Supplemental Materials

Mextli proteins use both canonical bipartite and novel tripartite binding modes to form eIF4E complexes that display differential sensitivity to 4E-BP regulation

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DNA constructs

The plasmids used for the expression of *Dm* elF4E, elF4G, CUP (full-length or fragments) and chimeric 4E-BP in *Escherichia coli* (*E. coli*) or *Dm* S2 cells have been described previously (Igreja and Izaurralde 2011; Peter et al. 2015). The plasmids for the expression of HA- or GFP-tagged *Dm* Mxt in S2 cells were obtained by inserting the corresponding cDNA into the EcoRI and ApaI sites of the pAc5.1B-λN-HA and pAc5.1B-EGFP vectors. For expression in *E. coli*, DNA fragments encoding *Dm* Mxt residues 577–620 (C+NC, bipartite) and 577–640 (C+NC+α3, tripartite) were inserted into the XhoI and BamHI restriction sites of the pnEA-NpM vector (Diebold et al. 2011), producing an N-terminally fused MBP cleavable by HRV3C protease. A cDNA fragment encoding the *Ce* Mxt elF4E-binding region [residues 471–507 (C+NC)] was inserted into the NdeI and NheI restriction sites of the pnEA-NpM vector. The cDNA encoding full length *Ce* elF4E3 and fragments thereof (residues 30–215, trunc 1; and residues 1–215, trunc 2) were inserted into the NdeI and XhoI restriction sites of the pnYC-NpH vector, generating N-terminal fusions with the hexahistidine (His_{6}) tag cleavable by HRV3C protease. The cDNA fragment coding for the elF4E-binding region of *Ce* elF4G (residues 315–491) was inserted into the NdeI and XhoI restriction sites of the pnEA-NpM vector. The cDNA encoding the B1 domain of immunoglobulin-binding protein G (GB1) (Cheng and Patel 2004) was inserted at the C-terminal end of the Mxt fragments by site-directed mutagenesis. All of the mutants used in this study were generated by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene) and are listed in Supplemental Table S1.
**NMR measurements**

All spectra were recorded at 298 K. The $^{15}$N-HSQC experiment for the GB1-stabilized Mxt peptide (residues 577–640) was recorded at 600 MHz on a Bruker AVIII-600 spectrometer. The $^{15}$N-TROSY experiments for the eIF4E–Mxt-GB1 complex were measured at 800 MHz on a Bruker AVIII-800 spectrometer. The $^{15}$N-labeled Mxt–GB1 protein was saturated with a 1.5-fold molar excess of unlabeled full-length *Dm* eIF4E. The molecular weight of the proteins used in the NMR-experiments was determined based on their tumbling behavior in solution. Both diffusion experiments (before and after the addition of full length *Dm* eIF4E to the Mxt–GB1 protein) were recorded at 600 MHz.

**Crystallization**

Crystals of *Dm* eIF4E (residues 69–248) copurified with a *Dm* Mxt fragment (residues 577–640) and supplemented with a 1.5-fold molar excess of m$^7$GpppG cap analog (New England BioLabs) were obtained by the hanging-drop vapor-diffusion method at 18°C. Crystals appeared one day after mixing the protein solution (12 mg/ml) with a reservoir solution containing 0.1 M MMT buffer and 23% PEG 1500. MMT buffer consists of a 1:2:2 molar ratio of DL-malic acid, 2-(N-morpholino)ethanesulfonic acid (MES), and Tris (pH 4.0), according to the PACT Suite Buffer protocols (Qiagen). Crystals of *Dm* eIF4E (residues 69–248) in complex with *Dm* Mxt (residues 577–640; 20 mg/ml) without the cap analog were obtained by the same method. Crystals appeared in one day over a reservoir of 0.1 M Bis-Tris (pH 6.0) and 23% PEG 3350. The complex of *Ce* eIF4E (residues 30–215), *Ce* Mxt (residues 471–507) and the m$^7$GpppG cap analog (New England BioLabs) was crystallized using the same method. Crystals appeared after two to three days over a
reservoir of 0.1 M sodium acetate (pH 5.0), 0.01 M ZnCl₂ and 18% PEG 6000. The same Ce complex but without the cap-analog was crystallized over a reservoir of 0.1 M Tris (pH 8.0), 0.2 M MgCl₂ and 17% PEG 6000, with crystals appearing after one day. For cryoprotection, crystals were soaked briefly in the reservoir solution supplemented with 10–15% glycerol followed by flash-freezing in liquid nitrogen.

Structure determination

Diffraction data were collected at a wavelength of 1.000 Å on a PILATUS 6M detector at the PXII beamline of the Swiss Light Source. Data were processed with XDS and scaled using XSCALE (Kabsch 2010). Crystals of the Dm eIF4E-Mxt complex and the cap analog diffracted to a resolution of 2.16 Å. The structure was solved by molecular replacement using PHASER (McCoy et al. 2007) with the Dm eIF4E structure (PDB-ID 4UE8) (Peter et al. 2015) as the search model. The molecular replacement solution was used to rebuild an initial model of Dm eIF4E using the PHENIX AutoBuild wizard (Terwilliger et al. 2008) to prevent model bias. To complete and improve the initial model of eIF4E, iterative cycles of model building and refinement were carried out using COOT (Emsley et al. 2010) and PHENIX (Afonine et al. 2012) against the unmodified native dataset. The Mxt peptide and the m⁷GpppG cap analog were then built into the weighted F₀-Fc difference density using COOT, and the structure of the complex was further refined using PHENIX. The stereochemical restraints library for the m⁷GpppG cap analog was generated using GRADE (Smart et al. 2011) for subsequent refinement. For the final model, 99.12% of all residues are in the favored regions of the Ramachandran plot and there are no outliers.
Crystals of the Dm eIF4E–Mxt complex without the cap analog diffracted to a resolution of 2.13 Å. The crystal belongs to space group P2₁ with a β-angle of 90.1° and further data analysis with PHENIX.XTRIAGE (Zwart et al. 2005) indicated the presence of translational pseudosymmetry, generating a strong peak in the Patterson map (37.3% of the origin). Nevertheless, the structure was solved by molecular replacement as described above with the difference being that the asymmetric unit contained four copies of the search model. The structures were rebuilt using the PHENIX AutoBuild wizard (Terwilliger et al. 2008) to prevent model bias. The initial models of eIF4E were then manually completed in COOT (Emsley et al. 2010) and refined using PHENIX (Afonine et al. 2012). The Mxt peptide was built into the weighted Fₒ-Fc difference density in COOT and the complex was refined using BUSTER with automated NCS restraints and TLS refinement (Bricogne et al. 2011; Smart et al. 2012). For the final model, 97.5% of all residues are in the favored regions of the Ramachandran plot and there are no outliers.

Crystals of the Ce eIF4E-Mxt complex without the cap analog diffracted to a resolution of 1.66 Å. Because of bound Zn²⁺ ions from the crystallization condition, there was a strong anomalous signal that was used to derive unbiased experimental phases. Zn²⁺-sites were identified with SHELX (Sheldrick 2010) and were used to build an initial model of the Ce eIF4E-Mxt complex using PHENIX AutoSol (Terwilliger et al. 2009). The model was manually completed and improved in COOT (Emsley et al. 2010) and refined using PHENIX (Afonine et al. 2012). Because the m⁷GpppG cap analog was not fully visible, an m⁷GTP molecule was placed into the weighted Fₒ-Fc difference density in COOT, and the Zn²⁺-sites were confirmed by examining the anomalous difference Fourier map. The final model was refined using
PHENIX and 99% of all residues are in the favored regions of the Ramachandran plot and there are no outliers.

Crystals of the Ce eIF4E-Mxt complex with no cap analog diffracted to a resolution of 1.95 Å. The structure was solved by molecular replacement using PHASER (McCoy et al. 2007). The experimentally phased structure of the Ce eIF4E-Mxt complex including the cap analog was used as the search model. To prevent model bias, the three complexes found in the asymmetric unit were rebuilt using the PHENIX AutoBuild wizard (Terwilliger et al. 2008). The models were improved in COOT (Emsley et al. 2010) and refined using PHENIX (Afonine et al. 2012). For the final model, 97.5% of all residues are in the favored regions of the Ramachandran plot and there are no outliers.

The stereochemical properties for all of the structures were verified using MOLPROBITY (Chen et al. 2010). Structural images were prepared using PyMOL (http://www.pymol.org). The diffraction data and refinement statistics are summarized in Table 1.

**ITC analysis**

*Dm* eIF4E (residues 69–248) used for ITC measurements was purified as described previously (Igreja et al. 2014). The ITC experiments were performed using a VP-ITC microcalorimeter (MicroCal) at 20°C as described previously (Igreja et al. 2014; Peter et al. 2015). The solution of wild-type eIF4E (residues 69–248, 1.0 or 2.0 µM) or eIF4E II-AA mutant (residues 69–248, 9.5 µM) in the calorimetric cell was titrated with tenfold concentrated solutions of GB1-stabilized Mxt peptides (residues 577–620, 20 µM; residues 577–640, 10 µM for eIF4E WT and 95 µM for eIF4E II-AA mutant) that were dissolved in the same buffer (20 mM Na-phosphate pH 7.0 and 150
mM NaCl). The titration experiments consisted of an initial injection of 2 µl followed by 28 injections of 10 µl at 240 s intervals. Each binding experiment was repeated three times. The thermodynamic parameters were calculated using a one-site binding model (Origin version 7.0). The datapoint of the first injection was removed for the analysis (Mizoue and Tellinghuisen 2004).
**Supplemental Table S1.** Mutants and constructs used in this study.

| Protein | Name of the construct | Fragments / mutations | Binding site / motif |
|---------|-----------------------|-----------------------|----------------------|
| **Dm eIF4E-2** | 4E | Full length | | |
| (1–248) (isoform C) | II-AA | I96A, I112A | Lateral surface | |
| P48598-2 | W106A | W106A | Dorsal surface | |
| NH-EE | N110E, H111E | | | |
| trunc | 69–248 | | | |
| **Dm eIF4G** | 4G | 578–650 | Extended eIF4E-binding region | |
| (1–1666) (isoform A) | Mxt | Full length | | |
| O61380 | C+NC+α3 | 577–640 | Tripartite eIF4E-binding region | |
| | C+NC | 577–620 | Bipartite eIF4E-binding region | |
| | C* | Y581A, L586A, L587A | Canonical motif mutant | |
| | Elbow1* | W596D | Elbow 1 loop mutant | |
| | Nc-L* | L598D | Non-canonical linker mutant | |
| | NC1* | W602D | Non-canonical motif mutants | |
| | NC2* | M605D | | |
| | IL-AA | 1612A, L613A | | |
| | C*+IL-AA | Y581A, L586A, L587A, 1612A, L613A | Canonical motif + auxiliary linker mutant | |
| | α3* | F625E | Auxiliary helix mutant | |
| | FY-EE | F625E, Y630E | | |
| | IS-RR | I583R, S590R | Arg mutant | |
| **CUP** | C+NC | 325–376 | eIF4E-binding region | |
| (1–1117) | 1–417 | | CUP N-terminal eIF4E-binding region | |
| Q9VMA3 | | | | |
| **Ce Mxt** | C+NC | 471–507 | eIF4E-binding region | |
| (1–507) | C* | Y579A, L584A, M585A | Canonical motif mutant | |
| Q9XW13 | NC 1* | I497D, I504D | | |
| | NC 2* | M493D, I497D, I504D | Non-canonical motif mutants | |
| | NC 3* | M493D | | |
| **Ce eIF4G** | 4G | 315–491 | Extended eIF4E-binding region | |
| (1–1156) | | | | |
| Q21531 | | | | |
| **Ce eIF4E3** | W68A | W68A | Dorsal surface mutant | |
| (1–251) | VI-AA | V58A, I74A | Lateral surface mutant | |
| | trunc 1 | 30–215 | | |
| | trucn 2 | 1–215 | | |
**Supplemental Table S2.** Thermodynamic parameters for the interaction of eIF4E with Mxt peptides.

| Mxt peptide | $K_D$ (M) | $\Delta H$ (kcal mol$^{-1}$) | $\Delta S$ (kcal mol$^{-1}$) | $\Delta G$ (kcal mol$^{-1}$) | Molar ratio |
|-------------|-----------|-----------------|-----------------|-----------------|-------------|
| 577–620     | 5.0 ± 3.4 × 10$^{-9}$ | -13.1 ± 0.4     | 1.9              | -11.2           | 0.98 ± 0.02 |
| 577–640     | 0.52 ± 0.09 × 10$^{-9}$ | -32.4 ± 0.6     | 19.9             | -12.5           | 1.00 ± 0.03 |

**eIF4E (69–248) II-AA mutant**

| Mxt peptide | $K_D$ (M) | $\Delta H$ (kcal mol$^{-1}$) | $\Delta S$ (kcal mol$^{-1}$) | $\Delta G$ (kcal mol$^{-1}$) | Molar ratio |
|-------------|-----------|-----------------|-----------------|-----------------|-------------|
| 577–640     | 0.3 ± 0.12 × 10$^{-6}$ | -18.3 ± 1.3     | 9.5              | -8.8            | 1.00 ± 0.02 |
Supplemental Table S3. Interface areas of 4E-BP-Elf4E complexes.
The covered surface areas on Elf4E by the indicated 4E-BPs or Dm Elf4G and the
individual motifs have been calculated using the PISA program from the CCP4 suite.

| Protein | motif | area (Å²) |
|---------|-------|-----------|
| *Dm Mxt* | Canonical helix (α1) | 471 |
| Dm Cyt | Canonical + auxiliary helix (α1+α3) | 655 |
| Complete 4E-binding region | 1498 |
| *Ce Mxt* | Canonical helix (α1) | 611 |
| Complete 4E-binding region | 1370 |
| *Dm Thor* | Canonical helix | 632 |
| Complete 4E-binding region | 1450 |
| *Dm CUP* | Canonical helix | 539 |
| *Dm 4E-T* | Canonical helix | 584 |
| Complete 4E-binding region | 1370 |
| *Dm Elf4G* | Canonical helix | 529 |
| *Hs 4E-BP1* | Canonical helix | 550 |
| Complete 4E-binding region | 1235 |

Supplemental Table S4. Antibodies used in this study.

| Antibody | Source | Catalog Number | Dilution | Monoclonal/ Polyclonal |
|----------|--------|----------------|----------|------------------------|
| Anti-HA-HRP (Western blot) | Roche | 12 013 819 001 | 1:5,000 | Monoclonal |
| Anti-HA (IP) | Covance | MMS-101P | 1:1,000 | Monoclonal |
| Anti-*Dm* Elf4E | In house | | 1:3,000 | Rabbit polyclonal |
| Anti-*Dm* Elf4G | In house | | 1:3,000 | Rabbit polyclonal |
| Anti-GFP | In house | | 1:2,000 | Rabbit polyclonal |
| Anti-*Dm* 4E-T | Kindly provided by P. Lasko | | 1:2,000 | Rabbit polyclonal |
| Anti-rabbit-HRP | GE Healthcare | NA934V | 1:10,000 | Polyclonal |
Supplemental Figure S1. Structure-based alignment of eIF4E sequences. (A) Western blot showing the interaction of HA-tagged *Dm* eIF4E (either wild-type or mutated) with endogenous eIF4G and GFP-tagged *Dm* CUP (residues 1–417). The inputs (0.6% for eIF4G, 3% for eIF4E and 2% for CUP) and immunoprecipitates (25% for eIF4G and 20% for eIF4E and CUP) were analyzed by western blotting using anti-HA, anti-eIF4G and anti-GFP antibodies. (B) Structure-based sequence alignment of eIF4E orthologous proteins from *Drosophila melanogaster* (*Dm*).
are eight eIF4E isoforms in *D. melanogaster*: 4E-1 (P48598) and 4E-2 (P48598-2; used in this study) are encoded by the same gene and result from alternative splicing, 4E-3 (Q9VSG), 4E-4 (Q9VRY0), 4E-5 (Q9VSB6), 4E-6 (Q9VAR1), 4E-7 (Q9W5B3) and 4E-HP (Q8T3K5) (Hernandez et al. 2005). *Dm* Mxt interacts with eIF4E-1, -2, -3, -4 and -7 in yeast two-hybrid assays (Hernandez et al., 2013). Conserved residues are highlighted with a black background and printed in white. Residues with >70% similarity are shown with a light gray background. Secondary structure elements for *Dm* eIF4E-2 (used in this study) are indicated above the sequences. The Trp residues coordinating the m$^7$GTP nucleotide are indicated by red asterisks. The lateral and dorsal binding surfaces (BS) are indicated by a line below the sequences. Residues mutated in this study are indicated with red open circles above the sequences. The dashed red box highlights residues N110 and H111 that coordinate interactions with the linker regions in the tripartite binding region of *Dm* Mxt. (C) Structure-based alignment of *Ce* eIF4E sequences. Five eIF4E proteins have been described in *C. elegans*: 4E-1 (IFE-1, O45551), 4E-2 (IFE-2, Q21693), 4E-3 (IFE-3, O61955, used in this study), 4E-4 (4E-HP, Q22888) and 4E-5 (IFE-5, P56570) (Jankowska-Anyszka et al. 1998). Symbols and labels are as in panel (B). Secondary structure elements for *Ce* eIF4E-3 are indicated above the sequences.
Supplemental Figure S2. Structure-based alignment of the eIF4E-binding region of Mextl proteins. (A) Structure-based sequence alignment of Mxt orthologous proteins from Drosophila species [Drosophila melanogaster (Dm), Drosophila willistoni (Dwi), Drosophila grimshawi (Dgr), Drosophila simulans (Dsi), Drosophila sechellia (Dse), Drosophila yakuba (Dya), Drosophila erecta (Der), Drosophila ananassae (Dan), Drosophila pseudoobscura (Dps), Drosophila persimilis (Dpe), Drosophila
virilis (Dvi), Drosophila mojavensis (Dmo)], from non-Drosophila Dipterans
[mosquitos: Aedes aegypti (Aae), Anopheles gambiae (Aga)], from non-Dipterans
Insects [bee: Apis mellifera (Ame); wasp: Nasonia vitripennis (Nv); ants: Atta
cephalotes (Ace), Solenopsis invicta (Sin); silmoth: Bombyx mori (Bmo); human
body louse: Pediculus humanus (Hm); beetle: Tribolium castaneum (Tcas)], from
non-Insects Arthropods [centipede: Strigamia maritime (Smm); flea: Daphnia pulex
(Dpu); mite: Tetranychus urticae (Tur)] and from Nematodes [worms:
Caenorhabditis brenneri (Cbe), Caenorhabditis elegans (Ce), Caenorhabditis
remanei (Cre), Caenorhabditis briggsae (Cbr)]. Invariant residues are highlighted
with a dark-blue background and printed in white. Residues with >70% similarity are
shown with a light blue color background. Secondary structure elements are indicated
above and below the sequences for the Dm and Ce proteins, respectively. Residues at
positions 2 and 9 of the canonical motifs of Mxt are indicated with a magenta
background. The canonical (C), non-canonical (NC) and auxiliary 4E-BMs are boxed
in black. Mxt residues in the motifs contacting eIF4E are indicated by red open
circles. (B) Secondary structure prediction for the eIF4E-binding region of Mxt
orthologous proteins using the Ali2D software (http://toolkit.tuebingen.mpg.de/). The
degrees of confidence for β-strands (in blue) and α-helices (in red) are indicated by
increasing color intensities. Species are the same as in panel (A).
Supplemental Figure S3. The tripartite Dm Mxt peptide is unfolded in solution. (A–C) Isothermal titration calorimetry data for the interactions of Dm eIF4E (residues 69–248, wild type or II-AA mutant) with the indicated Dm Mxt peptides. The thermodynamic parameters are shown in Table S2. (D) $^{15}$N-HSQC spectra of purified $^{15}$N-labeled Dm Mxt C+NC+α3 peptide C-terminally fused to GB1. The spectrum displays two distinct sets of peaks: a well dispersed spectrum with high intensity peaks corresponding to the folded GB1 protein (green) and a second spectrum with lower intensity and a limited $^1$H resonance dispersion, which is characteristic of disordered proteins (Mxt; blue). Diffusion experiments indicate that the Mxt-GB1 fusion protein is monomeric in solution (approximately 14 kDa). Black lines indicate overlapping spectra for Mxt and GB1. (E) $^{15}$N-TROSY spectra of purified $^{15}$N-labeled...
Dm Mxt C+NC+α3 peptide C-terminally fused to GB1 after addition of purified unlabeled Dm eIF4E. Two distinct sets of spectra are also identified. In the GB1 protein spectra (gray), the peaks do not show any chemical shift, suggesting that GB1 does not interact with eIF4E. The spectra corresponding to the Mxt peptide (red) gained tremendously in resonance dispersion upon addition of eIF4E. Many peaks left the disordered region of the spectra reflecting a disorder-to-order transition upon binding to eIF4E. The molecular weight of the eIF4E-Mxt-GB1 complex estimated by diffusion experiments corresponds to a 1:1 stoichiometry.
Figure S4

A Dm Mextl (cap-free complex)

Complex A  Complex B

Complex C  Complex D

B Dm Mextl (cap-free complex, complex A)

C Superposition cap-bound / cap-free complexes

Dm Mextl (cap-bound complex)

Dm Mextl (cap-free complex)

C e Mextl (cap-free complex)

Complex B

Complex A  Complex C

D Ce Mextl (cap-free complex)

E Ce Mextl (cap-free complex, complex A)

F Superposition cap-bound / cap-free complex

Ce Mextl (cap-bound complex)

Ce Mextl (cap-free complex)
Supplemental Figure S4. Structure of the *Dm* and *Ce* eIF4E-Mxt complexes in the absence of the cap analog. (A) Cartoon representation showing the cap-free crystal form of the *Dm* eIF4E-Mxt complex. The asymmetric unit of the crystal contained four complexes. (B) Overview of one *Dm* eIF4E–Mxt complex from the cap-free crystal form. The region of the Mxt peptide with structural similarity to other 4E-BPs is colored in yellow. The auxiliary linker and helix are colored in blue. Selected secondary structure elements are labeled in black for eIF4E and in color for Mxt. (C) Structural overlay of the *Dm* eIF4E–Mxt in the cap bound and cap-free state. The two complexes superimpose with an RMSD of 0.38 Å over 233 Cα atoms. (D) Cartoon representation showing the crystal form of the *Ce* eIF4E-Mxt complex in the absence of the cap analog. The asymmetric unit of the crystal contained three complexes. (E) Overview of one complex from the cap-free crystal form. (F) Structural overlay of the *Ce* Mxt-eIF4E complexes in the cap-bound (green) and cap-free (red) form. Selected secondary structure elements are labeled in black for eIF4E and in color for Mxt. The two complexes superimpose with an RMSD of 0.38 Å over 207 Cα atoms.
**Supplemental Figure S5.** Comparison of the *Dm* eIF4E-Mxt complex with the CUP and Thor complexes. (A) Overlay of the *Dm* eIF4E-Thor complex (PDB ID: 4UE8; Peter et al., 2015) and the *Dm* eIF4E-Mxt complex. The eIF4E molecule from the eIF4E-Thor complex has been removed for clarity. (B) Overlay of the elbow loops of *Dm* Mxt and *Dm* Thor reveals structurally similar residues, shown in colored sticks. (C) Overlay of the *Dm* eIF4E-CUP complex (PDB ID: 4AXG; Kinkelin et al., 2012) and the *Dm* eIF4E-Mxt complex bound to the cap analog. The eIF4E molecule from the eIF4E-CUP complex has been removed for clarity. (D–F) Close-up views of the
relative orientations of the non-canonical helices of \textit{Dm} Mxt, \textit{Ce} Mxt and \textit{Dm} CUP bound to the lateral hydrophobic pocket of eIF4E. Helices are represented as cylinders.

\textbf{Figure S6}

\textbf{A} \textit{Dm} Mxt

\textbf{B} \textit{Ce} Mxt

\textbf{Supplemental Figure S6}. Binding interfaces of \textit{Dm} and \textit{Ce} Mxt on eIF4E. (\textit{A}, \textit{B}) Surface representation of eIF4E in complex with \textit{Dm} and \textit{Ce} Mxt. Residues of eIF4E within a distance of 4 Å from the bound Mxt peptide are shown in color. Selected Mxt residues that mediate major surface contacts at the dorsal and lateral surfaces of eIF4E are shown in sticks. The residues present at positions 2 and 9 of the \textit{Dm} and \textit{Ce} canonical motifs are underlined. For \textit{Ce} Mxt, the contribution of the nc-L from the cap-free crystal structure is indicated in salmon.
Supplemental Figure S7. Validation of the eIF4E-Mxt interface. (A) *E. coli* lysates expressing MBP-tagged *Dm* Mxt fragments (WT or mutated), C-terminally fused to GB1, were incubated with SHN-tagged full length eIF4E. Protein complexes were
pulled down using amylose resin. Inputs (0.5%) and bound fractions (30%) were visualized on a SDS-PAGE gel followed by Coomassie staining. (B) The interaction of HA-tagged *Dm* Mxt (full length, either wild-type or mutated) with endogenous eIF4E was tested in *Dm* S2 cells. The proteins were immunoprecipitated using anti-HA antibodies. The inputs (2% for Mxt and 0.5% for eIF4E) and immunoprecipitates (15% for Mxt and 25% for eIF4E) were analyzed by western blotting using anti-HA and anti-eIF4E antibodies. (C–E) Western blotting showing the interaction of HA-tagged *Dm* eIF4E (full length, wild-type or mutants) with GFP-tagged *Dm* Mxt (full length, C), Thor (D) or CUP (1–417, E) and endogenous 4E-T and eIF4G (D,E). Input samples (1.5% for eIF4E, Thor and 4E-T; 10% for Mxt; 0.3% for eIF4G and 2% for CUP) and immunoprecipitates (15% for eIF4E; 30% for Mxt and 25% for Thor, 4E-T, eIF4G and CUP) were analyzed as described in (B). (F) Ni-NTA pulldown assay showing the association of His6-tagged *Ce* eIF4E (30–215) with MBP-tagged *Ce* Mxt eIF4E-binding region (471–507, either wild-type or carrying the indicated mutations). Samples were analyzed as described in (A). Protein mutants are described in Table S1. Inputs (0.2%) and bound fractions (12.5%) were analyzed on a SDS-PAGE gel followed by Coomassie staining. (G) Purified *Dm* eIF4E-eIF4G complexes (2µM) containing SHN-tagged eIF4E (full length, WT or the II-AA mutant) and GST-eIF4G (residues 578–650) were incubated with two-fold molar excess of the *Dm* Mxt tripartite peptide (C+NC+α3) C-terminally fused to GB1. MBP served as negative control. The eIF4E-interacting proteins were pulled down using Ni-NTA beads. The competitor proteins are labeled in blue and marked by blue dashed boxes. The black dashed box indicates the position of GST-eIF4G. Lanes 1–4 show the purified peptides and complexes (starting material, SM) used in the competition assay. (H) The amounts of GST-eIF4G bound to eIF4E in each experimental condition were
determined using the Image J software after Coomassie blue staining. To rule out the possibility that changes in the levels of GST-eIF4G resulted from variations in the loading volume, all values were normalized to the levels of SHN-eIF4E present in each condition. These values were set to 100% in the presence of MBP. The mean values ± SD from three independent experiments are shown.
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