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A New Paradigm for Translational Control: Inhibition via 5′-3′ mRNA Tethering by Bicoid and the eIF4E Cognate 4EHP

Park F. Cho,[1] Francis Poulin,[1,4,*] Yoon Andrew Cho-Park,[1,3] Ian B. Cho-Park,[1,3] Jarred D. Chicoine,[2] Paul Lasko,[2] and Nahum Sonenberg[1,*]

[1]Department of Biochemistry
McGill Cancer Center
McGill University
3655 Promenade Sir William Osler
Montréal, Québec H3G 1Y6
Canada

[2]Department of Biology
McGill University
1205 Avenue Dr. Penfield
Montréal, Québec H3A 1B1
Canada

Summary

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, simultaneous interactions of d4EHP with the cap structure and of Bcd 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.

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 multistep 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.

*Correspondence: fpoulin@berkeley.edu (F.P.); nahum.sonenberg@mcgill.ca (N.S.)

[3]These authors contributed equally to this work.

[4]Present address: Department of Integrative Biology, University of California, Berkeley, Berkeley, California 94720.

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.

Results

d4EHP Is a Cap Binding Protein

4EHP is evolutionarily conserved in metazoans and plants (Figure 1A). The *d4EHP* gene (GenBank: NM_176552; Gadfly: CG33100) encodes a 223 amino acid

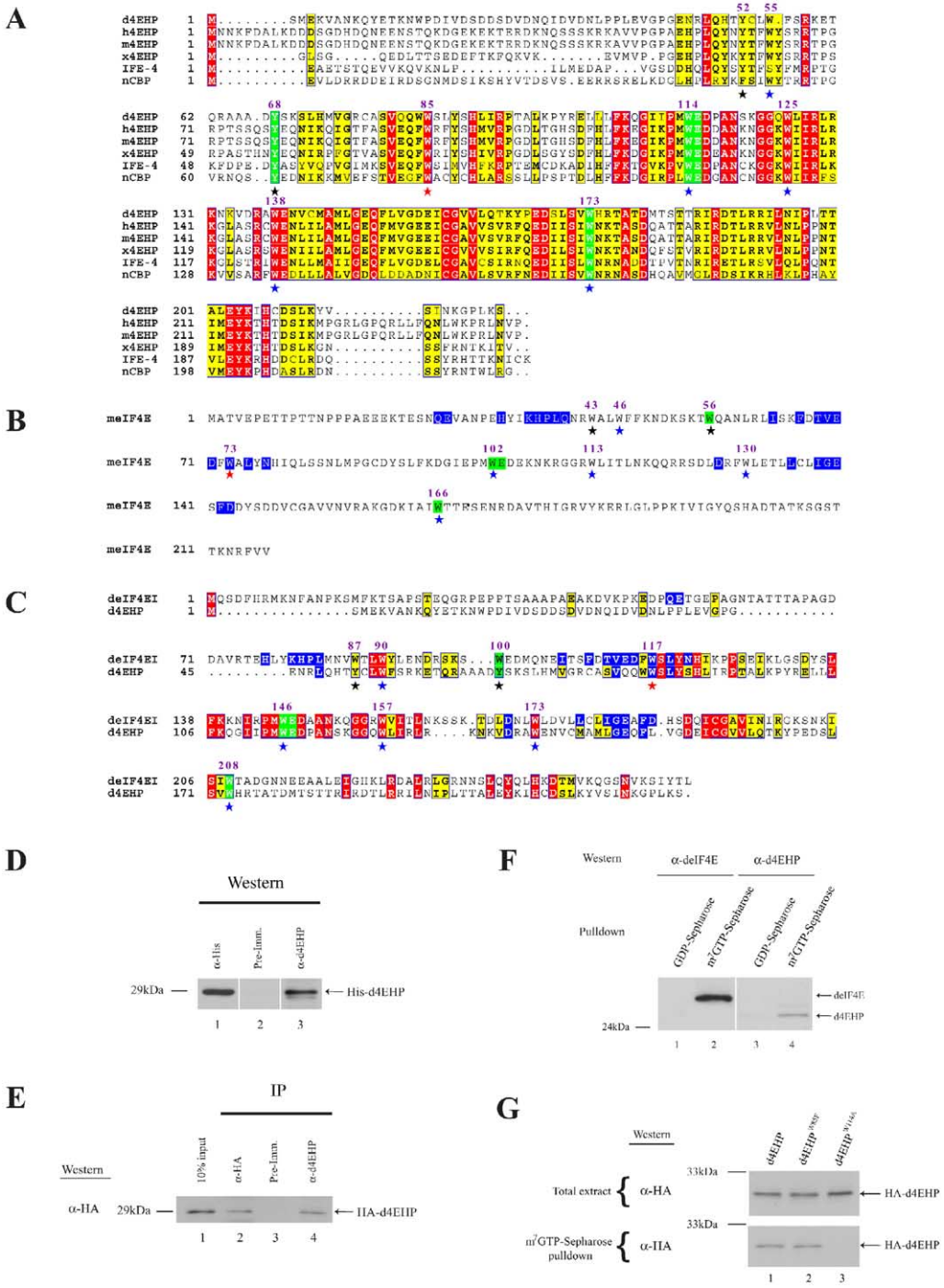


Figure 1. 4EHP Is an Evolutionarily Conserved Cap Binding Protein

(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 that 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.

(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.

(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 preimmune 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), preimmune serum (lane 3), or d4EHP antiserum (lane 4).

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 conserved in d4EHP (Figures 1A–1C), although two of eight conserved tryptophan residues in eIF4E are replaced by tyrosines in 4EHP (Figures 1A–1C). While one of these residues, Trp56 in eIF4E (Tyr68 in d4EHP; Figures 1A and 1B), 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 1D). The specificity of the antiserum was established by immunoprecipitation of HA-tagged d4EHP from transfected 293 cells (Figure 1E).

Like deIF4E, d4EHP binds to m^7GTP-Sepharose, but not to GDP-Sepharose (Figure 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 1G). Mutation of d4EHP Trp85, a residue to be discussed later in this report, does not affect cap binding (Figure 1G).

d4EHP Genetically Interacts with *cad*

d4EHP is uniformly distributed in early *Drosophila* embryos (see Supplemental Figure S1A available with this article online). To investigate its biological function, we produced mutants by imprecise excision of a P element inserted within the first exon of *d4EHP* (BG017013; Figure 2A; 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 2A). The resulting mutant is hypomorphic, as immunoblotting (Figure 2B) and immunostaining (Supplemental Figure S1B) detected the presence of small amounts of d4EHP in mutant embryo (7%, relative to wild-type; Figure 2B). This is most probably due to the presence of an in-frame AUG (AUG*) at the end of the first intron (Figure 2A) 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 2C, 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 2D). Expression of *syntaxin 1A* (*syx1A*), located in the second intron of *d4EHP* (Figure 2A), is not affected by the $d4EHP^{CP53}$ mutation (Supplemental Figure S2).

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 (Supplemental Figure S3).

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 2E), those from mothers homozygous for $d4EHP^{CP53}$ (subsequently termed $d4EHP^{CP53}$ mutant embryos) show Cad ectopically expressed at the anterior end (Figure 2H; 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 in $d4EHP^{CP53}$ mutant embryos (Figure 2I). In addition, *cad* mRNA expression levels (Supplemental Figure S4A) and localization (Figure 2J) 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.

d4EHP Interacts Biochemically with the Anterior Determinant Bcd

Next, we investigated whether d4EHP and Bcd interact in vivo. Extracts prepared from 0–2 hr wild-type embryos were treated with RNase and used to examine the interaction between Bcd and deIF4E or d4EHP (Figure 3A). Preimmune sera failed to precipitate deIF4E or d4EHP. Anti-deIF4E immunoprecipitated deIF4E, but not Bcd. In contrast, anti-d4EHP readily coimmunoprecipitated 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, $Bcd^{1\text{-}489}$ and $Bcd^{1\text{-}494}$, contain the homeobox domain that is critical for inhibiting anterior *cad* translation (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). To determine whether $Bcd^{1\text{-}489}$ and $Bcd^{1\text{-}494}$ differ in their ability to interact with d4EHP, HA-tagged deIF4EI (as a negative control) or d4EHP was transfected into 293 cells with each of the two FLAG-tagged Bcd variants. Immunoprecipitation with an anti-FLAG antibody

(F) d4EHP is a cap binding protein. Cap binding proteins were affinity purified from *Drosophila* S2 cell extracts using m^7GTP-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) containing transfected HA-tagged d4EHP (lane 1), $d4EHP^{W85F}$ (lane 2), and $d4EHP^{W114A}$ (lane 3) were incubated with m^7GTP-Sepharose, and the eluate was analyzed by Western blotting (bottom).

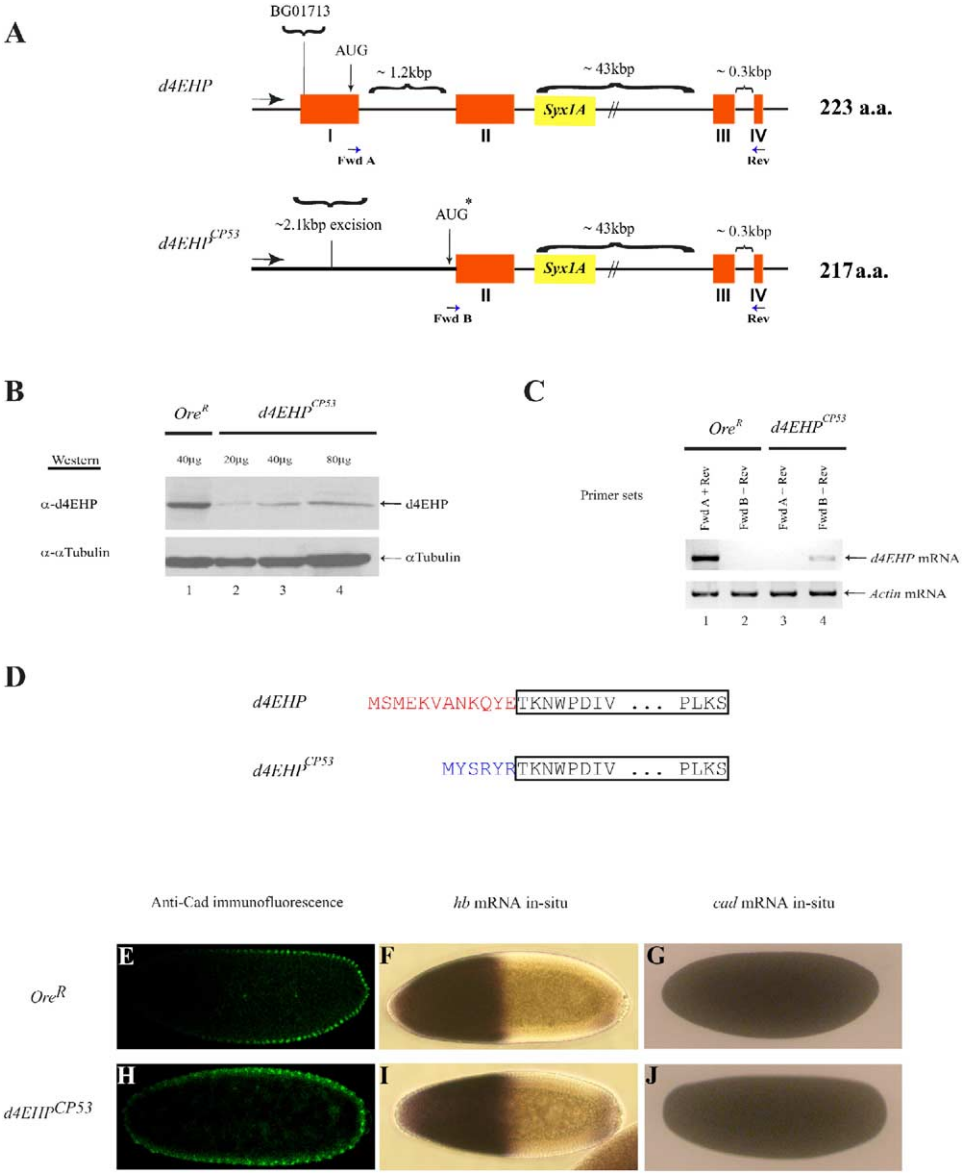


Figure 2. Characterization of *d4EHP*^{CP53} Mutant

(A) Schematic representation of the *d4EHP* gene. The *d4EHP* gene spans ~45 Kbp 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 *d4EHP*^{CP53} is an alternative translation start site that becomes active upon excision of exon I and part of intron I (*d4EHP*^{CP53} 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 *d4EHP*^{CP53} mutant. Wild-type (*Ore*^{R}) and *d4EHP*^{CP53} embryo extracts were analyzed by Western blotting using anti-d4EHP and anti-α-tubulin 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 *d4EHP*^{CP53} 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) *Ore*^{R} embryo displays wild-type Cad gradient (E), zygotic *hb* transcription (F), and *cad* distribution (G).

(H–J) 0–2 hr *d4EHP*^{CP53} 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 absence of surface nuclei that are evident in embryo images presented in Figures 4 and 6. Orientation of embryos is anterior left and dorsal up.

demonstrates that neither one of the Bcd spliced variants interacts detectably with deIF4EI, while both exhibit a comparable interaction with d4EHP (Figure 3B). We therefore used the Bcd$^{1\text{-}494}$ isoform for all subsequent experiments and will refer to it as Bcd for simplicity.

The similarity between 4EHP and eIF4E is not limited to the amino acids that participate in binding the 5′

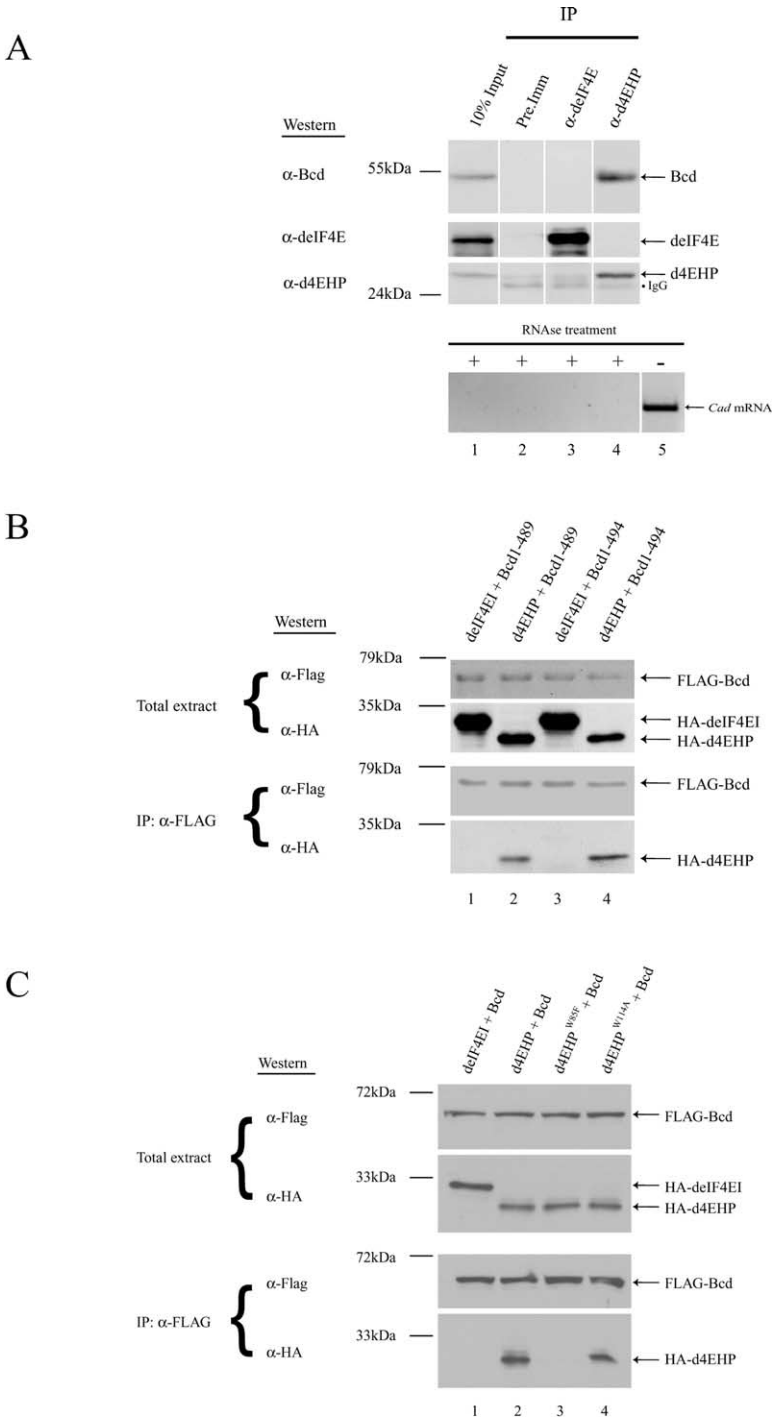


Figure 3. d4EHP Interacts with Bcd In Vivo and In Vitro

(A) d4EHP interaction with endogenous Bcd. 0–2 hr *Ore*^{R} embryo extract (lane 1) was immunoprecipitated (IP) using preimmune (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 Bcd$^{1-489}$ and Bcd$^{1-494}$ 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), d4EHP^{W85F} (lane 3), and d4EHP^{W114A} (lane 4). Extracts (top panels) were immunoprecipitated (IP) with anti-FLAG and analyzed by Western blotting (bottom panels).

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 (Figures 1B and 1C; 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 coimmunoprecipitated wild-type HA-d4EHP, but not HA-deIF4EI (Figure 3C). Mutation of Trp85 in d4EHP to Phe abrogated Bcd binding, while the W114A mutation, which affects cap binding, did not (Figure 3C). Taken together, these results de-

monstrate 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).

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 4A) 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 4B), $d4EHP^{CP53}$ embryos show Cad expression domains extending farther toward the anterior (Figure 4C). While transgene-derived expression of wild-type *d4EHP* ($d4EHP^{WT}$) rescued this effect (Figure 4D), the Bcd and cap binding mutants of d4EHP ($d4EHP^{W85F}$ and $d4EHP^{W114A}$, respectively) failed to establish a wild-type Cad gradient (Figures 4E and 4F), even though *cad* mRNA expression levels (Supplemental Figure S4A) and distribution (Supplemental Figure S4C–S4E) 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.

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 5A) 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 5B).

Transgenic fly lines carrying targeted *bcd* mutations were crossed into a *bcd* 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 5C). Consistent with our earlier results, endogenous Bcd coimmunoprecipitates 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

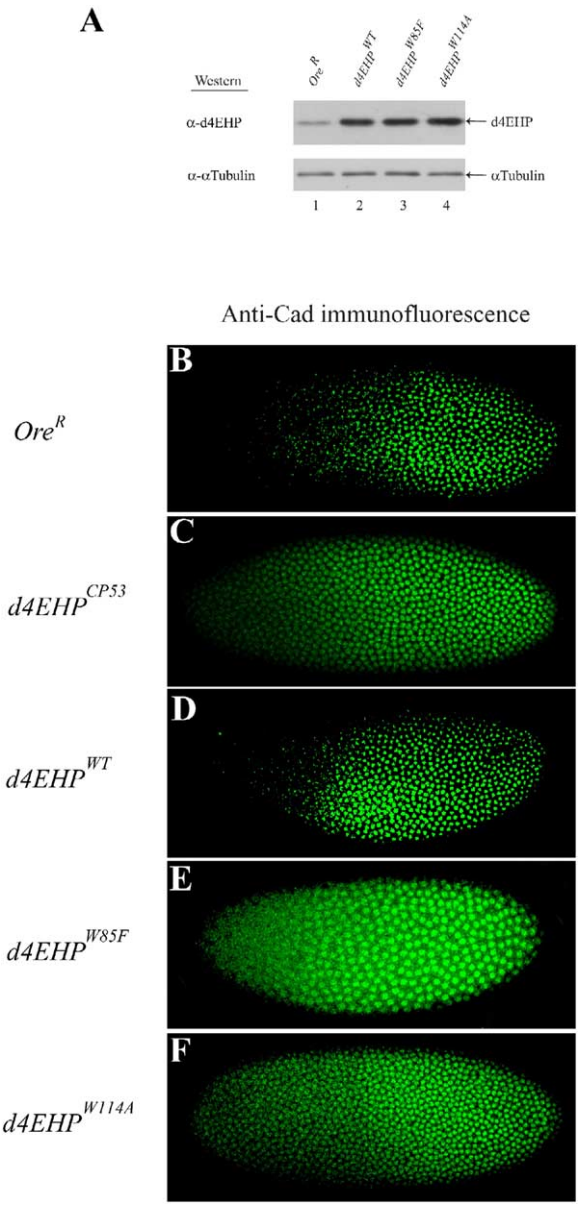


Figure 4. d4EHP Interaction with Bcd and the Cap Structure Is Crucial for *cad* mRNA Translation Inhibition

(A) Western blot analysis of d4EHP expression in transgenic embryos (top). Anti-α-tubulin was used as a loading control (bottom).
(B) Ore^R embryos display wild-type Cad gradient.
(C) $d4EHP^{CP53}$ mutant embryo display ectopic Cad expression at the anterior end.
(D) Expression of a $d4EHP^{WT}$ transgene rescues the $d4EHP^{CP53}$ mutant phenotype.
(E and F) Embryos from transgenic females expressing $d4EHP^{W85F}$ (E) and $d4EHP^{W114A}$ (F) in the $d4EHP^{CP53}$ mutant background show ectopic expression of Cad at the anterior end. Orientation of embryos is anterior left and dorsal up.

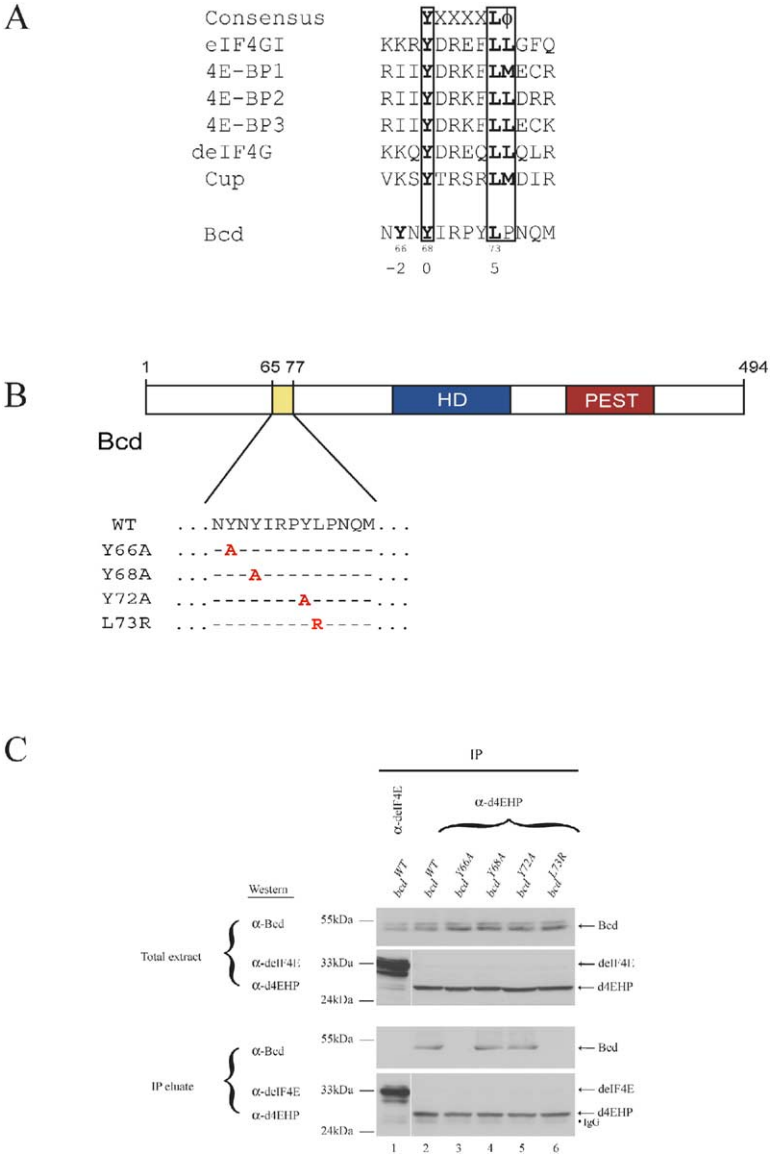


Figure 5. Bcd Contains a d4EHP Binding Motif

(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.

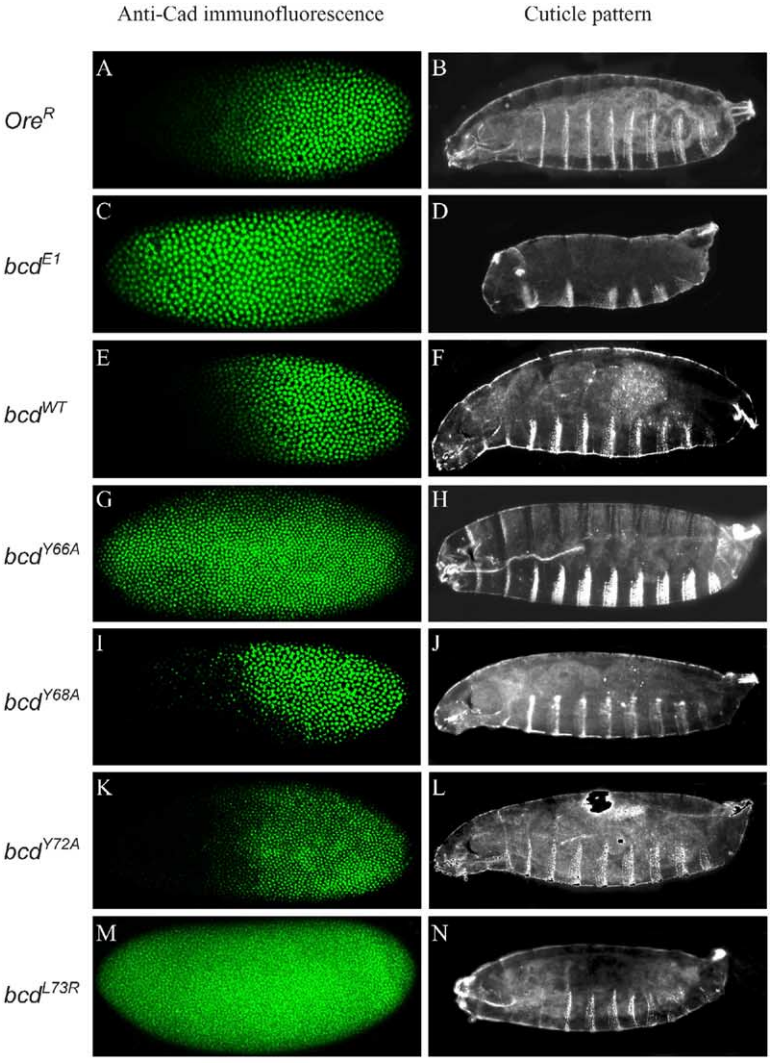
(C) In vivo interaction of Bcd mutants with d4EHP. 0–2 hr embryo extracts from females homozygous for the listed genotypes were immunoprecipitated (IP) with anti-deIF4E (lane 1) or anti-d4EHP (lanes 2–6). Eluted proteins were analyzed for the presence of Bcd, deIF4E, and d4EHP by Western blotting.

that of eIF4G or 4E-BP binding to eIF4E, requires a sequence motif that is distinct from the canonical YxxxxLϕ eIF4E-recognition motif.

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 (0–2 hr) show an anterior-to-posterior Cad gradient (Figure 6A) and normal cuticle segmentation pattern (Figure 6B), while in *bcd^{E1}* embryos, Cad is evenly distributed throughout the embryo (Figure 6C) and a *bcd* mutant cuticle pattern develops (Figure 6D; Driever and Nusslein-Volhard, 1988a). Transgene-derived expression of wild-type *bcd* (*bcd^{WT}*) rescued all mutant phenotypes associated with *bcd^{E1}* (Figures 6E and 6F). Embryos expressing forms of Bcd unaffected for d4EHP binding (*bcd^{Y68A}* or *bcd^{Y72A}*) exhibited both a normal Cad gradient (Figures 6I and 6K) and normal cuticle pattern (Figures 6J and 6L). In contrast, *bcd^{Y66A}* and *bcd^{L73R}* mutant embryos exhibit defects in anterior patterning (Figures 6H and 6N) and do not establish a Cad gradient (Figures 6G and 6M). 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 (Supplemental Figure S4A) and localization are normal in all the mutant embryos (Supplemental Figures S4F–S4K). Transgene-dependent Bcd expression levels were similar in all mutant transgenic lines (Figure 6O).

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% hatch-



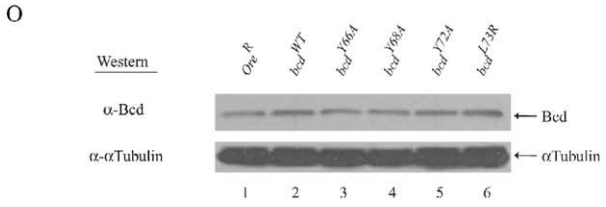


Figure 6. Functional Analysis of Mutant Bcd in Transgenic *Drosophila* Embryos

(A and B) *Ore*^{R} embryos display wild-type Cad gradient (A) and cuticle pattern (B).

(C and D) Embryos derived from homozygous *bcd*^{E1} 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 *bcd*^{WT} rescues the mutant Cad gradient (E) and cuticle pattern (F).

(G and H) Embryos derived from females expressing the mutant *bcd*^{Y66A} 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 *bcd*^{Y68A} gene demonstrate wild-type *cad* mRNA translation (I) and have normal cuticle pattern (J).

(K and L) Embryos derived from females expressing the mutant *bcd*^{Y72A} gene demonstrate wild-type Cad gradient (K) and have normal cuticle pattern (L).

(M and N) Embryos derived from females expressing the mutant *bcd*^{L73R} 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.

ing frequency, and all negative-control *bcd*^{E1} mutant embryos failed to hatch (Supplemental Table S1). Expression of *bcd*^{WT}, *bcd*^{Y68A}, and *bcd*^{Y72A} rescued the ability of *bcd*^{E1} embryos to hatch (Supplemental Table S1) 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 (Supplemental Table S1). 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.

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 antisense orientation in the 3′ UTR of the *Renilla reniformis* Luciferase (*rLuc*) reporter mRNA (Figure 7A). 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

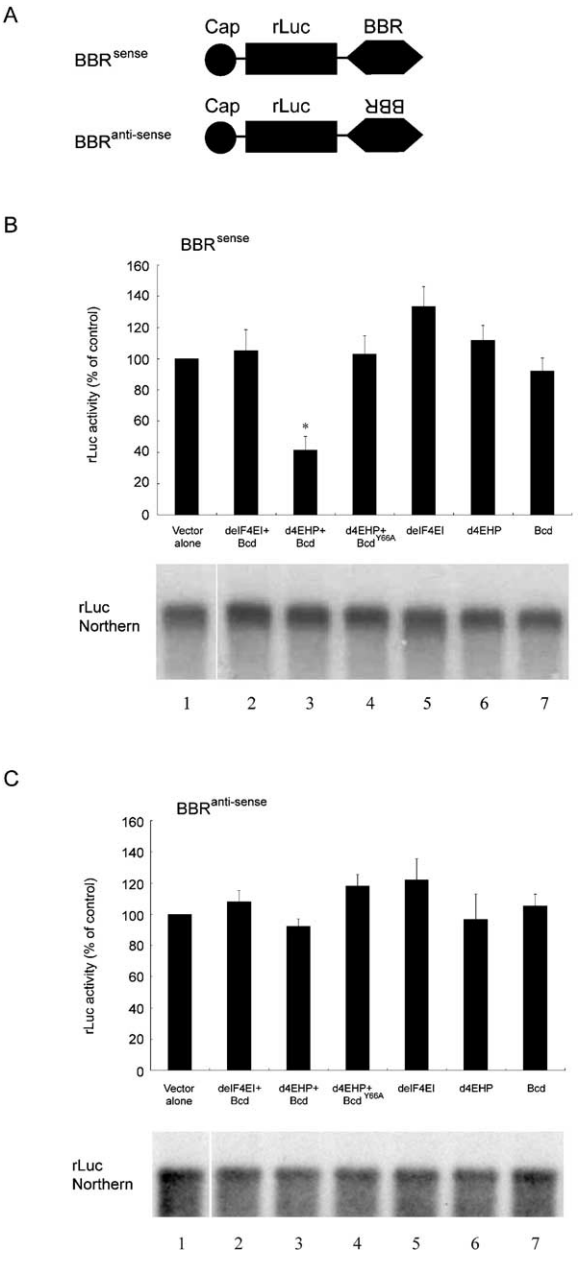


Figure 7. Bcd Specifically Represses Translation in the Presence of d4EHP

(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 (BBR^{sense}) or the antisense ($BBR^{antisense}$) orientation.

(B and C) In vitro translation of BBR^{sense} and $BBR^{antisense}$ reporters. In vitro translation of the BBR^{sense} (B) and the $BBR^{antisense}$ (C) reporter mRNAs (lane 1) was performed in the presence of deIF4EI and wild-type Bcd (lane 2), d4EHP and wild-type Bcd (lane 3), d4EHP and Bcd Y66A (lane 4), deIF4EI (lane 5), d4EHP (lane 6), and wild-type Bcd (lane 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 1 hr. Following this preincubation period, the extract was programmed with the reporter mRNA and incubated for an additional hour, and rLuc activity was then measured (Figures 7B and 7C). We first determined whether Bcd or d4EHP individually affect the translation of the BBR^{sense} and the $BBR^{antisense}$ 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 (Figures 7B and 7C, bottom panels).

Discussion

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 prebound 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).

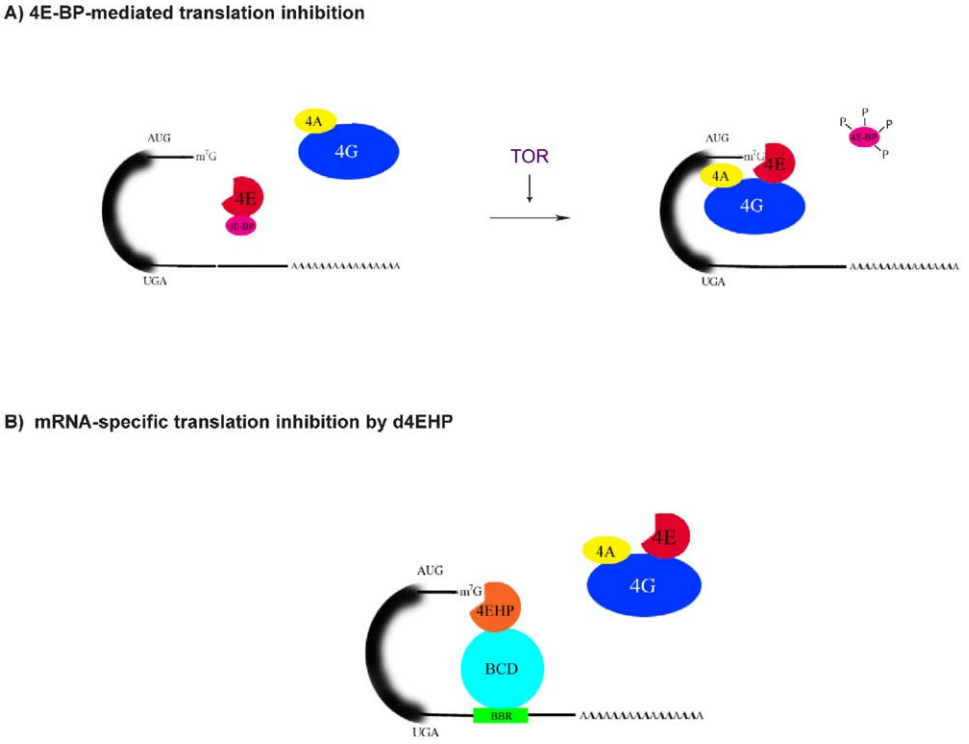


Figure 8. d4EHP:Bcd Translation Repression Model

(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.

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 8A). 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 8B). 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 germline development, based on our results and on a recent report that a mutant allele of *C. elegans 4EHP* (*ife-4*) shows a severe egg-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 5A) 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 1C).

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.

Experimental Procedures

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 *Eco*RI 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 *BamH*I/*Eco*RI and *Eco*RI/*Xho*I sites, respectively. To create pUASP-***d4EHP construct*** rescue vectors, d4EHP constructs were inserted into the pUASP vector using *Kpn*I/*BamH*I 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 antisense ($BBR^{antisense}$) 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 *Nde*I/*BamH*I restriction sites. Subsequently, a *Kpn*I/*Not*I cassette from the pKS-*nos* promoter-***Bcd construct***-Bcd 3′UTR vectors were transferred into the pCaSpeR4 vector. All constructs were fully sequenced.

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 2 hr 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.

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 0.22 μm 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), or mouse monoclonal anti-Bcd (Bcd mab23 ATCC; 1:50). This was followed by a 1 hr 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).

Cell Culture

Cationic lipid reagent (20 μl of Lipofectamine and 30 μl of Plus; Invitrogen) was diluted in serum-free media (Opti-MEM; Invitrogen) for transfection in Human Embryonic Kidney 293 cells (100 mm dish). Following a 3 hr 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 36 hr following the addition of serum-containing media. The cells were then lysed by repeated freeze/thaw cycles in 600 μl of lysis buffer (20 mM HEPES-KOH [pH 7.6], 200 mM KCl, 0.5 mM EDTA, 10% glycerol, 1% Triton X-100 and Protease Inhibitor Cocktail [Complete; Roche]) that contains RNase A (50 μg/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 0–2 hr embryo extract were prepared as described above.

Cap-Affinity Assay

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

Coimmunoprecipitation

For coimmunoprecipitation, 293 cell extract (200 μl; 6–10 μg/μl) was brought up to 1 ml with the lysis buffer and precleared for 1 hr at 4°C with 25 μl of Protein A Sepharose. The supernatant was immunoprecipitated for 1 hr at 4°C with 25 μl of anti-FLAG M2-Affinity Gel (Sigma). The resin was washed twice with lysis buffer and once with lysis buffer containing 300 mM KCl. Immunoprecipitates were eluted in 2× Laemmli sample buffer. For anti-HA, anti-deIF4E, and anti-d4EHP immunoprecipitations, 25 μl of Protein A Sepharose were preincubated for 2 hr with anti-HA (3 μl), anti-deIF4E (5 μl), and anti-d4EHP (5 μl). The resin was washed three times with the lysis buffer prior to immunoprecipitation as described above. Embryo extract was used at a concentration of 12 μg/μl.

P Element Excision and Transgenic Rescue Experiment

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.

RT-PCR

Total RNA was isolated from embryos using the RNAeasy kit (Qiagen) and then used to analyze various mRNAs by RT-PCR (for primer sequences, see Supplemental Table S2) using the One Step RT-PCR kit (Qiagen) according to the manufacturer's instructions.

In Vitro Transcription and Translation Assay

Plasmids were linearized with *Apa*I and transcribed using T7 RNA polymerase (MBI). Capped mRNA synthesis was performed using the RiboMAX system (Promega). Krebs-2 cell extract (12.5 μl) was incubated for 1 hr at 30°C with 300 ng of capped-mRNA encoding for individual proteins assayed herein. The extracts were subsequently programmed with 15 ng of the reporter mRNA (capped-$rLuc\text{-}BBR^{sense}$ or capped-$rLuc\text{-}BBR^{antisense}$) and incubated for an additional hour. Aliquots (2 μl) were assayed for luciferase activity using the Dual-Luciferase reporter assay system (Promega) in a Lumat LB 9507 bioluminometer (Berthold Technologies). ^{35}S-methionine labeling was performed as previously described (Brasey et al., 2003).

Supplemental Data

Supplemental Data include four figures and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/121/3/411/DC1/.

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References

Bellen, H.J., Levis, R.W., Liao, G., He, Y., Carlson, J.W., Tsang, G., Evans-Holm, M., Hiesinger, P.R., Schulze, K.L., Rubin, G.M., et al. (2004). The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. Genetics *167*, 761–781.

Brasey, A., Lopez-Lastra, M., Ohlmann, T., Beerens, N., Berkhout, B., Darlix, J.L., and Sonenberg, N. (2003). The leader of human immunodeficiency virus type 1 genomic RNA harbors an internal ribosome entry segment that is active during the G2/M phase of the cell cycle. J. Virol. *77*, 3939–3949.

Calero, G., Wilson, K.F., Ly, T., Rios-Steiner, J.L., Clardy, J.C., and Cerione, R.A. (2002). Structural basis of m7GpppG binding to the nuclear cap-binding protein complex. Nat. Struct. Biol. *9*, 912–917.

Chan, S.K., and Struhl, G. (1997). Sequence-specific RNA binding by bicoid. Nature *388*, 634.

Dinkova, T.D., Keiper, B.D., Korneeva, N.L., Aamodt, E.J., and Rhoads, R.E. (2005). Translation of a small subset of *Caenorhabditis elegans* mRNAs is dependent on a specific eukaryotic translation initiation factor 4E isoform. Mol. Cell. Biol. *25*, 100–113.

Driever, W., and Nusslein-Volhard, C. (1988a). The bicoid protein determines position in the *Drosophila* embryo in a concentration-dependent manner. Cell *54*, 95–104.

Driever, W., and Nusslein-Volhard, C. (1988b). A gradient of bicoid protein in *Drosophila* embryos. Cell *54*, 83–93.

Dubnau, J., and Struhl, G. (1996). RNA recognition and translational regulation by a homeodomain protein. Nature *379*, 694–699.

Gebauer, F., and Hentze, M.W. (2004). Molecular mechanisms of translational control. Nat. Rev. Mol. Cell Biol. *5*, 827–835.

Gingras, A.C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. *68*, 913–963.

Gross, J.D., Moerke, N.J., von der Haar, T., Lugovskoy, A.A., Sachs, A.B., McCarthy, J.E., and Wagner, G. (2003). Ribosome loading onto the mRNA cap is driven by conformational coupling between eIF4G and eIF4E. Cell *115*, 739–750.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev. *18*, 1926–1945.

Hernandez, G., Altmann, M., Sierra, J.M., Urlaub, H., Diez Del Corral, R., Schwartz, P., and Rivera-Pomar, R. (2005). Functional analysis of seven genes encoding eight translation initiation factor 4E (eIF4E) isoforms in *Drosophila*. Mech. Dev. *122*, 529–542.

Hershey, J.W.B., and Merrick, W.C. (2000). Pathway and mechanism of initiation of protein synthesis. In Translational Control of Gene Expression, N. Sonenberg, J.W.B. Hershey, and M.B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 33–88.

Hodel, A.E., Gershon, P.D., Shi, X., Wang, S.M., and Quiocho, F.A. (1997). Specific protein recognition of an mRNA cap through its alkylated base. Nat. Struct. Biol. *4*, 350–354.

Johnstone, O., and Lasko, P. (2001). Translational regulation and RNA localization in *Drosophila* oocytes and embryos. Annu. Rev. Genet. *35*, 365–406.

Kahvejian, A., Svitkin, Y.V., Sukarieh, R., M'Boutchou, M.N., and Sonenberg, N. (2005). Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. Genes Dev. *19*, 104–113.

Khaleghpour, K., Svitkin, Y.V., Craig, A.W., DeMaria, C.T., Deo, R.C., Burley, S.K., and Sonenberg, N. (2001). Translational repression by a novel partner of human poly(A) binding protein, Paip2. Mol. Cell *7*, 205–216.

Kobayashi, S., Amikura, R., Nakamura, A., and Lasko, P.F. (1999). Techniques for analyzing protein and RNA distribution in *Drosophila* ovaries and embryos at structural and ultrastructural resolution. In Advances in Molecular Biology: A Comparative Methods Approach to the Study of Oocytes and Embryos, J. Richter, ed. (New York: Oxford University Press), pp. 426–445.

Kuersten, S., and Goodwin, E.B. (2003). The power of the 3′ UTR: translational control and development. Nat. Rev. Genet. *4*, 626–637.

Lynch, J., and Desplan, C. (2003). Evolution of development: beyond bicoid. Curr. Biol. *13*, R557–R559.

Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995). The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. Mol. Cell. Biol. *15*, 4990–4997.

Marcotrigiano, J., Gingras, A.C., Sonenberg, N., and Burley, S.K. (1997). Cocrystal structure of the messenger RNA 5′ cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell *89*, 951–961.

Marcotrigiano, J., Gingras, A.C., Sonenberg, N., and Burley, S.K.

(1999). Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. Mol. Cell *3*, 707–716.

Matsuo, H., Li, H., McGuire, A.M., Fletcher, C.M., Gingras, A.C., Sonenberg, N., and Wagner, G. (1997). Structure of translation factor eIF4E bound to m7GDP and interaction with 4E-binding protein. Nat. Struct. Biol. *4*, 717–724.

Mazza, C., Ohno, M., Segref, A., Mattaj, I.W., and Cusack, S. (2001). Crystal structure of the human nuclear cap binding complex. Mol. Cell *8*, 383–396.

Nakamura, A., Sato, K., and Hanyu-Nakamura, K. (2004). *Drosophila* Cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. Dev. Cell *6*, 69–78.

Nelson, M.R., Leidal, A.M., and Smibert, C.A. (2004). *Drosophila* Cup is an eIF4E-binding protein that functions in Smaug-mediated translational repression. EMBO J. *23*, 150–159.

Niessing, D., Dostatni, N., Jackle, H., and Rivera-Pomar, R. (1999). Sequence interval within the PEST motif of Bicoid is important for translational repression of caudal mRNA in the anterior region of the *Drosophila* embryo. EMBO J. *18*, 1966–1973.

Niessing, D., Blanke, S., and Jackle, H. (2002). Bicoid associates with the 5′-cap-bound complex of caudal mRNA and represses translation. Genes Dev. *16*, 2576–2582.

Poulin, F., and Sonenberg, N. (2003). Translation mechanisms in eukaryotes. In Translation Mechanisms, J. Lapointe and L. Brakier-Gingras, eds. (Austin, TX: Landes Bioscience), pp. 280–297.

Pyronnet, S., Imataka, H., Gingras, A.C., Fukunaga, R., Hunter, T., and Sonenberg, N. (1999). Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. EMBO J. *18*, 270–279.

Raught, B., Gingras, A.C., and Sonenberg, N. (2000). Regulation of ribosomal recruitment in eukaryotes. In Translational Control of Gene Expression, N. Sonenberg, J.W.B. Hershey, and M.B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 245–294.

Richter, J.D., and Sonenberg, N. (2005). Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature *433*, 477–480.

Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W.J., and Jackle, H. (1996). RNA binding and translational suppression by bicoid. Nature *379*, 746–749.

Rom, E., Kim, H.C., Gingras, A.C., Marcotrigiano, J., Favre, D., Olsen, H., Burley, S.K., and Sonenberg, N. (1998). Cloning and characterization of 4EHP, a novel mammalian eIF4E-related cap-binding protein. J. Biol. Chem. *273*, 13104–13109.

Rubin, G.M., Hong, L., Brokstein, P., Evans-Holm, M., Frise, E., Stapleton, M., and Harvey, D.A. (2000). A *Drosophila* complementary DNA resource. Science *287*, 2222–2224.

Sachs, A. (2000). Physical and functional interactions between the mRNA Cap structure and the poly(A) tail. In Translational Control of Gene Expression, N. Sonenberg, J.W.B. Hershey, and M.B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 447–466.

Shatkin, A.J. (1976). Capping of eucaryotic mRNAs. Cell *9*, 645–653.

Sigrist, S.J., Thiel, P.R., Reiff, D.F., Lachance, P.E., Lasko, P., and Schuster, C.M. (2000). Postsynaptic translation affects the efficacy and morphology of neuromuscular junctions. Nature *405*, 1062–1065.

St Johnston, D., and Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. Cell *68*, 201–219.

Stebbins-Boaz, B., Cao, Q., de Moor, C.H., Mendez, R., and Richter, J.D. (1999). Maskin is a CPEB-associated factor that transiently interacts with eIF-4E. Mol. Cell *4*, 1017–1027.

Svitkin, Y.V., Imataka, H., Khaleghpour, K., Kahvejian, A., Liebig, H.D., and Sonenberg, N. (2001). Poly(A)-binding protein interaction with eIF4G stimulates picornavirus IRES-dependent translation. RNA *7*, 1743–1752.

Tautz, D. (1988). Regulation of the *Drosophila* segmentation gene hunchback by two maternal morphogenetic centres. Nature *332*, 281–284.

Tee, A.R., Tee, J.A., and Blenis, J. (2004). Characterizing the interaction of the mammalian eIF4E-related protein 4EHP with 4E–BP1. FEBS Lett. *564*, 58–62.

Thomson, T., and Lasko, P. (2004). *Drosophila* tudor is essential for polar granule assembly and pole cell specification, but not for posterior patterning. Genesis *40*, 164–170.

Wickens, M., Goodwin, E.B., Kimble, J., Strickland, S., and Hentze, M. (2000). Translational control of developmental decisions. In Translational Control of Gene Expression, N. Sonenberg, J.W.B. Hershey, and M.B. Mathews, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 295–370.

Wilhelm, J.E., Hilton, M., Amos, Q., and Henzel, W.J. (2003). Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. J. Cell Biol. *163*, 1197–1204.