4E-BPs require non-canonical 4E-binding motifs and a lateral surface of eIF4E to repress translation

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eIF4E-binding proteins (4E-BPs) are a widespread class of translational regulators that share a canonical (C) eIF4E-binding motif (4E-BM) with eIF4G. Consequently, 4E-BPs compete with eIF4G for binding to the dorsal surface on eIF4E to inhibit translation initiation. Some 4E-BPs contain non-canonical 4E-BMs (NC 4E-BMs), but the contribution of these motifs to the repressive mechanism—and whether these motifs are present in all 4E-BPs—remains unknown. Here, we show that the three annotated Drosophila melanogaster 4E-BPs contain NC 4E-BMs. These motifs bind to a lateral surface on eIF4E that is not used by eIF4G. This distinct molecular recognition mode is exploited by 4E-BPs to dock onto eIF4E-eIF4G complexes and effectively displace eIF4G from the dorsal surface of eIF4E. Our data reveal a hitherto unrecognized role for the NC 4E-BMs and the lateral surface of eIF4E in 4E-BP-mediated translational repression, and suggest that bipartite 4E-BP mimics might represent efficient therapeutic tools to dampen translation during oncogenic transformation.
he regulation of protein synthesis at the initiation step is a widespread and reversible mechanism to control gene expression in eukaryotes. During translation initiation, the small ribosomal subunit is recruited to mRNA by the eukaryotic initiation factor 4 (eIF4F) complex, which comprises the cap-binding protein eIF4E, the scaffolding protein eIF4G and the DEAD-box RNA helicase eIF4A. The eIF4E protein recognizes the mRNA m’GpppN cap structure and interacts with eIF4G, which promotes translation initiation via the recruitment of the 43S pre-initiation complex. eIF4F binds eIF4E through a conserved motif (or canonical eIF4E-binding motif, C 4E-BM) of sequence TyrX₇LeuΦ, where Φ is hydrophobic, and X is any amino acid.

The assembly of the eIF4E complex is regulated by a diverse group of eIF4E-binding proteins (4E-BPs), which share a similar C TyrX₇LeuΦ motif with eIF4G. Therefore, 4E-BPs bind to the same surface on eIF4E, sterically blocking its interaction with eIF4G and preventing translation initiation. The association of 4E-BPs with eIF4E is reversible and regulated by phosphorylation. Unphosphorylated or hypophosphorylated 4E-BPs exhibit a high affinity for eIF4E and repress translation, whereas hyper-phosphorylated 4E-BPs lose their affinity for eIF4E.

At a functional level, 4E-BPs play essential roles in the control of translation during development and regulate neuronal plasticity by repressing translation at a global or message-specific level. Through their inhibitory effect on translation, 4E-BPs negatively regulate cell proliferation and act as tumor suppressors. However, the 4E-BP anti-oncogenic function is compromised in many tumors, resulting in increased eIF4E activity and protein synthesis, which is required for tumorigenic transformation. Consequently, a detailed molecular understanding of the interaction between eIF4E and 4E-BPs is crucial to design or improve drugs that may be useful in pathologic conditions in which eIF4E activity and global translation are upregulated.

The C motifs of eIF4G and 4E-BPs adopt similar α-helical structures on binding to a conserved patch of hydrophobic residues on the dorsal side of the eIF4E cap-binding pocket. Additional surfaces on eIF4E also contribute to the interaction with eIF4G as well as with a subset of 4E-BPs by binding to residues that are carboxy terminal to the C motifs, which contain NC 4E-BMs. To date, NC motifs have only been identified and characterized in eIF4G, vertebrate 4E-BP1–3, and D. melanogaster CUP (ortholog of 4E-BP1–3) and D. melanogaster Thor (ortholog of 4E-BP1–3) and 4E-T (4E-transporter) bind to eIF4E through a bipartite sequence that contains a C motif and a NC motif. The newly identified NC motifs in Thor and 4E-T share no sequence similarity with their vertebrate counterparts or with CUP. Nevertheless, these motifs share an overlapping lateral binding surface on eIF4E with the NC motif of CUP, which is required for the binding of 4E-BPs but not of eIF4G. The binding to an eIF4E surface that is not used by eIF4G allows 4E-BPs to dock onto preexisting eIF4E–eIF4G complexes to begin to displace eIF4G from the dorsal surface. Our data reveal a hitherto unrecognized diversity of NC motifs and establish the relevance of these motifs in the mechanism by which 4E-BPs repress translation. More generally, our data indicate that bipartite 4E-BP mimics have a competitive advantage over eIF4G and might represent potent repressors for the treatment of malignancies, in which eIF4E activity is upregulated.

Results

4E-BPs bind to a lateral surface of eIF4E. To gain insight into the binding mode of different 4E-BPs to eIF4E, we compared the interaction of Dm CUP, Thor and 4E-T with Dm eIF4E (Fig. 1a). In coimmunoprecipitation and pull-down assays, we confirmed that all the proteins interacted with endogenous eIF4E in Dm Schneider (S2) cells (Supplementary Fig. 1a–e).

Dm CUP interacts with eIF4E through C and NC motifs. In particular, the CUP residues Tyr337, Leu332, Met333 and Arg336 in the C motif interact with residues on the dorsal surface of eIF4E, including Trp106 and Leu167 (Fig. 1b and Supplementary Fig. 2a,b). In addition, the CUP residues Leu364, Leu368, Met371 and Ile373 in the NC motif contact a lateral external surface that is centered at residues Ile96 and Ile112 (Fig. 1b and Supplementary Fig. 2a,b). To determine whether Thor, 4E-T and eIF4E also recognize the lateral surface of eIF4E, we substituted residues Ile96 and Ile112 with Ala (eIF4E mutant II-AA) and performed coimmunoprecipitation assays in S2 cells. As a control, we used an eIF4E mutant with a Trp106Ala substitution (W106A) on the dorsal binding surface, because this substitution abolishes the binding of CUP and eIF4G to eIF4E21,28,29. As expected, the W106A substitution strongly reduced the binding of eIF4E to endogenous eIF4G and to all three of the 4E-BPs (Fig. 1c–e, lanes 7). By contrast, the II-AA mutations disrupted the association of eIF4E with CUP, Thor and 4E-T but not with eIF4G (Fig. 1c–e, lanes 8). Thus, in contrast to eIF4G, 4E-BPs recognize and depend on the lateral surface to efficiently bind to eIF4E in cell lysates, in which eIF4G (or other 4E-BPs) is also present.

Identification of NC 4E-BMs in Thor and 4E-T. The coimmunoprecipitation assays shown in Fig. 1c–e indicate that similar to CUP, Thor and 4E-T contain NC motifs that interact with the lateral binding surface of eIF4E. In human 4E-BP1,2, the NC IPGVTS/T motif (located C-terminally to the C motif), increases the binding affinity of the proteins for eIF4E by approximately three orders of magnitude. However, the IPGVTS/T motif is not conserved across the animal kingdom (Supplementary Fig. 2c). Nevertheless, several hydrophobic residues are present in the corresponding region in Dm Thor (residues Pro76–Pro84; Supplementary Fig. 2c).

To determine whether the Thor residues 76–84 constitute a NC 4E-BM, we substituted Cys78, Leu79 and Leu80 with alanine (NC*) or deleted the motif (ΔNC, Supplementary Table 1). In

To shed light on the role of NC motifs in 4E-BP-mediated translational repression, we investigated whether different Dm 4E-BPs contain NC motifs and how these motifs contribute to the displacement of eIF4G from eIF4E. We show that similar to CUP, Thor (ortholog of 4E-BP1–3) and 4E-T (4E-transporter) bind to eIF4E through a bipartite sequence that contains a C motif and a NC motif. The newly identified NC motifs in Thor and 4E-T share no sequence similarity with their vertebrate counterparts or with CUP. Nevertheless, these motifs share an overlapping lateral binding surface on eIF4E with the NC motif of CUP, which is required for the binding of 4E-BPs but not of eIF4G. The binding to an eIF4E surface that is not used by eIF4G allows 4E-BPs to dock onto preexisting eIF4E–eIF4G complexes to begin to displace eIF4G from the dorsal surface. Our data reveal a hitherto unrecognized diversity of NC motifs and establish the relevance of these motifs in the mechanism by which 4E-BPs repress translation. More generally, our data indicate that bipartite 4E-BP mimics have a competitive advantage over eIF4G and might represent potent repressors for the treatment of malignancies, in which eIF4E activity is upregulated.
the coimmunoprecipitation assays, the deletion of the Thor residues 76–84 abolished the interaction with eIF4E (Fig. 2a, lane 12), whereas the alanine substitutions decreased the eIF4E binding (Supplementary Fig. 1d, lane 9, NC*). By contrast, alanine substitutions in the C motif (C*, Supplementary Table 1) also disrupted the interaction with eIF4E (Fig. 2a, lane 10). As a control, amino-acid substitutions of the flanking residues Arg81, Gly82 and Thr83 by alanine was ineffective (Fig. 2a, lane 12), whereas the alanine substitutions decreased the eIF4E binding (Supplementary Fig. 1d, lane 9, NC*). By contrast, the alanine substitutions decreased the eIF4E binding to eIF4E (Fig. 2b, lanes 10 and 11, and Supplementary Fig. 2d). Thus, a NC 4E-BM is also disrupted by alanine substitutions or deletions of various residues in this motif (Supplementary Table 1) caused a drastic reduction in the 4E-T binding to eIF4E (Fig. 2b, lanes 10 and 11, and Supplementary Fig. 1e), similar to the disruption of the C motif (C*, Fig. 2b, lane 9, and Supplementary Fig. 2d). Thus, a NC 4E-BM is also present in the Dm 4E-T that is conserved in Drosophila species.

4E-BPs and eIF4G display similar affinities for eIF4E. Next, we compared the binding efficiencies of the minimal eIF4E-binding regions of the 4E-BPs (C + NC, Supplementary Table 1) in pull-down assays. These regions were expressed with an amino-terminal MBP-tag and a C-terminal GB1-tag. In parallel, we analyzed the minimal eIF4E-binding fragment of eIF4G (residues 578–650), which includes the C motif and the SDVVL motif that was identified in Hs eIF4G (corresponding to Dm VKNVSL, Supplementary Fig. 2e), which plays an auxiliary function in stabilizing the eIF4E interaction with eIF4E. The bipartite C + NC regions of the three 4E-BPs and the eIF4G fragment pulled down the purified eIF4E at comparable levels (Fig. 2c).
To obtain information on the affinities and thermodynamic parameters, we performed isothermal titration calorimetry (ITC) experiments. The bipartite regions of all three of the 4E-BPs and elf4G exhibited comparable binding affinities for elf4E, with dissociation constants ($K_D$s) in the nanomolar range (Table 1 and Supplementary Fig. 3). The $K_D$ values obtained for Dm Thor and elf4G are comparable to those that have been reported for the human proteins. Notably, although the binding of all proteins to elf4E is enthalpically driven, the entropic penalties differ between these proteins, suggesting differences in the binding mechanisms. In particular, the interaction between CUP and elf4E displayed the highest entropic penalty, which is indicative of a lower degree of conformational freedom in the bound state. Thus, CUP may undergo larger disorder-to-order transitions on binding, which is consistent with the formation of two $\alpha$-helices. 4E-T and Thor exhibited lower entropic penalties, suggesting a more dynamic conformation in the bound state.

To understand the contribution of the NC 4E-BMs to the affinity of 4E-BPs for elf4E, we analyzed the binding of 4E-BP peptides containing only the C motifs or the complementary sequences comprising the linker (L) region between the two motifs and the NC motif (L + NC, Supplementary Fig. 4 and Supplementary Table 1). The affinities of the C motifs in isolation were one to three orders of magnitude lower than the C + NC peptides, indicating that the NC motifs contribute significantly to the overall affinity. Interestingly, the C motifs of all three 4E-BPs exhibited significant differences in binding affinities, with the affinity of the 4E-T peptide being approximately one and two orders of magnitude higher than those of the CUP and Thor peptides, respectively (Table 1 and Supplementary Fig. 4). The differences between 4E-BPs were more pronounced for the L + NC peptides, because only the CUP peptide interacted with elf4E at detectable levels. The binding of the CUP peptide (L + NC) was enthalpically driven, with a $K_D$ comparable to that of the C motif. These results indicate a similar contribution to the energetics of binding by the C and NC motifs of CUP.

Finally, we determined the affinities of the bipartite peptides (C + NC) for the elf4E II-AA mutant. The affinities of CUP and Thor peptides were reduced by one and two orders of magnitude, respectively (Table 1 and Supplementary Fig. 5). In contrast, 4E-T binding was not significantly affected perhaps reflecting the higher affinity of its C 4E-BM. Similarly, the mutations in the lateral surface of elf4E did not affect elf4G binding.

We conclude that although 4E-BPs and elf4G display similar affinities for elf4E, they use different binding modes. These differences can be mainly attributed to the linker regions and the NC motifs, consistent with their sequence diversity, although differences in affinities for the C motifs were also detected. Moreover, the results of the ITC experiments also indicate that the affinity of 4E-BPs for elf4E results from synergistic effects between the C and NC motifs.

**4E-BP NC motifs are sufficient to bind elf4E.** To further analyze the binding modes of the 4E-BPs and elf4G to elf4E, we performed pull-down assays with recombinant proteins that were expressed in *Escherichia coli*. In contrast to the experiments in cell lysates, the *in vitro* pull-down assays allowed us to investigate the interactions of the individual proteins in the absence of other 4E-BPs, which could compete for binding and obscure the interpretation of the results. We tested recombinant fragments of elf4G, CUP and 4E-T and full-length Thor for binding to either the elf4E wild-type (WT) or II-AA mutant (that is, with a disrupted lateral surface). elf4G and the 4E-BPs pulled down...
comparable amounts of WT and mutant eIF4E (Fig. 3a, lanes 9 and 10; Fig. 3b, lanes 10 and 11; and Fig. 3c,d, lanes 13 and 14), indicating that these proteins interact with the eIF4E mutant lacking a functional lateral binding surface in vitro. The results obtained in vitro contrast with the observation that the 4E-BPs did not interact with the II-AA mutant in cell lysates (Fig. 1e–g). One possible explanation for this difference is that cell lysates contain eIF4G, which blocks the dorsal surface of eIF4E, leaving only the lateral surface available for 4E-BPs. If the lateral surface is in addition mutated, then 4E-BPs may not be able to interact with eIF4E and displace bound eIF4G (see below).

The interaction of eIF4G and 4E-BPs with the eIF4E II-AA mutant is most likely mediated by their C motifs that bind to the dorsal surface of eIF4E. To confirm this assumption, we introduced mutations in the C motifs (C* mutants, Supplementary Table 1). Substitutions in the C motif of eIF4G abolished its interaction with either WT or mutant eIF4E (Fig. 3a, lanes 11 and 12). By contrast, the equivalent substitutions in the C motifs of 4E-BPs did not prevent their binding to eIF4E, reflecting a truly bipartite-binding mode (Fig. 3b, lane 12, and Fig. 3c,d, lane 15). However, the CUP and Thor C* mutants were strongly impaired in their binding to the eIF4E II-AA mutant (Fig. 3b, lane 13; Fig. 3c, lane 16), indicating that the C* mutants use the lateral surface of eIF4E. The 4E-T C* mutant showed reduced binding to both WT and mutant eIF4E (Fig. 3d, lanes 15 and 16).

Substitutions in the NC motifs (NC*) did not prevent the interaction of 4E-BPs with either WT or mutant eIF4E, most likely because the C motifs are sufficient for binding (Fig. 3b, lanes 14 and 15; Fig. 3c,d, lanes 17 and 18). The interaction of the three 4E-BPs with WT eIF4E was strongly reduced when the two motifs were mutated (C + NC*, Fig. 3b, lane 16, Fig. 3c,d, lanes 19). Remarkably, some residual binding to eIF4E was observed. These results suggest that the linker regions between the motifs in CUP and 4E-T and additional residues in Thor (which was full length) contact eIF4E and contribute to the interaction. The results obtained for the Thor NC* and C + NC* mutants were confirmed using a mutant with a deleted NC motif (ΔNC, Supplementary Fig. 6a).

Collectively, our results indicate that 4E-BPs interact with eIF4E using a bipartite-binding mode and recognize a lateral surface on eIF4E that is not used by eIF4G. Two main observations support these conclusions. First, mutations in the C motifs abolish the interaction of eIF4G but not of 4E-BPs with eIF4E. Second, mutations on the lateral surface of eIF4E abolish or reduce the binding of 4E-BPs to eIF4E when their binding to the dorsal surface is also compromised. Our results further indicate that the eIF4G residues downstream of the C motif, including the VKNVSI motif, do not use the binding surface centered at residues Ile96 and Ile112 and are not sufficient for binding to eIF4E when the C motif is mutated, which is in agreement with the proposed auxiliary role of these sequences. Finally, it is important to note that although mutations in the C motifs of Thor and 4E-T do not disrupt binding to eIF4E, a deletion of the C motif prevents binding (L + NC peptides, see ITC experiments). These results suggest that mutations in the C motifs of these proteins do not completely abolish binding to the eIF4E dorsal surface, or that the formation of an α-helical structure (which is likely maintained in the mutants) is indirectly required to facilitate the binding of the linker region and NC motifs.

**Table 1 | Thermodynamic parameters for the interaction of eIF4E with eIF4G and 4E-BP peptides.**

| Peptide + eIF4E | K_D (M) | ΔH (kcal mol^-1) | TΔS (kcal mol^-1) | ΔG (kcal mol^-1) | Molar ratio |
|----------------|---------|-----------------|-----------------|-----------------|-------------|
| eIF4G 578-680  | 17.1 ± 13 × 10^-9 | -16.5 ± 2 | 7.97 | -10.51 | 0.99 ± 0.08 |
| CUP C + NC    | 9.1 ± 0.5 × 10^-9 | -34.5 ± 0.35 | 23.86 | -10.78 | 1.05 ± 0.02 |
| CUP C         | 1.6 ± 0.1 × 10^-7 | -18.4 ± 0.04 | 7.70 | -9.13 | 1.01 ± 0.01 |
| CUP L + NC    | 1.03 ± 0.03 × 10^-7 | -18.5 ± 0.2 | 9.10 | -9.38 | 0.98 ± 0.01 |
| Thor C + NC   | 1.4 ± 0.3 × 10^-9 | -16.8 ± 1 | 4.90 | -11.87 | 0.95 ± 0.02 |
| Thor C        | 2.26 ± 0.06 × 10^-6 | -12.1 ± 2.8 | 2.24 | -9.82 | 1.06 ± 0.01 |
| Thor L + NC   | nb | nb | nb | nb | |
| 4E-T C + NC   | 5.6 ± 2.4 × 10^-9 | -22.8 ± 3 | 11.73 | -11.11 | 0.95 ± 0.01 |
| 4E-T C        | 1.6 ± 0.2 × 10^-8 | -18.6 ± 0.7 | 8.16 | -10.45 | 0.95 ± 0.01 |
| 4E-T L + NC   | nb | nb | nb | nb | |

| Peptide + eIF4E (II-AA) | K_D (M) | ΔH (kcal mol^-1) | TΔS (kcal mol^-1) | ΔG (kcal mol^-1) | Molar ratio |
|-------------------------|---------|-----------------|-----------------|-----------------|-------------|
| eIF4G 578-680           | 40 ± 9.5 × 10^-9 | -16.24 ± 0.04 | 6.32 | -9.93 | 1.03 ± 0.01 |
| CUP C + NC              | 5.0 ± 0.8 × 10^-8 | -18.6 ± 0.7 | 8.79 | -9.84 | 0.98 ± 0.01 |
| Thor C + NC             | 4.7 ± 0.3 × 10^-7 | -7.6 ± 0.3 | -0.91 | -8.46 | 0.97 ± 0.01 |
| 4E-T C + NC             | 8.8 ± 2 × 10^-9 | -12.9 ± 0.93 | 2.16 | -10.80 | 1.03 ± 0.04 |

C, canonical; eIF4, eukaryotic initiation factor 4; L, linker; nb, no binding; NC, non-canonical.

See Supplementary Figs 3–5.

**4E-BPs use the eIF4E lateral surface to compete with eIF4G.** The observation that 4E-BPs can bind to the eIF4E II-AA mutant in vitro (that is, in the absence of competition) but not in cell lysates (that is, in the presence of eIF4G) suggests that 4E-BPs are not able to compete with eIF4G for binding to eIF4E when the lateral binding surface is disrupted.

To further investigate the role of the lateral binding surface of eIF4E in the competition mechanism, we performed competition assays using preassembled eIF4E-eIF4G complexes containing either eIF4E WT or the II-AA mutant and GST-tagged eIF4G (residues 578–650). eIF4G formed stable complexes both with WT and mutant eIF4E (Fig. 4a–c, lanes 4 and 5, respectively). These preassembled eIF4E-eIF4G complexes were challenged with increasing amounts of peptides containing the C and NC (C + NC) motifs of 4E-BPs or the same eIF4G fragment. Proteins that were associated with eIF4E were recovered by eIF4E pull-down assays.

The CUP, 4E-T and Thor C + NC peptides displaced eIF4G from the complex and associated with eIF4E (Fig. 4a–c, lanes 7–10 versus 6, Supplementary Figs 6b–d and 7a–c). The CUP and 4E-T peptides were able to effectively displace eIF4G when present at
two- and onefold molar excess, respectively. Under the same conditions, the 4E-BP C+NC peptides did not efficiently displace eIF4G from complexes that contained the eIF4E II-AA mutant (Fig. 4a–c, lanes 13 versus 12, Supplementary Figs 6b–d and 7a–c). Thus, binding to the lateral surface is required for 4E-BPs to effectively compete with eIF4G. In agreement with this conclusion, peptides containing only the 4E-BP C motifs did not displace eIF4G from eIF4E, although they were tested at the highest molar concentration (Fig. 4a–c, lanes 11 versus 10 and Supplementary Fig. 7a–c).

In striking contrast to the 4E-BP peptides, the eIF4G peptide hardly competed with GST-eIF4G for binding to eIF4E, irrespective of whether eIF4E was WT or mutated (Fig. 4d, lanes 5–11, Supplementary Figs 6b and 7d). Mechanistically, our results
indicate that 4E-BPs are more efficient competitors than is eIF4G and must bind to the lateral surface of eIF4E to effectively displace eIF4G from preassembled eIF4E–eIF4G complexes.

**4E-BPs use the NC motifs to compete with eIF4G.** Given that binding of 4E-BPs to the lateral surface of eIF4E is required for competition with eIF4G and that peptides containing only the 4E-BP C motifs cannot compete with eIF4G (Fig. 4), we next investigated the requirement for NC motifs. To this end, we performed competition assays using preassembled eIF4E–eIF4G complexes and excess 4E-BP peptides lacking either the C or NC motifs. The WT CUP C + NC peptide interacted with eIF4E and efficiently displaced preassembled eIF4G (Fig. 5a, lane 9 versus 6). Peptides containing either the C or the NC motifs of CUP did not compete with eIF4G (Fig. 5a, lanes 7 and 8), although these peptides bind to eIF4E in the absence of eIF4G (Fig. 5b, lanes 6 and 7), which is in agreement with the ITC experiments.

Similar results were obtained for Thor. Notably, deleting the non-canonical motif in the context of full-length Thor was sufficient to abolish its ability to compete with eIF4G (Fig. 5c, lane 10 versus 7), although in the absence of eIF4G this deletion mutant interacted with eIF4E (Supplementary Fig. 6a, lanes 17 and 18). Mutations in the canonical motif also abolished competition, as expected (Fig. 5c, lane 9). We conclude that 4E-BPs require both canonical and non-canonical motifs to compete with eIF4G for eIF4E binding. Thus, the non-canonical motifs play an essential role in the competition mechanism.

**4E-BPs exhibit a kinetic competitive advantage over eIF4G.** Given that the 4E-BPs and eIF4G display similar affinities for eIF4E, the differences in the ability to efficiently displace prebound eIF4G in competition assays are likely explained by the binding kinetics and the bipartite-binding mode. To obtain additional information on the ability of 4E-BPs to compete with eIF4G, we challenged preassembled eIF4E–eIF4G complexes with five- to tenfold molar excess of 4E-BP and eIF4G peptides and monitored the amount of eIF4G remaining bound to eIF4E over time.

In the absence of competitors, eIF4G remained bound to eIF4E, as expected (Fig. 5d, lane 4). In the presence of a tenfold molar excess of eIF4G peptide, we observed a 50% dissociation of prebound eIF4G after 4 h at 4 °C (Fig. 5d and Supplementary Fig. 7e). In the presence of a fivefold molar excess of CUP and 4E-T peptides (C + NC), we observed a 50% eIF4G dissociation in 2.5 ± 0.5 and 22 min, respectively, whereas the half-life of the eIF4E–eIF4G complexes in the presence of tenfold molar excess of Thor was 37 ± 9 min. (Fig. 5e–g, and Supplementary Fig. 7f). The simplest explanation of these results is that the bipartite-binding mode and the binding to an eIF4E surface that is not used by eIF4G confer on 4E-BPs a kinetic competitive advantage because they can bind preassembled eIF4E–eIF4G complexes without the need for prior eIF4G dissociation.

**Figure 4 | 4E-BPs require binding to the lateral surface of eIF4E to compete with eIF4G.** (a–d) Purified eIF4E–eIF4G complexes (2 μM) containing SHN-tagged eIF4E (full length, WT or II-AA mutant) and GST-eIF4G (residues 578–650) were incubated with increasing amounts of CUP (a), 4E-T (b), Thor (c) and eIF4G (d) peptides fused C terminally to GB1. The 4E-BP peptides contained the canonical and non-canonical motifs (C + NC) or only the C and are described in Supplementary Table 1. MBP served as a negative control. The proteins that were bound to eIF4E were pulled down using Strep-Tactin beads. The competitor peptides are labeled in blue, and their positions are highlighted by blue, dashed boxes. The black dashed boxes indicate the position of preassembled GST-eIF4G. Numbers above the lanes indicate fold molar excess of the competitor peptides. The corresponding quantification of the competition assays is shown in Supplementary Fig. 7a–d (n = 2). Asterisks indicated a contaminant protein.
elf4G competes with 4E-BPs bound to the elf4E II-AA mutant. Next, we asked whether elf4G could compete with 4E-BPs when their binding to the lateral surface of elf4E was disrupted. For this purpose, preassembled complexes containing elf4E (WT or II-AA mutant) bound to GST-4E-BP fragments were challenged with excess amounts of MBP-elf4G (residues 578–650). Proteins that were bound to elf4E were recovered via elf4E pull down. MBP-elf4G did not displace CUP, Thor, 4E-T or elf4G bound to WT elf4E (Fig. 6a, lane 6 versus 5, and Fig. 6b, lanes 8, 10 and 14). In contrast, MBP-elf4G partially displaced CUP (Fig. 6a, lane 8 versus 7) and completely displaced full-length Thor (Fig. 6b, lane 12 versus 11) bound to the elf4E II-AA mutant. These observations indicate that elf4G can compete with 4E-BPs for binding to elf4E only when their interaction with the lateral surface of elf4E is impaired. Thus, the dissociation of 4E-BPs from the lateral surface of elf4E (for instance, on phosphorylation) may be sufficient for their dissociation from elf4E to allow elf4G to resume translation (Fig. 6c).

The non-canonical motifs mediate translational repression. To determine the role of non-canonical motifs in translational repression, we tested whether 4E-BPs repressed the expression of a firefly luciferase (F-Luc) reporter when coexpressed in S2 cells. A short un capped and unadenylated RNA served as a transfection control (control RNA). To rule out the possibility that the inhibition of F-Luc expression resulted from changes in the F-Luc mRNA levels, we analyzed these levels by northern blotting and determined translation efficiencies (Fig. 7a,b).

The CUP N-terminal fragment or full-length Thor inhibited the expression of the F-Luc reporter in a dose-dependent manner (Fig. 7a–d). 4E-T caused mRNA degradation when overexpressed and was excluded from the analysis (C1 and E1, unpublished results). Mutations in either the canonical or non-canonical motifs as well as the combined mutations suppressed CUP- and Thor-mediated repression (Fig. 7a,b). The mutant proteins were expressed at levels that were comparable to the highest tested level for the WT protein (Fig. 7c,d, WB). Thus, both the canonical and non-canonical motifs are required for Thor and CUP to repress translation in a cellular context, which is in agreement with the competition assays.

The non-canonical motifs regulate elf4E localization. 4E-BPs are nucleocytoplasmic shuttling proteins that transport elf4E to the nucleus26,33–35. Although elf4E nuclear functions are not clearly understood, the nuclear retention/import of elf4E could contribute to the efficient inhibition of cap-dependent translation. In addition, human 4E-T can also induce the accumulation of elf4E in mRNA processing bodies or P-bodies36. To determine whether the non-canonical motifs contribute to the regulation of elf4E subcellular distribution mediated by 4E-BPs, we analyzed the localization of endogenous elf4E by immunofluorescence in S2 cells expressing WT or mutant 4E-BPs (Fig. 8).

At a steady-state, CUP and Thor distributed evenly throughout the cytoplasm (Fig. 8a,e). By contrast, 4E-T accumulated in cytoplasmic foci, which correspond to P-bodies as judged by the colocalization with the P-body marker Trailer hitch (Fig. 8i and Supplementary Fig. 7g). Endogenous elf4E was also evenly distributed in the cytoplasm in cells overexpressing WT CUP and Thor as well as the mutant versions of these proteins (Fig. 8a–h, middle panels). In contrast, in cells expressing 4E-T, elf4E was detected in P-bodies (Fig. 8i). Thus, 4E-T can drag elf4E into P-bodies. Accordingly, the number of elf4E-positive P-bodies was reduced in cells overexpressing 4E-T mutants (C*, NC*, C+ NC*; Fig. 8j–l), although the mutants still localized to P-bodies. Thus, both the canonical and non-canonical motifs of 4E-T are required to induce the accumulation of elf4E in P-bodies.

Next, we treated S2 cells with Leptomycin B (LMB), a drug that inhibits nuclear export by CRM1, which has been shown to export elf4E26,33,35. The LMB treatment induced the nuclear accumulation of CUP and 4E-T proteins (Fig. 8m,u) and a partial nuclear accumulation of Thor (Fig. 8q). Concomitantly, endogenous elf4E accumulated in the nucleus (Fig. 8m,u, middle panels). elf4E nuclear accumulation was dependent on binding to the 4E-BPs because this accumulation was strongly reduced in cells expressing the 4E-BP mutants (Fig. 8n–p,r–t,v–x). None of the 4E-BPs required binding to elf4E to translocate to the nucleus in the LMB-treated cells (Fig. 8m–x, left panels). Taken together, our data indicates that both the canonical and non-canonical motifs are required for 4E-BPs to regulate elf4E subcellular distribution.

Discussion
In this study, we show that similar to CUP, Thor and 4E-T employ a bipartite interface that is composed of canonical and non-canonical motifs to bind to the dorsal and lateral surfaces of elf4E, respectively. While the dorsal binding surface of elf4E is also used by elf4E5,7,17, the lateral binding surface is only used by 4E-BPs and is required for 4E-BPs to displace elf4F from preassembled elf4E–elf4G complexes. Based on these results, we propose that the lateral surface of elf4E provides an exclusive docking surface for 4E-BPs on elf4E–elf4G complexes. After docking, 4E-BPs can begin to displace elf4E by establishing interactions with the elf4E dorsal surface via their own canonical motifs, further stabilizing their association with elf4E (Fig. 6c).

The ability to bind laterally to the side of elf4E that is not used by elf4G enable 4E-BPs to displace elf4E even when their binding affinities are similar and under conditions in which 4E-BPs are not in great excess compared with elf4G. Indeed, by docking to preassembled elf4E–elf4G complexes, the 4E-BPs increase their local concentration and can rapidly dissociate bound elf4E, inhibiting ongoing translation. Our model also provides one possible explanation for why elf4G is a poor competitor compared with 4E-BPs. Indeed, elf4G will not bind elf4E unless the prebound elf4G or 4E-BPs dissociate. In this
context, it will be of interest to determine the contribution of the canonical and non-canonical motifs to the association ($K_{on}$) and dissociation ($K_{off}$) rate constants of 4E-BP proteins.

How can elf4G bind back to elf4E to resume translation? We show that elf4G can displace 4E-BPs when their binding to the lateral surface of elf4E is impaired. Although in our studies this interaction was impaired by mutations, in vivo this impairment could be achieved by posttranslational modifications such as phosphorylation. Indeed, it is well established that the phosphorylation of 4E-BPs reduces their affinity for elf4E [2,8]. Thus, it will be of interest to dissect the impact of phosphorylation on the interaction of 4E-BPs with either the lateral or dorsal surfaces of elf4E.

Owing to their lack of conservation, it has remained unclear whether non-canonical motifs are present in all 4E-BPs. Our data indicate that the non-canonical motifs are intrinsic to the ability of 4E-BPs to compete with elf4G and thus are likely to be present in all 4E-BPs that repress translation. At the functional level, non-canonical motifs have been proposed to play an auxiliary role and have been mainly implicated in the regulation of the affinity of elf4E for the mRNA cap structure through allosteric effects [7,17,27,28,38]. Specifically, the binding of the 4E-BP,2 non-canonical motifs to elf4E increases the affinity for the cap structure [9,22,38]. Here, we show that the non-canonical motifs are essential, not auxiliary, for 4E-BP function in inhibiting translation. Given the diversity of non-canonical motifs and their different modes of interaction with elf4E, it is possible that their binding to the lateral surface of elf4E modulates the affinity for the cap in different ways, thereby mediating different effects. For example, by increasing the affinity of elf4E for the cap structure, 4E-BPs may stabilize translationally repressed mRNA targets as observed for CUP [39]. Alternatively, by decreasing the affinity of elf4E for the mRNA cap, 4E-BPs may destabilize the repressed mRNA target through decapping and subsequent decay.

In summary, our current understanding of 4E-BPs role in translational repression is predominately based on the study of the low-molecular-weight 4E-BPs of the 4E-BP1–3 family. The identification of additional, high-molecular-weight 4E-BPs together with the characterization of their interaction mode with elf4E reveals an unexpected sequence diversity of the elf4E-binding regions and of the functional mechanisms. The functional diversity of 4E-BPs is further enhanced by the presence of additional domains in the high-molecular-weight 4E-BPs. These additional domains link elf4E binding with other cellular processes, such as mRNA decay, as described for CUP and 4E-T [36,39]. Understanding the molecular basis for the interaction of diverse 4E-BPs with elf4E will provide valuable insight into the variety of mechanisms that are employed by these proteins to regulate gene expression. These studies promise to uncover novel therapeutic strategies to selectively target dysregulated translation in cancer.

**Methods**

**DNA constructs.** The plasmids expressing the luciferase reporters, control RNA and GFP- or HA-tagged elf4G, elf4E, Tral and CUP (WT or mutated) have been previously described [39–42]. The plasmids expressing HA-Thor-V5 and GFP-Thor were obtained by inserting the corresponding DNA into the EcoRV and XhoI sites of the pAc5.1-L-HA or pAc5.1-GFP vectors, respectively. A plasmid expressing HA-4E-T was obtained by inserting the corresponding DNA (CG32016 isoform B)

**Figure 6 | elf4G competes with 4E-BPs when their binding to the lateral surface of elf4E is impaired.** (a,b) Purified complexes containing SHN-tagged elf4E bound to either elf4G or the indicated 4E-BPs were incubated with MBP or MBP-elf4G-GB1. The amount of elf4G or 4E-BP proteins that were associated with elf4E was determined by pull down using Strep-Tactin beads. The complexes contained GST-elf4G (residues 578–650), GST-CUP (residues 311–440), GST-Thor (full length) or GST-4E-T (residues 1–58). (c) Competition model: elf4E (blue circle) contains a dorsal and a lateral surface that bind to the C and NC motifs of 4E-BPs (shown in orange), respectively. The dorsal surface also binds to the canonical motif of elf4G (shown in green). The elf4E lateral binding surface provides a docking site for the non-canonical motifs of 4E-BPs when elf4G is bound to the dorsal surface of elf4E via its canonical motif (1). After docking, 4E-BPs displace elf4G from the dorsal surface of elf4E and repress translation (2).Phosphorylation (P) of 4E-BPs destabilizes their association with elf4E (3). Therefore, elf4G can bind to elf4E and translation resumes (4). In humans, 4E-BP1–3 the phosphorylation sites are located in the linker region between the 4E-BMs and in the sequences N-terminal to the canonical motif (not shown). Dephosphorylation of 4E-BPs is required for binding to elf4E (5). Symbols are as in Fig. 3e.
WB can be found in Supplementary Fig. 9.

The DNA fragment encoding a truncated His6-eIF4E (residues 69–248) that was used in the ITC experiments and normalized to those of the control RNA. The normalized F-Luc mRNA levels were used to normalize the F-Luc activity to obtain the translation efficiencies, which were set to 100 in cells expressing the HA peptide.

The red, dashed lines indicate the F-Luc levels for the maximum repression activity that was exhibited by the 4E-BPs. The black, dashed lines indicate the F-Luc levels expected in the absence of repression. (c, d) Upper panels show northern blot (NB) analyses of representative RNA samples corresponding to the experiments that are shown in (a, b), respectively. The lower panels show the expression of the 4E-BP proteins analyzed by WB. The original northern and western blots can be found in Supplementary Fig. 9.

**Figure 7 | Role of non-canonical motifs in translational repression.** (a, b) S2 cells were transfected with plasmids expressing a F-Luc reporter (F-Luc) and HA-CUP (N-Term, residues 1–402) or HA-Thor (full length, either WT or the indicated mutants). The F-Luc mRNA levels were analyzed by northern blotting and were normalized to those of the control RNA. The normalized F-Luc mRNA levels were used to normalize the F-Luc activity to obtain the translation efficiencies, which were set to 100 in cells expressing the HA peptide. The mean values ± s.d. from three independent experiments are shown.

Protein expression and purification. Unless indicated otherwise, all the proteins were expressed in E. coli BL21 Star (DE3) cells (Invitrogen) that were grown in LB medium overnight at 20 °C. The lysis buffers were supplemented with DNsase (5 μg ml⁻¹), lysozyme (1 mg ml⁻¹) and protease inhibitor cocktail (Roche). The truncated His₆-eIF4E (residues 69–248) that was used in the ITC experiments and in Figs 2c and 5b was purified in lysis buffer containing 50 mM HEPES (pH 7.2), 300 mM NaCl, 2 mM β-mercaptoethanol using Ni²⁺-affinity chromatography (HisTrap HP 5 ml, GE Healthcare) and eluted with a gradient of 20–500 mM imidazole. After the cleavage of the His₆-tag with HRV3C protease (homemade), the protein was further purified using a heparin column (HiTrap HP 5 ml, GE Healthcare) and eluted with a gradient of 20–500 mM imidazole. To obtain the preassembled eIF4E–eIF4G complexes used in Figs 4 and 5d–g, Coimmunoprecipitation assays and western blotting. The coimmunoprecipitations were performed as previously described41. For the pull downs using m⁹GTP beads, 25 μl of immobilized γ-aminophenyl-m⁹GTP (C₉₋₁₀-spacer—Jena Bioscience) beads was added to the cell lysates and the mixtures were rotated for 1 h at 4 °C. The beads were washed three times with NET buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA and 0.1% Triton X-100). The bound proteins were eluted with 2 × SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and analyzed by western blotting (WB). All of the WB experiments were developed with the ECL western blotting detection system (GE Healthcare) as recommended by the manufacturer. The antibodies used in this study are listed in Supplementary Table 2.

(f) The coimmunoprecipitation assay was performed as previously described41. Equal amounts of S2 cell lysates were immunoprecipitated with antibodies to CUP, Thor, C+NC*, or HA. Bound complexes were eluted with 2 × SDS–PAGE sample buffer and analyzed by western blotting with antibodies to eIF4G or eIF4E.

Figure 8 | The non-canonical motifs are required for 4E-BP-mediated eIF4E nuclear import. (a-x) S2 cells expressing HA-tagged versions of CUP (a-d, m-p), Thor (e-h, q-t) or 4E-T (i-l, u-x) or the corresponding 4E-BMs mutants (indicated on the left) were treated with LMB for 12 h (+ LMB) or with methanol as control (− LMB). The cells were fixed and the localization of the HA-tagged proteins and endogenous eIF4E was determined by indirect immunofluorescence using anti-HA and anti-eIF4E antibodies. The merged pictures show the HA signal in green and the eIF4E signal in red. Scale bar, 5 μm.
The eIF4E-associated proteins were pulled down, eluted and analyzed as described above. 

**ITC analysis.** The ITC experiments were performed using a VP-ITC microcalorimeter (MicroCal) at 20 °C. The solution of eLF4G (residues 69–248) or eLF4G (residues 1–200) in the calorimeter cell was titrated with tenfold concentrated solutions of GB1-stabilized peptides corresponding to 4E-BPs C + NC (10 μM), C (50 μM), L + NC (100 μM CUP, 200 μM Thor and 4E-T) or eLF4G (residues 578–650, 20 μM) that were dissolved in the same buffer (20 mM Na-phosphate (pH 7.2) and 150 mM NaCl). The titration experiments consisted of an initial injection of 2 μL followed by 28 injections of 10 μL at an interval of 240 s. Each binding experiment was performed twice. The thermodynamic parameters were estimated using a one-site binding model (Origin version 7.0), whereby the datapoint of the first injection was removed for the analysis.

**Translation repression assays.** S2 cells were transfected in 6-well plates using Effectene transfection reagent (Qiagen) according to the manufacturers protocol. The transfection mixtures contained: 0.1 μg of F-Luc reporter plasmid (F-Luc-V5), 0.3 μg of control RNA reporter, and increasing amounts of plasmids expressing HA-CUP (fragment 1–402; 0.05–0.2 μg) and HA-Thor (full length, 0.1–0.5 μg). The plasmids expressing the corresponding mutants or the HA peptide control were transfected at the highest concentration. In all the experiments, the cells were collected three days after transfection. The F-Luc activity was measured using the Dual-Lucerase reporter assay system (Promega). The northern blotting was performed as previously described. The F-Luc mRNA levels were determined by northern blotting and were normalized to those of the control RNA. The normalized F-Luc mRNA levels were then used to normalize the F-Luc activity to obtain translation efficiencies.

**Immunofluorescence.** S2 cells expressing HA-tagged versions of CUP, Thor and 4E-T or the indicated mutants were treated with Leptomycin B (100 nM; Sigma) or indicated control for 12 h. Cells were fixed with 4% paraformaldehyde for 10 min. The cells were then permeabilized with 0.1% Triton X-100 in PBS (10 min) and stained with affinity-purified monoclonal anti-HA (Covance 1:1,000) and polyclonal anti-eLF4G (1:2,000) antibodies in PBS containing 1% BSA for 1 h. Alexa Fluor 594-labeled goat anti-rabbit and 488-labeled anti-mouse antibodies (Invitrogen) were used at dilutions of 1:1,000 and 1:2,000, respectively. The cells were mounted using Fluoromount-G (Southern Biotech). The images were acquired at room temperature using a confocal microscope (TCS SP2; Leica) that was fitted with a Plan-Apochromat × 100 NA 1.40 oil immersion objective and a series of three photomultipliers (Hamamatsu Photonics) control with the Leica confocal software (version 2.61). The images were prepared using Photoshop (Adobe).

**References**

1. Jordan, J. R., Hellen, C. U. & Pestova, T. V. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat. Rev. Mol. Cell Biol.* **11**, 113–127 (2010).

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

3. Hershey, P. E. et al. The Cap-binding protein eIF4E promotes folding of a functional domain of yeast translation initiation factor eIF4G1. *J. Biol. Chem.* **274**, 21297–21304 (1999).

4. Mader, S., Lee, H., Pause, A. & Sonenberg, N. The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4G and the translational repressors 4E-binding proteins. *Mol. Cell Biol.* **15**, 163–173 (1995).

5. Marcatri ograno, J., Gingras, A. C., Sonenberg, N. & Burley, S. K. Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. *Mol. Cell* **3**, 707–716 (2003).

6. Altman, M., Schmitz, N., Bernst, C. & Trachsel, H. A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E. *EMBO J.* **16**, 1114–1121 (1997).

7. Matsuo, H. et al. Structure of translation factor eIF4E bound to m7GDP and interaction with 4E-binding protein. *Nat. Struct. Biol.* **4**, 717–724 (1997).

8. Gingras, A. C. et al. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev.* **13**, 1422–1437 (1999).

9. Martineau, Y., Azar, R., Bousquet, C. & Pyronnet, S. Anti-oncogenic potential of the mammalian target of rapamycin (mTOR) signalling: specific role of eukaryotic initiation factor 4E-binding protein 2. *Eur. J. Biochem.* **323**, 62–69 (2016).

10. Bidinosti, M. et al. Postnatal deamidation of 4E-BP2 in brain enhances its association with raptor and alters kinetics of excitatory synaptic transmission. *Mol. Cell* **37**, 797–808 (2010).

11. Dowling, R. J. et al. mTORC1-mediated cellular proliferation, but not cell growth, controlled by the 4E-BPs. *Science* **328**, 1172–1176 (2010).

12. Chokkaz, C. G. et al. Antioxidant defence via deregulated eIF4E-dependent translational control. *Nature* **493**, 371–377 (2013).

13. Hsieh, A. C. et al. The translational landscape of mTOR signalling steers cancer initiation and metastasis. *Nature* **485**, 53–61 (2012).

14. Lasko, P., Cho, P., Poulin, F. & Sonenberg, N. Constraining mechanisms of regulating translation of specific Drosophila germline mRNAs at the level of 5'-cap structure binding. *Biochem. Soc. Trans.* **33**, 1544–1546 (2005).

15. Faivre, S., Kroemer, G. & Raymond, E. Current development of mTOR inhibitors as anticancer agents. *Nat. Rev. Drug Discov.* **5**, 671–688 (2006).
16. Jia, Y., Polonovsky, V., Bitterman, P. B. & Wagner, C. R. Cap-dependent translation initiation factor eIF4E: an emerging anticancer drug target. Med. Res.Rev. 32, 786–812 (2012).
17. Gross, J. D. et al. Ribosome loading onto the mRNA cap is driven by conformational coupling between eIF4G and eIF4E. Cell 115, 739–750 (2003).
18. Gosselin, P. et al. The translational repressor 4E-BP called to order by eIF4E: new structural insights by SAXS. Nucleic Acids Res. 39, 3496–3503 (2011).
19. Luketina, S., Bah, A., Lin, H., Sonenberg, N. & Forman-Kay, J. D. Interaction of the eukaryotic initiation factor 4E with 4E-BP2 at a dynamic bipartite interface. Structure 21, 2186–2196 (2013).
20. Mizuno, A. et al. Importance of C-terminal flexible region of 4E-Binding protein in binding with eukaryotic initiation factor 4E. FEBS Lett. 582, 3439–3444 (2008).
21. Nelson, M. R., Ledal, A. M. & Smibert, C. A. Drosophila cup is an eIF4E-binding protein that functions in Smaug-mediated translational repression. EMBO J. 23, 150–159 (2004).
22. Paku, K. S. et al. A conserved motif within the flexible C-terminus of the translational regulator 4E-BP is required for tight binding to the mRNA cap-binding protein eIF4E. Biochem. J. 441, 237–245 (2012).
23. Umenaga, Y., Paku, K. S., In, Y., Ishida, T. & Tomoo, K. Identification and function of the second eIF4E-binding region in N-terminal domain of eIF4G: comparison with eIF4E-binding protein. Biochem. Biophys. Res. Commun. 414, 462–467 (2011).
24. Nakamura, A., Sato, K. & Hanayo-Nakamura, K. Drosophila cup is an eIF4E-binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. Dev. Cell 6, 69–78 (2004).
25. Wilhelm, J. E., Hilton, M., Amos, Q. & Henzel, W. J. 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 (2003).
26. Zappavigna, V., Piccioni, F., Vilaescusa, J. C. & Verrotti, A. C. cup is a nucleocytoplasmatic shuttling protein that interacts with the eukaryotic translation initiation factor 4E to modulate Drosophila ovary development. Proc. Natl Acad. Sci. USA 101, 14800–14805 (2004).
27. Kinkel, K., Veith, K., Grunwald, M. & Bono, F. Crystal structure of a minimal eIF4E-Cup complex reveals a general mechanism of eIF4E regulation in translational repression. RNA 18, 1624–1634 (2012).
28. Ptushkina, M. et al. Cooperative modulation by eIF4G of eIF4E-binding to the mRNA 5’ cap in yeast involves a site partially shared by p20. EMBO J. 17, 4798–4808 (1998).
29. Pyronnet, S. et al. Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. EMBO J. 18, 270–279 (1999).
30. Kubacka, D. et al. Investigating the consequences of eIF4E2 (4EHP) interaction with 4E-transporter on its cellular distribution in HeLa cells. PLoS ONE 8, e72761 (2013).
31. Cheng, Y. & Patel, D. J. An efficient system for small protein expression and refolding. Biochem. Biophys. Res. Commun. 317, 401–405 (2004).
32. Siepenkov, S. V., Korneeva, N. L. & Rhoads, R. E. Kinetic mechanism for assembly of the mGpppG.eIF4E.eIF4G complex. J. Biol. Chem. 283, 25227–25237 (2008).
33. Dostie, J., Ferraiuolo, M., Pause, A., Adam, S. A. & Sonenberg, N. A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA 5’ cap-binding protein, eIF4E. EMBO J. 19, 3142–3156 (2000).
34. Rong, L. et al. Control of eIF4E cellular localization by eIF4E-binding proteins, 4E-BPs. RNA 14, 1318–1327 (2008).
35. Sukarieh, R., Sonenberg, N. & Pelletier, J. The eIF4E-binding proteins are modifiers of cytoplasmic eIF4E relocalization during the heat shock response. Am. J. Physiol. Cell Physiol. 296, C1207–C1217 (2009).
36. Ferraiuolo, M. A. et al. A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay. J. Cell Biol. 170, 913–924 (2005).
37. Wolff, B., Sangerl, J. J. & Wang, Y. Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. Chem. Biol. 4, 159–167 (1997).
38. Siddiqui, N. et al. Structural insights into the allosteric effects of 4EBP1 on the eukaryotic translation initiation factor eIF4E. J. Mol. Biol. 415, 781–792 (2012).
39. Igreja, C. & Izaurralde, E. CUP promotes deadenylation and inhibits decapping of mRNA targets. Genes Dev. 25, 1955–1967 (2011).
40. Zekri, L., Kuzuooglu-Ozturk, D. & Izaurralde, E. GW182 proteins cause PABP dissociation from silenced mRNA targets in the absence of deadenylation. EMBO J. 32, 1052–1065 (2013).
41. Trittshler, F. et al. Similar modes of interaction enable Trailer Hitch and EDC3 to associate with DCIP1 and Me31B in distinct protein complexes. Mol. Cell Biol. 28, 6695–6708 (2008).
42. Behm-Ansmant, I. et al. mRNA degradation by miRNAs and GW182 requires both CCR4-NOT deadenylase and DCIP1-DCIP2 decapping complexes. Genes Dev. 20, 1885–1898 (2006).
43. Diebold, M. L., Fribourg, S., Koch, M., Metzger, T. & Romier, C. Deciphering correct strategies for multiprotein complex assembly by co-expression: application to complexes as large as the histone octamer. J. Struct. Biol. 175, 178–188 (2011).
44. Studier, F. W. Protein production by auto-induction in high density shaking cultures. Protein Expr. Purif. 41, 207–234 (2005).
45. Mizioe, L. S. & Tellinghuisen, J. The role of backlash in the “first injection anomaly” in isothermal titration calorimetry. Anal. Biochem. 326, 125–127 (2004).