions are tightly regulated (Gebauer and Hentze, 2004; Johnstone and Lasko, 2001; St Johnston and Nusslein-Volhard, 1992; Wickens et al., 2000). In *Drosophila*, local morphogen concentrations are important for the establishment of polarity and subsequent organization of both the antero-posterior and dorso-ventral axes of the embryo. A key morphogen for establishing antero-posterior pattern is the transcription factor Hunchback (Hb); when maternal Hb is allowed to accumulate inappropriately, posterior segmentation is blocked (Lehmann and Nusslein-Volhard, 1987; Struhl et al., 1992; Wharton and Struhl, 1991). Two modes of translational control have been proposed for the establishment of the Hb gradient: translational silencing via deadenylation (Wreden

et al., 1997) and inhibition at the initiation step in a cap-dependent manner (Chagnovich and Lehmann, 2001).

d4EHP, an eIF4E-like cap-binding protein that does not interact with deIF4G and d4E-BP, was shown to inhibit the translation of *cad* mRNA by interacting simultaneously with the cap and Bicoid (Bcd, chapter 2). While many embryos (~41%) produced by females homozygous for the $d4EHP^{CP53}$ mutation showed anterior patterning defects consistent with mislocalized Cad, some (~7%) also exhibited patterning defects such as missing anterior abdominal segments that cannot be readily explained by ectopic Cad. Since inhibition of *hb* mRNA translation has been linked in one study to the cap structure (Chagnovich and Lehmann, 2001) and since these additional phenotypes could be consistent with inappropriate regulation of Hb, we investigated the role of d4EHP in Hb expression. Embryos (0-2h) from females homozygous for the $d4EHP^{CP53}$ mutation were collected and immunostained using anti-Hb antibody. For simplicity, embryos will subsequently be referred to by their maternal genotype. Remarkably, similar to pum^{680} and $brat^{fs1}$ mutant embryos (Sonoda and Wharton, 2001; Tautz, 1988), in $d4EHP^{CP53}$ mutant embryos the Hb expression domain extended substantially toward the posterior (62 ± 1.7 % egg length (EL); the anterior tip is indicated as 0%) than in wild-type embryos (49 ± 0.5 % EL), and its posterior boundary was much less sharply defined (compare Figure 3.1A to 3.1C). Normal Hb expression was restored to $d4EHP^{CP53}$ mutant embryos by transgene-derived expression of wild-type *d4EHP* ($d4EHP^{WT}$) (Figure 3.1E-F), but not by expression of a mutant form of *d4EHP* ($d4EHP^{W114A}$), which cannot bind to the cap structure (Figure 3.1G-H). Interestingly, expression of another mutant form of d4EHP ($d4EHP^{W85F}$), which cannot bind Bcd, fully rescued the mutant Hb phenotype (Figure 3.1I-J), suggesting that d4EHP affects Hb expression differently than that of Cad. Distributions of Nos, Pum,

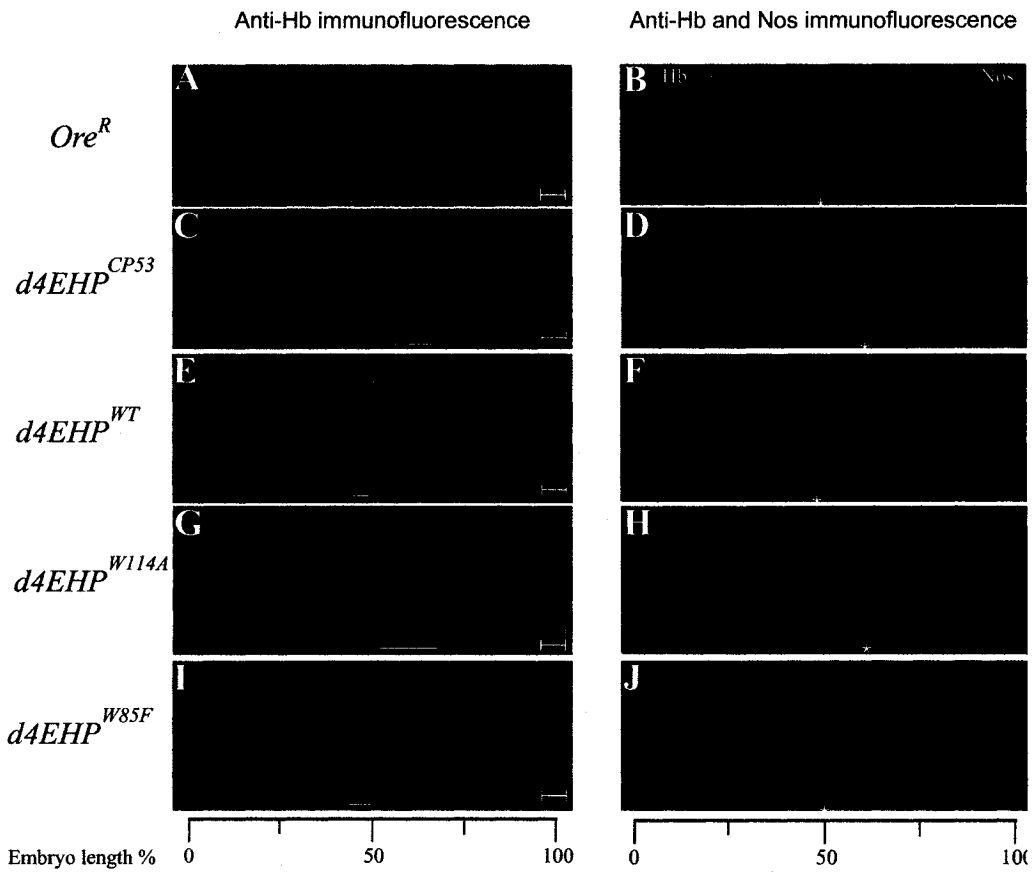

Figure 3.1 d4EHP interaction with the cap structure is required for hb translation inhibition.

A and B. OreR embryos display normal Hb gradient. C and D. d4EHPCP53 mutant embryo show ectopic Hb expression in the posterior half. E and F. Expression of d4EHPWT transgene in the d4EHPCP53 mutant background rescues the mutant phenotype. G and H. Embryos derived from females expressing d4EHPW114A fail to repress Hb translation. I and J. Embryos derived from females expressing d4EHPW85F show wild-type Hb distribution pattern. Nos is used as an internal control for staining intensity. The anterior tip is indicated as 0%. The posterior boundary of Hb expression is indicated by a white asterisk and line. Orientation of embryos is anterior left and dorsal up.

and Brat were unaffected in *d4EHP*^{CP53} mutant embryos (Figure 3.2). Taken together, these data demonstrate that d4EHP plays a key role in establishing the posterior boundary of Hb expression in a manner that requires its cap-binding activity. Furthermore, as d4EHP^{W85F} retains this function, d4EHP must influence Hb expression via a new interaction that does not require Bcd.

We reasoned that Brat might be a candidate partner protein for d4EHP, so we investigated whether d4EHP and Brat physically interact *in vivo*. Extracts prepared from 0-2h Oregon-R (Ore^{R}) embryos were treated with RNase and used to examine the interaction between Brat and d4EHP. Western blotting analysis using antibodies against d4EHP and Brat (Figure 3.3) demonstrates that while anti-d4EHP co-immunoprecipitated endogenous Brat (Figure 3.4A; lane 3), pre-immune serum did not (lane 2). To further demonstrate the specificity of this interaction, HA-tagged deIF4EI and the RNA-binding protein La (as negative controls) were transfected in 293 HEK cells along with FLAG-tagged full-length Brat. While anti-FLAG antibody immunoprecipitated wild-type HA-d4EHP together with FLAG-Brat (Figure 3.4B, lane 2), deIF4EI and La failed to co-immunoprecipitate (lanes 1 and 3). Similarly, other RNA-binding proteins such as hnRNP U and HuR, and a d4EHP mutant (W173A), in which a tryptophan that is part of the hydrophobic core and thus affects protein folding is replaced, also failed to interact with Brat (data not shown), demonstrating that Brat interacts specifically with d4EHP.

To identify the d4EHP sequence that is responsible for this interaction, number of residues found on the convex dorsal surface of d4EHP were mutated and tested via co-immunoprecipitation. Despite our efforts, however, we were unable to identify a point mutant of d4EHP that abrogated the interaction (data not shown). To circumvent this problem, we took advantage of our unique knowledge that, unlike d4EHP, deIF4EI

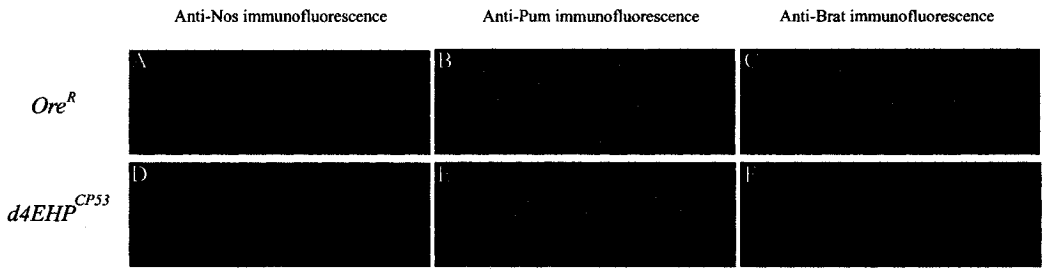

Figure 3.2 Distribution pattern of the components of the NRE-complex.
Nos, Pum and Brat distribution in OreR (A-C) and d4EHPCP53 mutant (D-F) embryos were visualized via immunofluorescence. Antibodies were used at 1:500 dilutions. Orientation of embryos is anterior left and dorsal up.

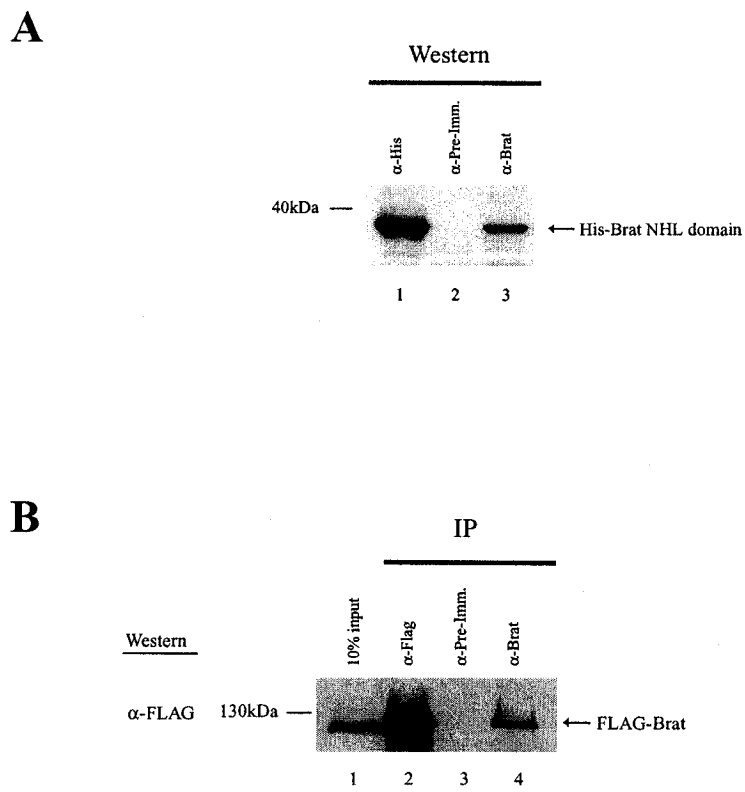

Figure 3.3 Characterization of anti-Brat antibody.
A. Brat antiserum detects the recombinant Brat NHL domain. Recombinant His-tagged Brat NHL domain (lane 1) is detected in a Western blot with a Brat antiserum (lane 3), but not with pre-immune serum (lane 2). B. Brat antiserum immunoprecipitates Brat from cell extracts. FLAG-tagged Brat was transfected in HEK293 cells (lane 1) and immunoprecipitated with an anti-FLAG antibody (lane 2), pre-immune serum (lane 3), or Brat antiserum (lane 4).

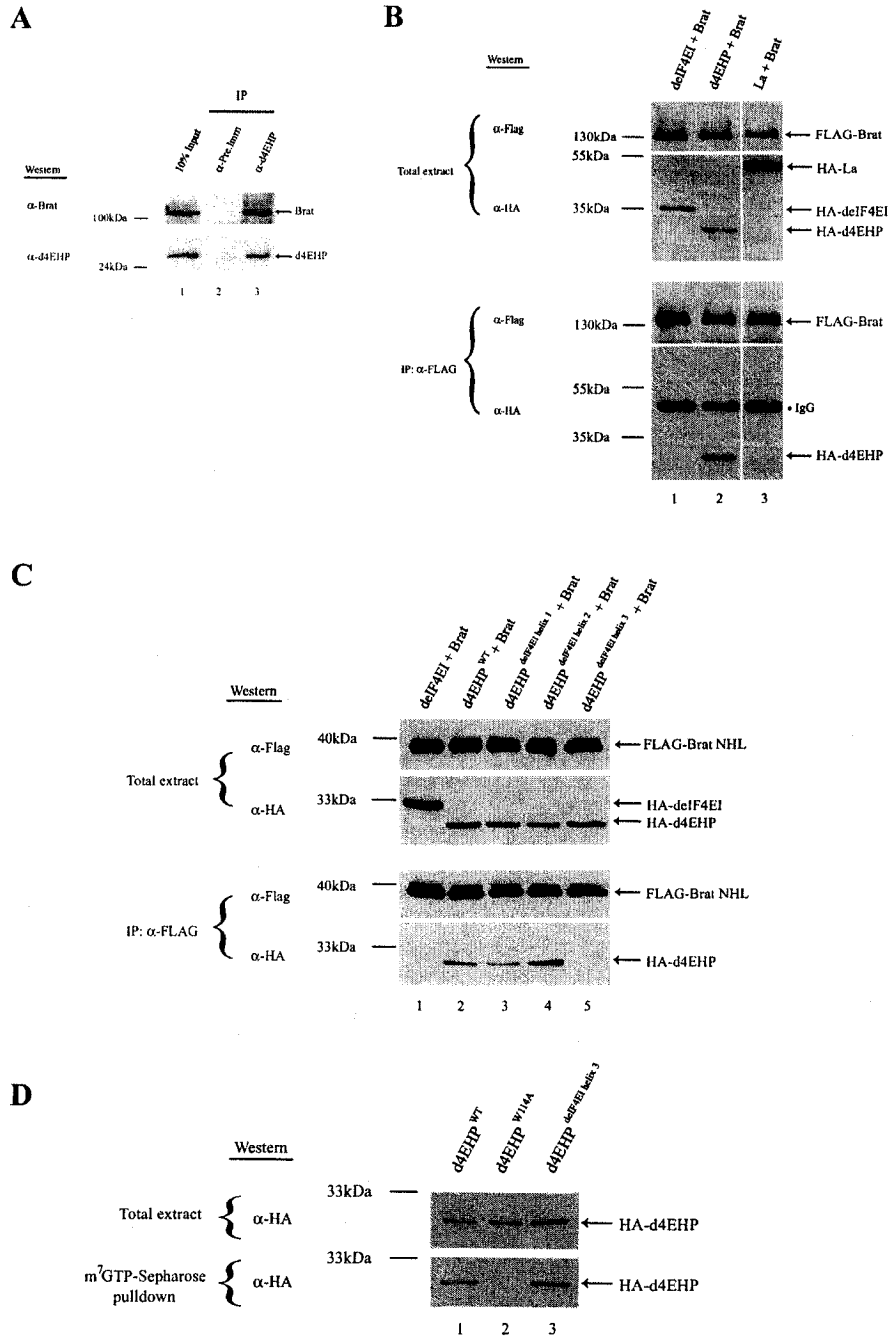

Figure 3.4 Characterization of the d4EHP:Brat interaction.
A. d4EHP interacts with Brat in vivo. OreR embryo (0-2 hr) extract (lane 1) was immunoprecipitated using pre-immune (lane 2), or anti-d4EHP (lane 3) antisera. Eluted proteins were analyzed by Western blotting for the presence of Brat (top panel) and d4EHP (bottom panel). B. d4EHP interacts specifically with Brat. FLAG-tagged Brat was transfected in 293 cells together with HA-tagged deIF4EI, d4EHP or La. C. The d4EHP:Brat interaction is mediated by the 3rd dorsal α -helix of d4EHP. Flag-tagged Brat wild-type NHL domain was transfected in 293 cells together with HA-tagged deIF4EI, d4EHP or d4EHP/deIF4EI chimeras. B and C. Proteins from cell extracts were immunoprecipitated (IP) with an anti-FLAG antibody and analyzed by Western blotting with antibodies against FLAG and HA. D. The d4EHPdeIF4EI helix 3 mutant interacts with the cap. 293 cell extracts (top panel) containing transfected HA-tagged d4EHP (lane 1), d4EHPW114A (lane 2) and d4EHPdeIF4EI helix 3 (lane 3), were incubated with m7GTP-Sepharose, and the eluate was analyzed by Western blotting (bottom panel).

does not interact with Brat (Figure 3.4C, lane 1). Therefore, by creating mutants of d4EHP which have one of their three dorsal α-helices replaced with that of deIF4EI, we sought to uncover the region of d4EHP that mediates this interaction. Indeed, while helix 1 and 2 mutants failed to disrupt the binding (lanes 3 and 4), substitution of the d4EHP helix 3, spanning residues 179 to 194, significantly abrogated the interaction (lane 5). Consistently, helix 3 is the least conserved of all α-helices between d4EHP and deIF4EI (Figure 2.1C). It is important to note that the structure of d4EHP is not affected by the substitution, since d4EHP$^{\text{deIF4EI helix 3}}$ still binds to the cap (Figure 3.4D, lane 3). In summary, our data demonstrate that Brat interacts with d4EHP on its convex dorsal surface and that this interaction is mediated by the d4EHP 3rd α-helix.

A C-terminal domain of Brat termed the NHL domain is both necessary and sufficient to inhibit *hb* mRNA translation (Sonoda and Wharton, 2001). The NHL domain contains two large surfaces (defined as top and bottom), that can support protein-protein interactions (Edwards et al., 2003). While the top surface of the NHL domain binds to Pum and Nos, the bottom surface does not interact with any known protein (Edwards et al., 2003; Sonoda and Wharton, 2001). Although the Brat NHL domain contains an amino acid sequence that conforms to the YxxxxxxLΦ d4EHP-binding motif (chapter 2), the d4EHP:Brat interaction does not require this motif, since a deletion mutant of Brat that lacks it can still interact with d4EHP and the d4EHP W85F mutant (Figure 3.5). This sequence is most probably masked from interaction with d4EHP because it is located in the hydrophobic core of the NHL domain(Edwards et al., 2003). To determine whether the d4EHP:Brat interaction requires the NHL domain, a Brat mutant that lacks the domain (Brat ΔNHL) was engineered and used in a co-immunoprecipitation experiment (Figure 3.6A). While wild-type Brat was readily

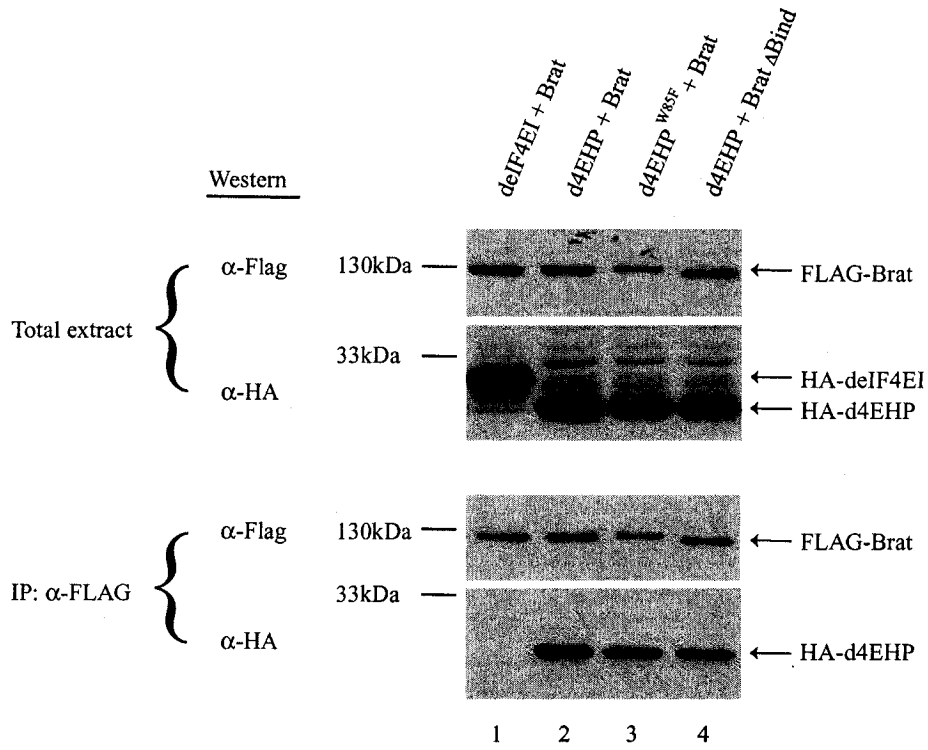

Figure 3.5 The d4EHP:Brat interaction does not require the YxxxxxxLϕ d4EHP-binding motif.

FLAG-tagged Brat wild-type or ΔBind mutant were transfected in 293 cells with HA-tagged deIF4EI, d4EHP wild-type and d4EHP W85F mutant and cell extracts were subjected to Western blotting (Total extract). Cell extracts were immunoprecipitated (IP) with an anti-FLAG antibody and analyzed by Western blot (IP panels).

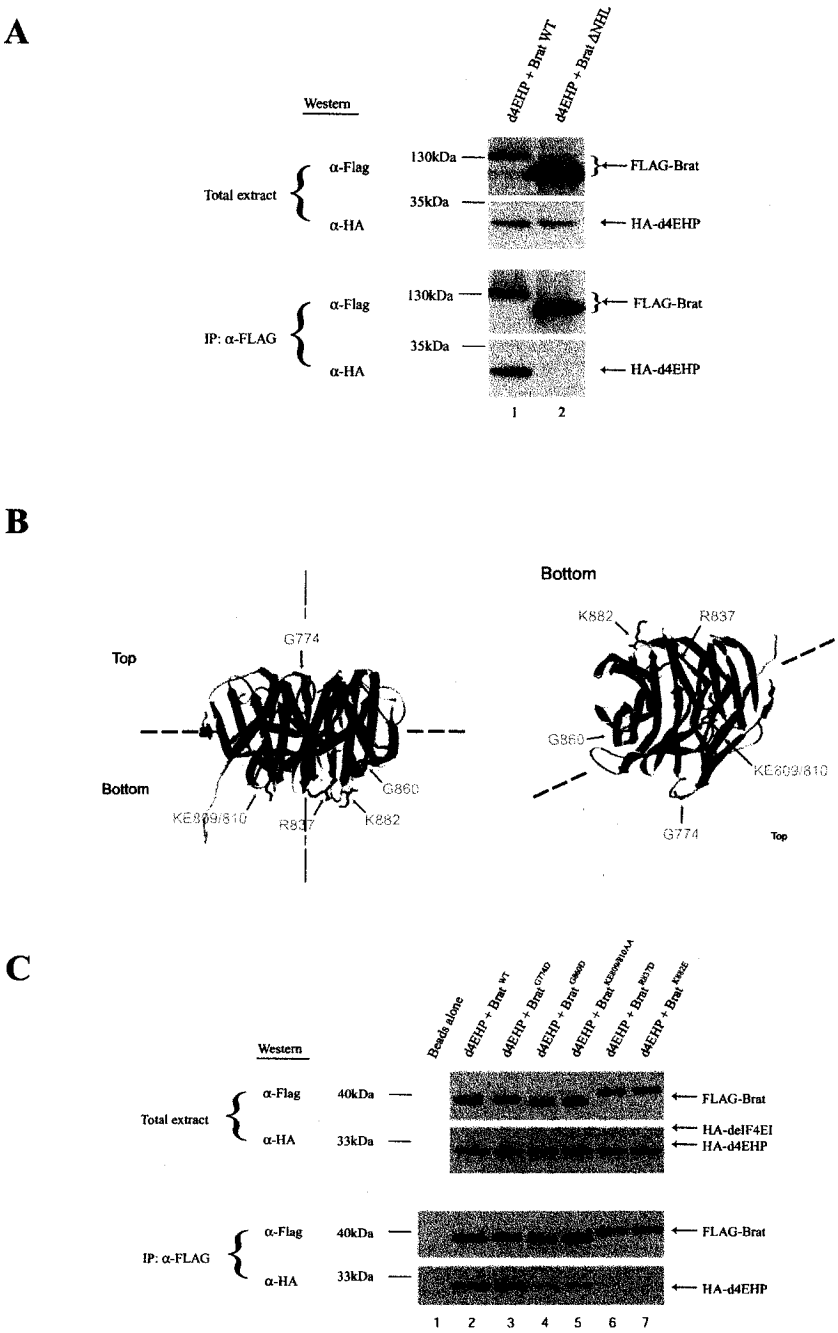

Figure 3.6 Brat interacts with d4EHP via its NHL domain

A. d4EHP interacts with Brat C-terminal NHL domain. FLAG-tagged Brat wild-type or ΔNHL mutant were transfected in 293 cells with HA-tagged d4EHP and cell extracts were subjected to Western blotting (Total extract). Cell extracts were immunoprecipitated (IP) with an anti-FLAG antibody and analyzed by Western blotting. B. Ribbon diagrams of the Brat NHL domain(Edwards et al., 2003). The positions of select surface residues are indicated. C. Interaction of Brat mutants with d4EHP. FLAG-tagged wild-type (lane 2) or mutants of the Brat NHL domain (lanes 3-7) were transfected in HEK293 cells together with HA-tagged d4EHP and cell extracts were subjected to Western blotting (Total extract). Cell extracts were immunoprecipitated (IP) with an anti-FLAG antibody and eluted proteins were analyzed for the presence of FLAG-Brat and HA-d4EHP by Western blotting (IP panels).

co-immunoprecipitated with d4EHP, the Brat ΔNHL mutant was not (compare lanes 1 and 2). Thus, we conclude that the NHL domain is the site of d4EHP interaction. To further characterize this interaction, point mutations were designed to replace residues on the two surfaces of the NHL domain (Figure 3.6B), and the mutant proteins were tested for their ability to interact with d4EHP. Mutation of a top surface residue that affects Brat interaction with Pum (G774A; Figure 3.6C, lane 3)(Sonoda and Wharton, 2001) did not affect the d4EHP:Brat interaction. However, when residues on the bottom surface were mutated, the d4EHP:Brat interaction was either significantly reduced (G860D and KE809/810AA; lanes 4 and 5), or abrogated (R837D and K882E; lanes 6 and 7; Note that the charge differences caused R837D and K882E mutant proteins to migrate slower in the gel). Importantly, the Brat NHL R837D mutant can assemble into an NRE-complex (see below; Figure 3, lane 4), demonstrating that the mutation we examined specifically affected the d4EHP interaction.

Brat binds to both Pum and Nos to recruit NRE and inhibits *hb* mRNA translation (Sonoda and Wharton, 2001). Since d4EHP interacts physically with Brat, we asked whether d4EHP can be co-purified with the NRE-complex *in vitro*. Incubation of recombinant components of the NRE-complex (Brat, Pum, Nos and NRE) together with HA-tagged d4EHP resulted in the retention of d4EHP on glutathione-Sepharose beads through the GST-Pum RNAB fusion protein (Figure 3.7, lane 2). The association of Brat with d4EHP was dependent on the ability of d4EHP to bind to Brat, since addition of Pum/Nos/NRE alone or in combination with the Brat R837D mutant failed to capture it (lanes 3 and 4). Thus, by interacting with Brat, d4EHP can associate with the NRE-complex.

To investigate the biological significance of the d4EHP:Brat interaction, we studied the effects of Brat mutants which are defective for d4EHP binding in

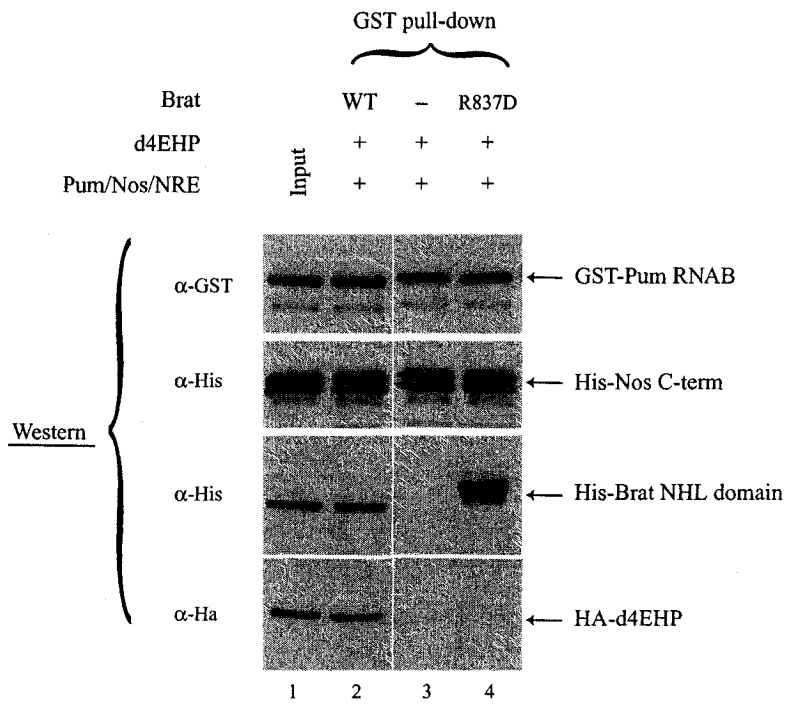

Figure 3.7 d4EHP interacts with the NRE-complex in vitro.
Samples containing in vitro translated HA-tagged d4EHP and purified components of the NRE-complex were used to perform an in vitro GST pull-down experiment. Eluted proteins were analyzed for the presence of GST-Pum RNAB, His-Nos C-term, His-Brat NHL domain and HA-d4EHP by Western blotting.

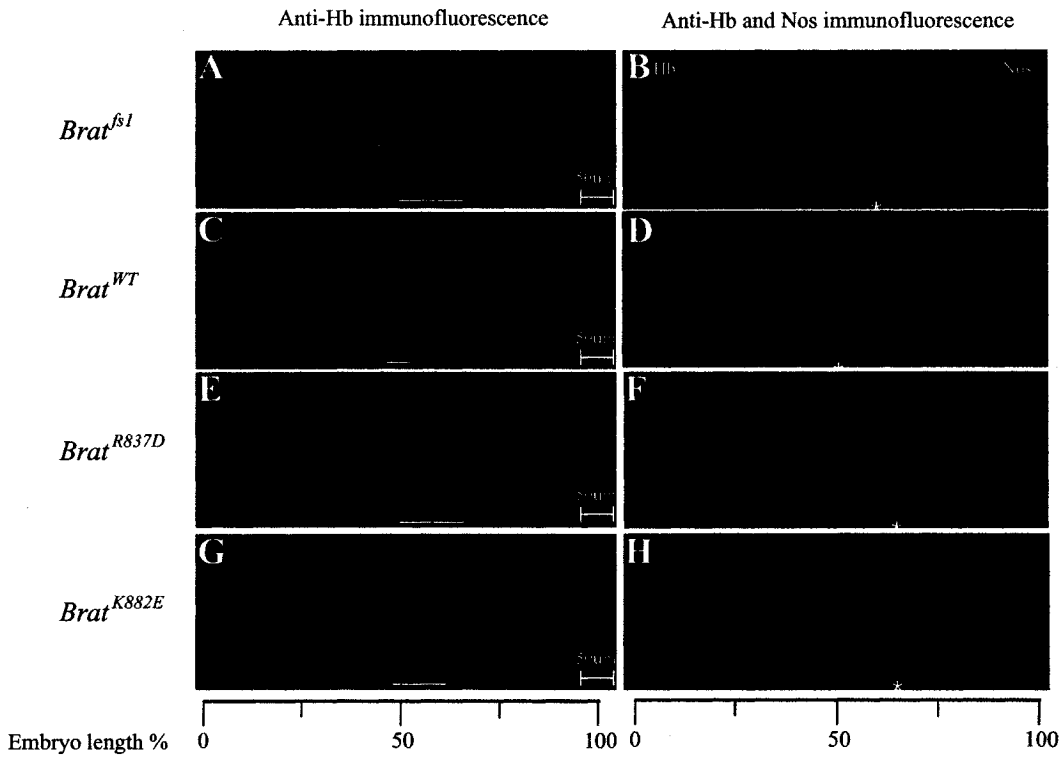

Figure 3.8 Functional analysis of Brat mutants in transgenic Drosophila embryos. A and B. Embryos derived from homozygous bratfs1 females show a shift of the Hb expression boundary towards the posterior. C and D. Embryos derived from females expressing bratWT in the bratfs1 mutant background show wild-type Hb distribution pattern. E-H. Embryos derived from females expressing mutant bratR837D and bratK882E genes show ectopic Hb expression. Nos is used as an internal control for staining intensity. The anterior tip is indicated as 0%. The posterior boundary of Hb expression is indicated by a white asterisk and line. Orientation of embryos is anterior left and dorsal up.

Table 3.1 Abdominal segmentation defects in Brat mutant embryos

	No. of abdominal segments								
	0	1	2	3	4	5	6	7	8
$Brat^{fs1}$/Df(2L)TE37C-7	2	10	62	20	5	1			
$Brat^{fs1}$/Df(2L)TE37C-7; $Brat^{WT}$									100
$Brat^{fs1}$/Df(2L)TE37C-7; $Brat^{R837D}$						7	24	39	30
$Brat^{fs1}$/Df(2L)TE37C-7; $Brat^{K882E}$							20	41	39

Each entry is the percentage of embryos derived from females of the indicated genotype (*left*) bearing the indicated number of abdominal segments (*above*). Seventy to one-hundred embryos were scored in each case.

Drosophila embryos. As previously shown (Sonoda and Wharton, 2001), $brat^{fs1}$ mutant embryos exhibit a significant expansion of the Hb expression domain towards the posterior (Figure 3.8A-B) and display abdominal segmentation defects (Table 3.1). When a $brat^{WT}$ transgene is expressed in the $brat^{fs1}$ mutant background, normal Hb distribution and a wild-type cuticle pattern is observed (Figure 3.8C-D and Table 3.1). Consistent with the biochemical interaction data, mutant forms of Brat that affect the d4EHP:Brat interaction ($brat^{R837D}$ and $brat^{K882E}$) show posteriorly-extended Hb expression (Figure 3.8E-H) and abdominal segment deletions; albeit weaker than that observed for $brat^{fs1}$ mutant (Table 3.1). Taken together, our data strongly argue for the critical requirement of the d4EHP:Brat interaction in *hb* regulation.

3.3 Discussion

We have demonstrated here that through its interaction with Brat, d4EHP defines the posterior boundary of Hb expression. The d4EHP:Brat interaction is mediated via residues on the bottom surface of the Brat NHL domain (Figure 3.6A). Thus, similar to the model established for *cad*, a simultaneous interaction of d4EHP with the cap and Brat results in mRNA circularization and renders *hb* translationally inactive (Figure 3.9). Since the interaction between Brat and d4EHP does not involve the canonical 4EHP-binding motif (YxxxxxxLΦ), it is possible that d4EHP interacts indirectly with Brat (see model, Figure 3.9). Although d4EHP plays a crucial role in defining the posterior boundary of the Hb domain, it is probably not the only protein that controls *hb* translation, as the distribution of Hb does not extend all the way to the posterior pole in *d4EHP* or *brat* mutants. However, it must be noted that neither the *d4EHP* nor the *brat* mutant alleles used in this study are complete loss-of-function alleles.

Our data strongly support a requirement for the 5' cap structure in regulation of the endogenous *hb* mRNA. An earlier study that assessed translation of NRE-containing mRNAs after injection into *Drosophila* embryos supports the conclusion that the cap structure is functionally significant (Chagnovich and Lehmann, 2001). Another study reported that Nos and Pum are able to repress expression of an engineered transgene containing an internal ribosome entry site (IRES) and a hairpin loop designed to block cap-dependent translation (Wharton et al., 1998). These results were used to conclude that translational repression of *hb* is cap-independent. However, the phenotypic assay used in that study was indirect. Nos-dependent deadenylation has also been shown to be important in establishing the Hb gradient (Wreden et al., 1997). To reconcile all these data, we propose that in the more posterior regions of the *Drosophila* embryo where Nos is abundant, translational silencing might predominantly

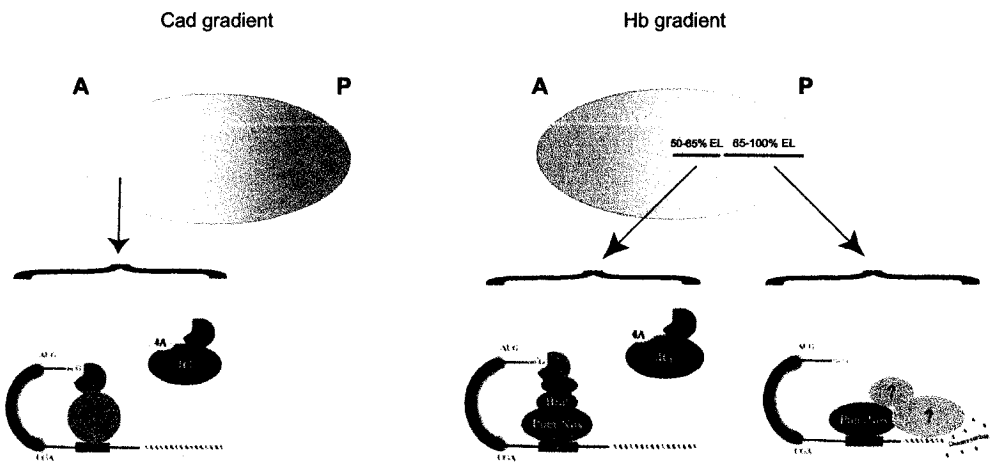

Figure 3.9 Model of the d4EHP:Brat translational inhibitory complex.
Hb expression is regulated by two distinct translational inhibitory mechanisms. In regions of the embryo where Nos is abundant, Hb translation silencing could predominantly occur via deadenylation. In the central region where Nos is at threshold concentrations, d4EHP activity controls the precise expression of Hb. In a manner analogous to that proposed for cad, d4EHP binds directly to the cap structure to recruit the NRE-complex (via Brat) and inhibits hb mRNA translation. By preventing eIF4F from binding to the cap, the d4EHP:Brat interaction renders the mRNA translationally inactive.

occurs via deadenylation, which may not be a cap-dependent process (Chagnovich and Lehmann, 2001). However, in the central region where Nos is at threshold concentrations, d4EHP activity governs the precise expression of Hb (see model, Figure 3.9).

The emerging new concept of a common inhibitory mechanism which regulates *cad* and *hb* mRNA translation simplifies our understanding of how the anterior-posterior axis is organized during early *Drosophila* embryogenesis. By regulating two classical maternal morphogenetic gradients, d4EHP plays a critical role in early *Drosophila* embryo development. Since d4EHP and some of its interacting partners are evolutionarily conserved in higher eukaryotes and because cap-dependent translation regulation plays such an important role in eukaryotic gene expression (Richter and Sonenberg, 2005), we predict that the 4EHP-dependent translational inhibitory mechanism is widespread throughout the animal kingdom.

3.4 Experimental Procedures

3.4.1 Plasmids

Cloning of *d4EHP* was described in chapter 2. Brat cDNA (RE16276; Research Genetics) was obtained from the Berkeley *Drosophila* Genome Project (Rubin et al., 2000). All constructs reported herein were produced using the polymerase chain reaction (PCR). For *d4EHP/deIF4EI* chimera and *brat*, PCR-amplified wild-type and mutant cDNAs were introduced into the pcDNA3-3HA and pcDNA3-N-term-FLAG vectors using *EcoRI/XhoI* and *EcoR*V/*Not*I sites, respectively. For recombinant protein expression, Brat NHL domain and Nos C-term domain (Nos C-term) were subcloned into the pProEx-His vector using *SalI/Not*I and *EcoRI/Xho*I sites, respectively, and Pum RNA-binding domain (Pum RNAB) into the pGEX 6p-1 vector using *EcoRI/SalI* sites. NRE from *hb* mRNA, flanked by *Xba*I sites, was introduced into the 3'UTR of pcDNA3-rLuc-Δ*Apa*I reporter vector. To create pCaSpeR4-*nos* promoter-*Brat* wild-type and mutant rescue vectors, Brat constructs were inserted into the pKS-*nos* promoter vector using *NheI/Not*I sites. Subsequently, a *Kpn*1/*Not*I cassette from pKS-*nos* promoter-*Brat* wild-type and mutant vectors were transferred into the pCaSpeR4 vector. All inserts were fully sequenced.

3.4.2 Recombinant Protein Purification

E. coli BL21(DE3) transformed with the pProEx-Brat NHL domain, pProEx-Nos C-term and pGEX-Pum RNAB constructs were used to produce His-Brat NHL domain, His-Nos C-term and GST-Pum RNAB fusion proteins as previously described. TALON™ Metal Affinity resin (BD Bioscience) and Glutathione Sepharose™ 4B resin (Amersham Pharmacia) were used according to the manufacturer's instructions.

3.4.3 Anti-Brat antibody and Western Blotting Analysis

An anti-Brat antibody (#3187) was raised in a New Zealand White rabbit injected with recombinant His-Brat NHL domain protein and used for Western blotting (1:3000). Cell culture, coimmunoprecipitation and Western blotting was performed as previously described.

3.4.4 Transgenic rescue experiment

Transgenic flies were generated by P-element mediated germline transformation of *yw* recipients using pCaSpeR-*nos* promoter-*Brat* wild-type and mutant rescue vectors. Transformed *brat* lines were crossed to the $brat^{fs1}$ mutant and tested for the rescue of mutant phenotypes. pUASp-*d4EHP* transgenic lines and antibody staining were performed as previously described (Kobayashi et al., 1999). Hb, and Nos, Pum and Brat immunostainings were visualized using AlexaFluor® 546 goat anti-rat IgG secondary and AlexaFluor® 488 goat anti-rabbit IgG secondary, respectively (1:500; Molecular Probes) using confocal laser scanning microscope. Embryo images were analyzed for Hb gradient using Zeiss LSM data acquisition software.

3.4.5 In vitro transcription/translation and binding assay

pcDNA3-3HA-d4EHP and pcDNA3-rLuc-Δ*Apa*I-NRE vectors were linearized with *Apa*I and transcribed using T7 RNA polymerase (MBI). Nuclease treated rabbit reticulocyte lysate (Promega) was incubated for 1h at 30°C with 300ng of HA-d4EHP mRNA. Subsequently, the extract was supplemented with components of the NRE-complex and the experiments of Figure 3.7 were performed as previously described (Sonoda and Wharton, 1999; Sonoda and Wharton, 2001).

3.5 Acknowledgments

We thank P. Macdonald (anti-Hb), N. Dostatny (pKS-*nos*-X-Bcd 3'UTR vector) and Z. Lev (*brat*^{fs1}) for reagents; J. Laliberte for confocal microscopy; Y. R. Lee, H. Beili and C. Lister for technical assistance; F. Poulin, M. Bidinosti, J. Chicoine, M. Ferraiuolo, B. Raught, A-C. Gingras, L. Huck, K. Madjar and other members of our labs for valuable discussions, and support during the course of this study. Y.A.C-P. was supported by the Canadian Institute of Health Research (CIHR) Burroughs Wellcome scholarship to medical students. N.S. is a CIHR Distinguished Scientist and a Howard Hughes Medical Institute (HHMI) International Scholar. This work was supported by grants from the CIHR to N.S. and P.L, and by a grant from the NIH (#5R01 GM66157-03) to N.S.

CHAPTER 4 - Conclusion

4.1 *General Discussion*

As described in this thesis, d4EHP functions as a translational inhibitor. However, unlike other translational repressors such as 4E-BPs, which act to repress overall translational output, d4EHP inhibits the cap-dependent expression of a specific transcript during early embryogenesis via a novel mechanism. Through a series of experiments, we have shown in this thesis that the unique spatio-temporal feature of the d4EHP-dependent translational control mechanism is provided by a coordinated protein-protein interaction between d4EHP and *trans*-acting RNA-binding regulatory proteins that contain the positional information. Furthermore, we have here provided substantial evidences to support the idea that the mRNA circularization plays an important role in regulating eukaryotic gene expression. As such, while chapter 2 of this thesis provides a thorough biochemical description of d4EHP, whose interaction with the anterior determinant Bcd leads to the inhibition of the anterior *cad* mRNA expression, chapter 3 describes the mechanism by which d4EHP interacts with Brat, a component of the NRE-complex, to repress, albeit partially, the translation of the posterior *hb* mRNA. The key feature of the d4EHP-dependent translational inhibitory mechanism is the ability of d4EHP to directly compete with eIF4E to bind to the mRNA 5' cap structure. Hence, unlike other translational repression mechanisms that recruit the eIF4E:Cap complex by directly competing with eIF4G for eIF4E-binding, the competition that underlies the d4EHP-dependent translational inhibition occurs at the level of the cap-recognition step. In support of this notion, when compared to wild-type, we have observed a substantial increase in ribosome recruitment to *cad* mRNA in $d4EHP^{CP53}$ mutant embryo extracts (Appendix 1).

Embryo development in eukaryotes requires the activity of various maternal determinants, whose spatial and temporal expressions are strongly regulated (Johnstone

and Lasko, 2001; St Johnston and Nusslein-Volhard, 1992; Wickens et al., 2000). For instance, in the case of Hb, it was recently shown that while other morphogen gradients such as Bcd show embryo-to-embryo variability, Hb gradient displays high reproducibility and is defined more precisely than the size of one nucleus at its posterior most expression domain (Houchmandzadeh et al., 2002). Indeed, Hb is so tightly regulated that even changes in external developmental cue such as temperature fail to affect it (Houchmandzadeh et al., 2002). As we have demonstrated in this thesis, by regulating the expression of *cad* and *hb* mRNAs, d4EHP plays a central role in early *Drosophila* embryo development; when maternal Cad and Hb are allowed to accumulate inappropriately in the anterior and posterior half of an embryo, as a result of reduced d4EHP expression, anterior-posterior axis patterning gets disrupted. This is consistent with an earlier report that showed that ectopic Cad expression at the anterior end of an embryo results in a partial failure of head involution and segmentation (Mlodzik et al., 1990). Similarly, persistence of Hb at the posterior end of an embryo was also shown to affect abdominal segmentation (Hulskamp et al., 1989; Irish et al., 1989; Lehmann and Nusslein-Volhard, 1987; Struhl et al., 1992; Tautz, 1988; Wharton and Struhl, 1991). The observed *cad* and *hb* phenotypes in various early mutant embryos are reflected in an altered distribution of the segmentation gene *fushi tarazu* (*ftz*)(Hulskamp et al., 1989; Macdonald and Struhl, 1986; Mlodzik et al., 1990). In support of our proposed model, and highlighting the critical role of d4EHP in regulating the translation of both *cad* and *hb* mRNAs, we also observed a significant alteration in the pattern of *ftz* mRNA distribution in d4EHP^{CP53} mutant embryos (unpublished observation). Therefore, we conclude that any changes that affect the expression of d4EHP, and consequently the precision of Cad and Hb expression boundaries, will result in an alteration of the embryonic fate map and prevent further

development from taking place.

In conclusion, d4EHP is a cap-binding inhibitor of translation and an important developmental factor that plays a key role in establishing the anterior-posterior axis polarity in early *Drosophila* embryo. Through our study of d4EHP and the novel inhibitory mechanism it mediates, we have gained an important understanding of how certain mRNA 3'UTR translational control elements communicate with the 5' end to regulate gene expression in eukaryotes.

4.2 *Future Directions*

Whether a d4EHP-like translational inhibitory mechanism exists in higher eukaryotes remains unknown. However, to further understand the implication of this novel translational regulatory mechanism in human, we used the information we have presented in this thesis to identify two promising candidates that may undergo similar control.

4.2.1 *hThrRS is a novel h4EHP interacting protein*

E.coli Threonyl-tRNA synthetase (ThrRS) encoded by the *thrS* gene, is a homodimeric enzyme that aminoacylates $tRNA^{Thr}$. In addition, ThrRS has the ability to bind to its own mRNA, immediately upstream of the initiator AUG, to inhibit its own synthesis (Romby and Springer, 2003). ThrRS does so by recognizing two stem-loop structures in the 5'UTR of ThrRS mRNA, which mimic the anticodon arm of *E.coli* $tRNA^{Thr}$ (Jenner et al., 2005; Romby et al., 1996; Torres-Larios et al., 2002). Since the two stem-loop structures are found in proximity to the ribosome binding site, their binding to ThrRS prevent ribosome recruitment (Jenner et al., 2005).

In collaboration with Drs. Wongi Seol and Sung-Hoon Kim at Seoul National

University (Seoul, South Korea), we have recently identified h4EHP in a yeast two-hybrid screen, using the human homolog of ThrRS (hThrRS) as bait (Seol W and Kim SH, personal communications). In order to identify the residue important for the interaction and to show that the interaction is direct, purified recombinant h4EHP protein containing the phosphorylation site for the heart muscle kinase (HMK) was labeled to high specific activity and used as a "Far-Western" probe on a membrane that was transferred with different GST-tagged mutants of hThrRS. As shown in Appendix 2A, whereas the wild-type, and NΔ16 and NΔ35 N-terminal deletion mutants of hThrRS interact strongly with h4EHP (Appendix 2A, lanes 1-3), NΔ52 and NΔ69 deletion mutants of hThrRS abrogated the h4EHP:hThrRS interaction (lanes 4-5). This is most probably due to the presence of a putative YxYxxxxLΦ 4EHP-binding motif in the N-terminal appendage of hThrRS, between residues 41 to 49 (Appendix 2B). To further delineate the binding site, mutants of hThrRS that carries mutations in the putative 4EHP-binding motif were created and tested via Far-Western (Appendix 2A). Similar to Bcd, we have discovered that the tyrosine residue at -2 position and the methionine-tyrosine residues at +5/6 position of the hThrRS putative 4EHP-binding motif are critical for the interaction, since their replacement to alanine significantly abrogated the interaction (Appendix 2A, lanes 6 and 8). Surprisingly, however, unlike Bcd, the second tyrosine at -0 position of hThrRS motif is also found to be important for the interaction (lane 7). Whether the difference in binding motif for h4EHP versus d4EHP would result in different function for h4EHP still remains unknown. Moreover, further analysis will be required to understand the role of the h4EHP:hThrRS interaction. In summary, we have here demonstrated that h4EHP directly interacts with hThrRS. The interaction is mediated by a putative 4EHP-binding motif that is found in the N-terminal appendage of hThrRS.

4.2.2 *miRNA-mediated Translation Repression and h4EHP*

While there are numerous examples of translational regulation that depend on sequence elements found in the mRNA 3'UTR, the molecular mechanism through which they interact with the 5' end of an mRNA and block protein synthesis still remains unknown. Since, d4EHP functions as a cap-binding protein that interacts with various 3'UTR regulatory mRNP complexes to inhibit translation, h4EHP may be the missing puzzle in some of these inhibitory mechanisms.

Translational control by small noncoding RNAs called micro RNA (miRNA) may be dependent on the activity of h4EHP. miRNAs are single stranded RNAs of ~ 22 nucleotides in length that are generated from endogenous hairpin-shaped transcripts (Kim, 2005; Zamore and Haley, 2005). RNA-RNA duplex, formed as a result of a miRNA-3'UTR target sequence binding, is central to the miRNA-dependent translational repression mechanism. Specifically, in human, the short RNA-duplex is known to recruit a family of proteins called Argonaute (Ago), whose Piwi domain contain an RNase H like endonuclease that is used to cleave target RNAs (Kim, 2005; Zamore and Haley, 2005). Interestingly, when miRNAs pair only partially with their targets, they cannot direct mRNA cleavage. Instead, they block translation at the initiation step, in cap-dependent manner (Doench et al., 2003; Humphreys et al., 2005; Pillai et al., 2005). Subsequently, it was proposed that as a consequence of the miRNA-directed translation inhibition, target mRNAs are localized to the site of mRNA degradation and storage called "P-bodies" (Coller and Parker, 2004; Ferraiuolo et al., 2005; Liu et al., 2005; Sen and Blau, 2005; Sheth and Parker, 2003).

What would be the role of h4EHP in miRNA-dependent translation repression? Although much remains unknown, there are several evidences that point toward a role

of h4EHP in miRNA-induced translational control of gene expression. To begin with, we reasoned that since the cap structure is present in all nuclear transcribed mRNA, to achieve the kind of specificity that is demanded of the miRNA-induced translation inhibition mechanism, h4EHP must first associate with a known component of the microribonucleoproteins (miRNPs) complex. Indeed, in collaboration with Dr. Witold Filipowicz at the Friedrich Miescher Institute for Biomedical Research (Basel, Switzerland), we have recently discovered that h4EHP interacts with the human Argonaute proteins (hAgos; Yoshida M, personal communications). This interaction is of particular importance, since hAgos, an established component of miRNPs and the RNAi induced silencing complex (RISC), when tethered to an mRNA reporter, are able to mimic the repressive effect of miRNAs in HeLa cells (Pillai et al., 2004; Pillai et al., 2005). In addition, it was recently demonstrated by Mr. Kfir Madjar, a graduate student in the Sonenberg lab, that h4EHP also interacts with a protein called 4E-Transporter (4E-T; Madjar K, personal communications). 4E-T is an eIF4E-binding protein that was recently shown to localize to P-bodies and was shown to be important for its formation (Ferraiuolo et al., 2005). In support of the above data, we also observed that when the h4EHP protein level is reduced by RNA interference (RNAi), the number of cytoplasmic P-bodies increased dramatically (Ferraiuolo M, personal communications). Since P-bodies are functionally linked to miRNA (Liu et al., 2005; Sen and Blau, 2005), the effect of h4EHP knock-down by RNAi on P-body biogenesis and the h4EHP:4E-T interaction provide yet another series of evidences to support the idea that h4EHP may be an important component of the miRNPs. Therefore, similar to the d4EHP-repression model we have outlined in this thesis, we propose that h4EHP functions to bridge the 5' cap structure and the miRNA-binding sequence elements in the mRNA 3'UTR via mRNA circularization to inhibit the translation of specific transcripts.

4.3 *Conclusion*

Although we have here annotated d4EHP as a translational inhibitor that acts during early *Drosophila* embryo development, much work remains to be done to fully understand its role at the organism-level, at different developmental time-frame: what would be its function after embryogenesis? Furthermore, while it is likely that h4EHP also functions as a translational inhibitor, since human homologs of Bcd and Brat are lacking, the exact mechanism by which it will function as a translational repressor still remains to be answered. It is our hope that the next few years will bring us much insight into how 4EHP-dependent translational control integrates into higher level regulatory networks.

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ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

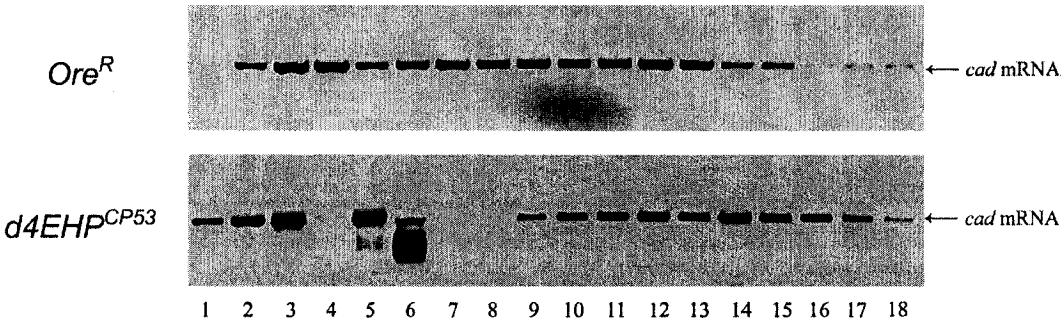
1) **Cloning and characterization of d4EHP.** d4EHP is a highly conserved cap-binding protein. However, despite its homology to deIF4E, d4EHP does not interact with any of the known eIF4E-binding proteins such as d4E-BP and deIF4G.

2) **d4EHP functions as a cap-binding inhibitor of translation.** It inhibits the translation of specific transcripts by interacting with various regulatory proteins such as Bcd and Brat (as part of the NRE-complex), and with the cap structure. Therefore, the d4EHP-dependent translational repression mechanism occurs via mRNA circularization.

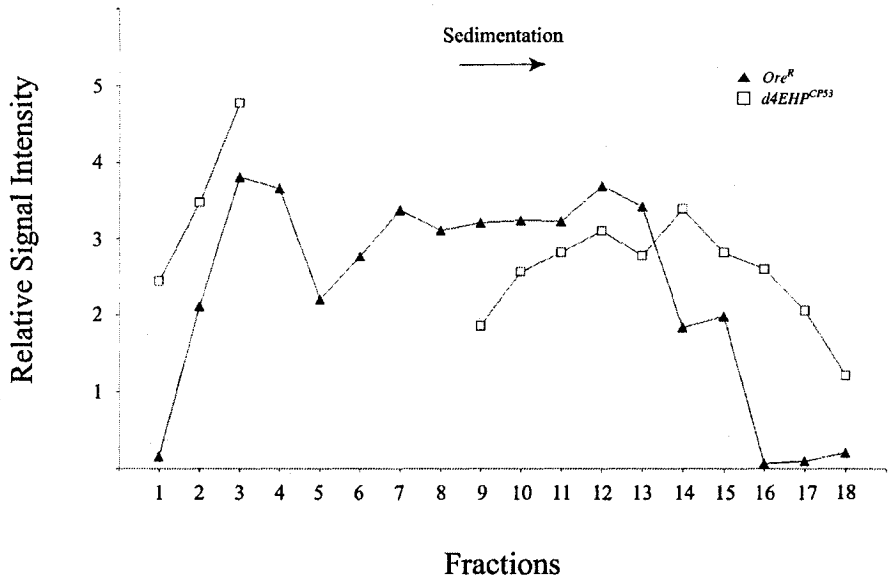
3) **d4EHP is an important developmental factor.** By regulating the synthesis of Cad and Hb at the anterior and posterior end of an embryo, respectively, d4EHP plays a key role in establishing the anterior-posterior axis polarity in *Drosophila* embryo.

4) We have here demonstrated that while the 4EHP-binding motif (YxYxxxxLΦ) is highly similar to that of eIF4E-recognition motif (YxxxxLΦ), it is functionally divergent. Furthermore, since the motif is found in all 4EHP-binding proteins known to date, it can readily be used in bioinformatic screens to identify additional 4EHP interacting proteins in various organisms.

A



B

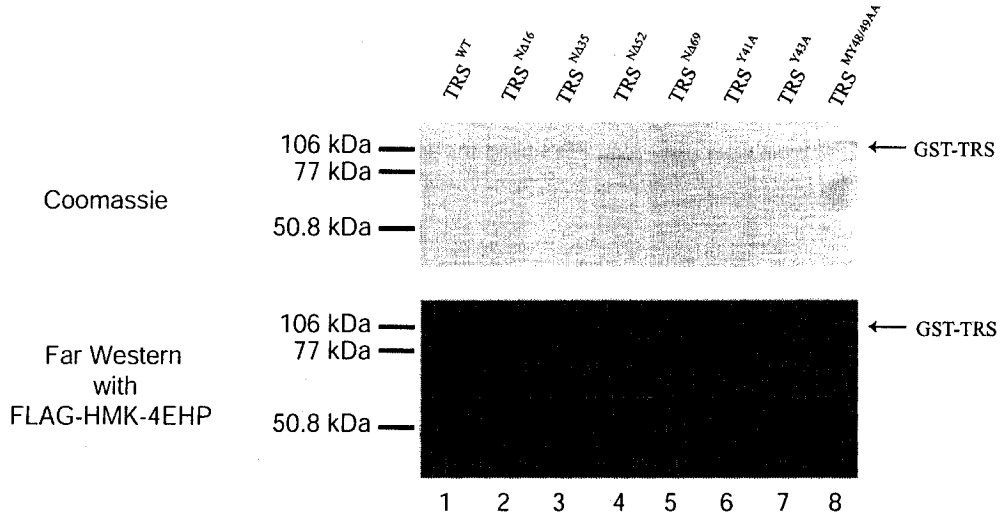


Appendix 2: Increased ribosomal recruitment to *cad* mRNA in *d4EHP^{CP53}* embryos

A. Wild-type (*Ore^R*) and *d4EHP^{CP53}* embryo extracts were subjected to sucrose gradient sedimentation, and fractions were analyzed by RT-PCR of *cad* mRNA. Sucrose gradient sedimentation was preformed as described (Clark et al., 2000), except for the following modifications: 14ml of 10-50% sucrose gradient containing 20mM Hepes, pH 7.5, 5mM $MgCl_2$ and 100mM KCl was used.

B. Quantitation of *cad* mRNA signal observed in (A). Some of the RNA samples used for detecting *cad* mRNA were fully or partially degraded during manipulation (lanes 4 and 6-8 of *d4EHP^{CP53}*).

A



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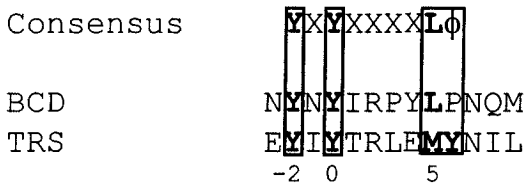

Appendix 2 hThrRS contains a 4EHP binding motif

A. Far-Western analysis of the h4EHP:hThrRS interaction. Purified recombinant h4EHP protein containing the phosphorylation site for the heart muscle kinase (HMK) was labeled to high specific activity and used to probe a membrane that was transferred with different GST-tagged mutants of hThrRS.

B. Alignment of 4EHP binding motifs from Bcd with hThrRS amino acids 41 to 49.
ϕ denotes any hydrophobic amino acid and X any amino acid.