eIF3d is an mRNA cap-binding protein that is required for specialized translation initiation

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Eukaryotic mRNAs contain a 5′ cap structure that is crucial for recruitment of the translation machinery and initiation of protein synthesis. mRNA recognition is thought to direct interactions between eukaryotic translation initiation factor 4E (eIF4E) and the mRNA cap. However, translation of numerous capped mRNAs remains robust during cellular stress, early development, and cell cycle progression despite inactivation of eIF4E. Here we describe a cap-dependent pathway of translation initiation in human cells that relies on a previously unknown cap-binding activity of eIF3d, a subunit of the 800-kilodalton eIF3 complex. A 1.4 Å crystal structure of the eIF3d cap-binding domain reveals unexpected homology to endonucleases involved in RNA turnover, and allows modelling of cap recognition by eIF3d. eIF3d makes specific contacts with the cap, as exemplified by cap analogue competition, and these interactions are essential for assembly of translation initiation complexes on eIF3-specialized mRNAs such as the cell proliferation regulator c-Jun (also known as JUN). The c-Jun mRNA further encodes an inhibitory RNA element that blocks eIF4E recruitment, thus enforcing alternative cap recognition by eIF3d. Our results reveal a mechanism of cap-dependent translation that is independent of eIF4E, and illustrate how modular RNA elements work together to direct specialized forms of translation initiation.

The rate-limiting step of translation initiation is the recognition of the 5′ cap structure by eIF4E3,4. eIF4E activity is highly regulated by extracellular stimuli, predominantly through steric hindrance by eIF4E-binding proteins (4E-BPs)5,6. The translational efficiencies of mRNAs range in sensitivity to 4E-BP inhibition7–9, and these differences have conventionally been addressed by categorizing translation into cap-dependent versus cap-independent pathways10. However, the mechanisms underlying mRNA sensitivity to active eIF4E levels remain enigmatic as all cellular mRNAs maintain the same 5′ cap structure11.

Recently, we discovered a new translation pathway driven by RNA interactions with eIF3 that is used by a subset of cell proliferation mRNAs, with the prototype member being the mRNA encoding the early response transcription factor c-Jun. eIF3-specialized translation is cap-dependent and requires recruitment of eIF3 to an internal stem–loop structure in the 5′ untranslated region (UTR). However, the translational efficiency of a subset of these mRNAs is unaffected by eIF4E inactivation11–13, suggesting that cap recognition may proceed by a non-canonical mechanism (Supplementary Table 1).

To understand how cap recognition occurs during eIF3-specialized translation, we examined whether c-Jun mRNA uses the canonical eIF4E cap-binding complex during initiation. We programmed in vitro translation extracts from human 293T cells with capped and polyadenylated c-Jun mRNA, and isolated the 48S complex to assess the presence of the eIF4F factors (eIF4G1, eIF4A1 and eIF4E) (Fig. 1a, b). Unexpectedly, although c-Jun mRNA translation initiation complexes contain eIF3 and the small ribosomal subunit, they are depleted of all eIF4F components. By contrast, eIF4F is readily detectable in 48S initiation complexes formed on a canonical eIF4E-dependent mRNA, ACTB12 (Fig. 1b). In agreement with the absence of eIF4F, c-Jun levels are unaffected by cell treatment with the mTOR inhibitor INK128 (ref. 7), which inactivates eIF4E, or with eIF4A inhibitors13 (Extended Data Fig. 1). These results indicate that c-Jun mRNA translation occurs independently of eIF4F and that the process of eIF3-specialized translation is fundamentally distinct at the initial stage of 5′ cap recognition. eIF3-specialized translation requires recognition of an internal RNA stem–loop for efficient translation. Therefore, we asked whether eIF3
might also be involved in 5′ cap recognition. In agreement with the previously demonstrated RNA-binding capability of eIF3, the four eIF3 RNA-binding subunits, eIF3a, eIF3b, eIF3d and eIF3g, provide RNase protection to internally 32P-labelled c-Jun 5′ UTR RNA after UV254-induced crosslinking2 (Fig. 1c). By contrast, when the 32P label is placed in the 5′ cap of c-Jun mRNA, RNase protection is observed with a single subunit of eIF3, corresponding to eIF3d (Fig. 1c, Extended Data Fig. 2a). We confirmed subunit identity by limited proteolysis and mass spectrometry, and defined a C-terminal region of eIF3d that is responsible for protection of the 5′ mRNA terminus (Extended Data Fig. 2). The mapped C-terminal region of eIF3d is broadly conserved throughout plant, fungal and animal phylogeny (Fig. 2a, Extended Data Table 1, Extended Data Fig. 3). The structure of eIF3d therefore reveals a new cap-binding domain.

To understand how eIF3d recognizes the 5′ RNA terminus, we determined a 1.4 Å crystal structure of the conserved C-terminal domain of eIF3d from Nasonia vitripennis (65% identical, 84% similar to human eIF3d) using sulfur anomalous dispersion for phase determination (Extended Data Table 1, Extended Data Fig. 3). The structure of eIF3d reveals a complex fold that forms a cup-shaped architecture with a positively charged central tunnel that is negatively charged at its base (Fig. 2b). Remarkably, despite no significant sequence homology, the structural topology of eIF3d is nearly identical to the DXO proteins, a recently described family of 5′ cap-endonucleases involved in RNA quality control14–16 (Fig. 2c, Extended Data Fig. 4). In contrast to DXO, eIF3d contains a unique insertion of ~15 highly conserved amino acids between strand β5 and helix α6. The eIF3d-specific insertion folds down along the front face of the domain, making loosely packed charged interactions that close off the RNA binding tunnel (Extended Data Fig. 5). We term this insertion an ‘RNA gate’, as the sequence clashes with the path of single-stranded RNA (ssRNA) bound to DXO15 and must undergo a conformational change for eIF3d to become competent for RNA recognition (Fig. 2d). We determined the structure of eIF3d in two additional crystal forms, and confirmed the RNA gate exhibits a closed conformation regardless of crystal packing (Extended Data Fig. 6). As eIF3d does not bind all capped RNAs17,18, we postulate that the RNA gate regulates cap recognition to prevent promiscuous mRNA binding before assembly of eIF3d into the full eIF3 complex. We tested this model using c-Jun mRNA, and verified that eIF3d cap-recognition only occurs in the context of a full eIF3 complex and requires previous eIF3-sequence-specific RNA interactions with the eIF3-recruitment stem–loop (Extended Data Fig. 7). Allosteric communication between eIF3 subunits during initial RNA recruitment likely facilitates eIF3d RNA gate opening to allow 5′ end recognition. The structure of eIF3d therefore reveals a new cap-binding protein and explains the ability of the eIF3 complex to protect the 5′ end of mRNA (Fig. 1c).

To validate the structural finding that eIF3d is a cap-binding protein, we examined the ability of eIF3 to bind the c-Jun mRNA 5′ cap in the presence of competitor ligands. eIF3d cap recognition is sensitive to m7GDP competition but resistant to GDP, indicating that, analogous to eIF4E1, eIF3d specifically interacts with the 5′ cap and requires a mature methylated cap structure for recognition (Fig. 3a). Using the DXO–RNA structure as a template15, we modelled a capped ssRNA along the basic binding groove shared between eIF3d and DXO and identified two conserved helices (α5 and α11) likely to be involved in cap recognition (Fig. 3b). We purified recombinant eIF3 containing helix α5- or α11-mutated eIF3d and demonstrated that both mutants have markedly reduced ability to crosslink to the c-Jun mRNA cap (Fig. 3c). eIF3d-mutated complexes retain wild-type levels of RNA-binding, indicating that these residues specifically coordinate 5′ mRNA cap recognition (Extended Data Fig. 8). We next introduced haemagglutinin (HA) epitope-tagged wild-type or mutant eIF3d into 293T cells, and measured the assembly of 48S initiation complexes on c-Jun

Figure 2 | Structure of eIF3d reveals a conserved cap-binding domain. a, Cartoon schematic and phylogenetic conservation of eIF3d amino acid sequence according to physicochemical property similarity. Peptides in the cap-binding domain as identified by limited proteolysis are mapped below. b, Structure of the eIF3d cap-binding domain. α-helices are coloured in blue and β-strands in magenta. c, Topological maps of the eIF3d cap-binding domain and the DXO cap-endonuclease domain15. d, Structures comparing the eIF3d cap-binding domain with its gate insertion to DXO (PDB 4J7L).
To identify the eIF4F inhibitory element, we constructed luciferase
plex. In support, the 5' mRNA contains a 5' cap structure provides an alternative
cap-binding surface inhibit eIF3d cap-binding activity is required for efficient 48S
initiation complex formation on specific mRNAs. a Phosphorimage of SDS–PAGE gel resolving RNase-protected 32P-cap-labelled c-Jun 5' UTR RNA crosslinked to eIF3 in the presence of competitor ligands (m7GDP, GDP). b, Electrostatic surface view of the eIF3d cap-binding domain coloured by charge, with a zoomed view of ssRNA and cap analogue modelled according to their positions bound to DXO15. Positive charge is coloured blue, negative charge is in red, and the RNA gate is removed for clarity. c, Phosphorimage of SDS–PAGE gel resolving RNase-protected 32P-cap-labelled c-Jun 5' UTR RNA crosslinked to wild-type (WT) or helix α5- or helix α11-mutant eIF3d. Helix α5- or helix α11-mutant eIF3d: D249Q/V262I. d, Incorporation of c-Jun and ACTB mRNA into initiation complexes by wild-type, helix α5-, or helix α11-mutant eIF3d as measured by quantitative RT–PCR. The mRNA–ribosome association is expressed as the ratio of the quantity of mRNA transcripts to 18S RNA and normalized to the wild-type sample. The results are representative of three independent experiments and given as the mean ± s.d. from a representative quantitative RT–PCR experiment performed in duplicate.

Extended Data Fig. 8). These results demonstrate that cap binding by eIF3d is required for efficient initiation complex formation during eIF3-specialized translation.
eIF3d recognition of the 5' cap structure provides an alternative cap-dependent translation mechanism from canonical eIF4F cap recognition. Perplexingly, when the RNA stem–loop element that recruits eIF3 to the c-Jun mRNA is deleted, translation is inhibited even though the mRNA contains a 5' cap5. We proposed that an RNA element within the c-Jun mRNA blocks recruitment of the eIF4F complex. In support, the 5' cap of c-Jun mRNA crosslinks less efficiently to purified eIF4E than that of the ACTB mRNA (Extended Data Fig. 9). To identify the eIF4F inhibitory element, we constructed luciferase reporters to test deletions in the c-Jun 5' UTR (Fig. 4a). Deletion of the 5' 153 nucleotides, but not the initial 67 nucleotides, was sufficient to allow c-Jun mRNA translation to occur independently of the eIF3-recruitment stem–loop, suggesting that canonical cap dependent translation is no longer blocked (Fig. 4b). We confirmed by western blot analysis of the 48S initiation complex formed on c-Jun mRNA with a 5' 153-nucleotide truncation, 293T, total protein from 293T in vitro translation extracts. The result is representative of three independent experiments. For gel source data, see Supplementary Fig. 1. d, Model for eIF3d-directed cap–dependent mRNA translation. An eIF4F-inhibitory RNA element ensures that mRNA translation occurs through an eIF3-specialized pathway.

Figure 4 | An RNA element inhibits eIF4F recruitment and directs mRNAs to use an eIF3-specialized translation pathway. a, Schematic of c-Jun 3' UTR truncation-luciferase (Luc) reporter mRNAs. SL, stem–loop. b, Luciferase activity from in vitro translation of mRNAs containing truncations of the c-Jun 5' UTR, with or without the internal eIF3-recruitment stem–loop sequence. The results are given as the mean ± s.d. of three independent experiments, each performed in triplicate. c, Western blot analysis of initiation factors in 48S translation initiation complexes formed on c-Jun mRNA with a 5' 153-nucleotide truncation. 293T, total protein from 293T in vitro translation extracts. The result is representative of three independent experiments. For gel source data, see Supplementary Fig. 1. d, Model for eIF3d-directed cap–dependent mRNA translation. An eIF4F-inhibitory RNA element ensures that mRNA translation occurs through an eIF3-specialized pathway.

mRNA by quantitative RT–PCR19,20. Mutations to the predicted eIF3d cap-binding surface inhibit c-Jun mRNA incorporation into translation complexes, while the control ACTB mRNA is unaffected (Fig. 3d, Extended Data Fig. 8). These results demonstrate that cap binding by eIF3d is required for efficient initiation complex formation during eIF3-specialized translation.

While considerable advances have been made in the structural understanding of eIF3 bound to the ribosome, direct localization of eIF3d in a 48S complex remains unclear25. Thus, understanding how eIF3d functions and assemblies within the full translation initiation complex will have important mechanistic implications in how cap recognition links to mRNA ribosomal recruitment. Our discovery of
elf3 as a cap-binding protein now reveals a new translation pathway independent of elf4E, and adds another layer of cap-dependent translation.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Information Atomic coordinates and structure factors for the reported crystal structures are deposited at the Protein Data Bank (PDB) under accession codes 5K4B (crystal form 1), 5K4C (crystal form 2), and 5K4D (crystal form 3). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.H.D.C. (jcate@lbl.gov).

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METHODS

Cells and transfections. Human 293T cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Seradigm). The cells were obtained from the University of California, Berkeley, Cell Culture Facility, which authenticates cells by STR profiling and tests for mycoplasma contamination. Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen), following the manufacturer’s protocol, and polysome or immunoprecipitation analyses were performed at 48 h after transfection. For INK128 (Cayman Chemical) cell treatment, 293T cells were incubated with the indicated concentration of INK128 for ~14–16 h before cell lysis.

Plasmids. To generate the elf3d expression plasmids, elf3d was amplified from human cDNA and inserted into pcDNAs/FRT. A 39-nucleotide linker followed by the HA epitope tag (YPYDVPDYA) was subsequently inserted before the elf3d stop codon. The wild-type c-Jun 5′ UTR luciferase reporter plasmid was previously described2. To generate the c-Jun in vitro transcription template, the 5′ UTR, ORF and 3′ UTR were separately amplified from human cDNA and stitched together downstream of a T7 promoter by Gibson cloning into pcDNA4. The ACTB in vitro transcription template was constructed by addition of a T7 promoter during amplification of the full mRNA from human cDNA and inserted into pcDNA4.

Western blot. Western blot analyses were performed using the following antibodies: anti-elf3d (Bethyl A301-758A), anti-elf4A1 (Cell Signaling 2490), anti-elf6F1G1 (Cell Signaling 2858), anti-rrpS19 (Bethyl A304-002A), anti-elf4E (Bethyl A301-154A), anti-rrpLP0 (Bethyl A302-882A), anti-HA epitope tag (Pierce 21183), anti-c-Jun (Cell Signaling 9165), anti-Hsp90 (BD Biosciences 610418), and anti-4E-BP1 (Cell Signaling 9644).

In vitro RNA transcription and labelling. Unlabelled RNAs were in vitro transcribed, polyadenylated, and capped as previously described2. For internal radiolabelling of RNAs, in vitro transcription was performed in the presence of 0.1 μM [α-32P]GTP. RNAs were purified by phenol–chloroform extraction and ethanol precipitation.

In vitro translation. In vitro translation extracts were made from human 293T cells as previously described21. lysates were nuclease-treated with 18 g/ml of anti-HA antibody-conjugated agarose (Roche) and 100 μM m7GDP/GDP or GDP/GTP. lysates were incubated with Ni-NTA agarose resin (QIAGEN) for 1 h at 4 °C with gentle rocking. Resin was washed with lysis buffer supplemented to 1.5 M NaCl and eluted by gravity-flow chromatography at 4 °C with lysis buffer supplemented to 300 mM imidazole. The eluted fraction was diluted to ~50 mM imidazole and 5% glycerol, concentrated to ~50 mg/ml by precipitation with 80% glycerol and stored in liquid nitrogen for storage at ~80 °C.

Crystallization and structure determination. Initial crystals of human elf3d were grown at 18°C by hanging drop vapour diffusion, but diffracted poorly. Analogous elf3d cap-binding domain sequences were cloned from PCR and cloned into a modified pET vector to express an N-terminal 6× His (KSSHHHHHHGGSH)-MBP-TEV fusion protein as previously described26. Extensive expression trials were conducted to determine optimal N- and C-terminal domain boundaries and identified a minimal stable human elf3d cap-binding domain S161–F527. Recombinant protein was expressed in BL21-RIL DE3 E. coli cells co-transformed with a pRA2E1 rRNA plasmid (Agilent). E. coli was grown in 2× YT media at 37°C to an OD600 of ~0.5, cooled at 4°C for 15 min, induced with addition of 0.5 mM IPTG and then incubated with shaking for ~20 h at 16°C. Pelleted cells were washed with PBS and then lysed by sonication in lysis buffer (200 mM HEPES-KOH pH 7.5, 400 mM NaCl, 10% glycerol, 30 mM imidazole, 1 mM TCEP) in the presence of EDTA-free Complete Protease Inhibitor (Roche). Following centrifugation for 30 min at 23,000 g and 4 °C, clarified lysate was incubated with Ni-NTA agarose resin (QIAGEN) for 1 h at 4°C with gentle rocking. Lysates were centrifuged at 4 °C to remove the MBP tag. Recombinant elf3d was isolated from free MBP by diluting with gel-filtration buffer (200 mM HEPES-KOH pH 7.5, 250 mM NaCl, 1 mM TCEP) and passing over a 5 ml Ni-NTA column (QIAGEN) connected in line with a 5 ml MBP-Trap column (GE Life Sciences) before additional purification by size-exclusion chromatography on a Superdex 75 16/60 column. Final purification of elf3d was concentrated to ~20–50 mg/ml, used immediately for cryostalllography, or flash frozen in liquid nitrogen for storage at ~80°C.

Crystallography. The characteristic crystal form of human elf3d was grown at 18°C by hanging drop vapour diffusion, but diffracted poorly. Analogous elf3d cap-binding domain sequences were cloned from a panel of highly homologous animal sequences, with the equivalent domain from the parasitic wasp N. vitripennis (S172–F337) producing the best crystals. Optimized N. vitripennis elf3d crystals were grown in 2 μl hanging drops set at a 1:1 ratio over 300 μl of reservoir liquid: 200 mM (NH4)2SO4, 100 mM Bis-Tris 6.5, 23–27% PEG-3350 (crystal form 1), 1.6–1.8 M ammonium citrate, pH 7.0 (crystal form 2), or 200 mM NaCl, 100 mM Bis-Tris 6.5, 23–27% PEG-3350 (crystal form 3). elf3d crystals (crystal forms 1 and 2) were cryoprotected by covering the drop with a layer of saturated paratone-N or NVH oil (Hampton) and crystals were transferred into the oil emulsion and cleaned using a Koazk cat whisker as previously described29, or cryoprotected by transferring to a reservoir solution supplemented with 20% ethylene glycol (crystal form 3). Crystals were harvested with a nylon loop and then flash-frozen in liquid nitrogen. X-ray diffraction data were collected under cryogenic conditions at the Lawrence Berkeley National Laboratory Advanced Light Source (beamline 8.3.1).

Data were processed with XDS and AIMLESS30 using the SSRl software suite (A. Maciag, Stanford SSRl). elf3d crystals belonged to the orthorhombic space group P21_21_21, and contained either two copies per asymmetric unit (crystal form 1) or one copy (crystal form 2), or the space group P21, and contained two copies per asymmetric unit (crystal form 3). Experimental phase information was collected from a native crystal using sulfur single-wavelength anomalous dispersion. Data were collected at a minimal accessible wavelength (~7,235 eV) and iterative data

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sets were completed and merged from independent portions of an exceptionally large eIF3d crystal. After $\sim 90 \times$ multiplicity, anomalous signal was detected to $\sim 2.4$ Å, and a clear phase solution was obtained at $\sim 120 \times$ multiplicity. 35 sites were identified with HySS in PHENIX\(^{31}\) corresponding to 32 sulfur atoms in eIF3d and 3 chloride positions. Phases were extended to the native eIF3d data set processed to $\sim 1.40$ Å using SOLVE/RESOLVE\(^{32}\), and model building was completed in Coot\(^{33}\) before refinement with PHENIX. X-ray data for refinement were extended according to an $I/\sigma$ resolution cut-off of $\sim 1.5$, CC* correlation and $R_{	ext{sym}}$ parameters, and visual inspection of the resulting map.\(^{34}\) A completed eIF3d cap-binding domain from crystal form 1 was used as a search model to determine phases for crystal form 2 and 3 using molecular replacement. Final structures were refined to stereochemistry statistics for Ramachandran plot (favoured/allowed), rotamer outliers, and MolProbity score as follows: crystal form 1, 96.8%/3.2%, 0.2% and 1.40; crystal form 2, 97.3%/2.7%, 0% and 1.29; crystal form 3, 97.4%/2.6%, 0.9% and 1.26.

**Recombinant eIF4E protein purification and RNA crosslinking.** Full-length human eIF4E was cloned and expressed using the same protocol as for eIF3d. eIF4E–RNA crosslinking was performed as described for eIF3–RNA crosslinking, but using 25 nM RNA with normalized counts per million and the indicated concentration of eIF4E. RNase treatment was performed using 4 U RNase R and 250 U RNase T1.

27. Lee, A. S., Burdeinick-Kerr, R. & Whelan, S. P. A ribosome-specialized translation initiation pathway is required for cap-dependent translation of vesicular stomatitis virus mRNAs. *Proc. Natl. Acad. Sci. USA* **110**, 324–329 (2013).

28. Kranzusch, P. J. et al. Structure-guided reprogramming of human cGAS dinucleotide linkage specificity. *Cell* **158**, 1011–1021 (2014).

29. Kranzusch, P. J. et al. Ancient origin of cGAS-STING reveals mechanism of universal 2′,3′-cGAMP signaling. *Mol. Cell* **59**, 891–903 (2015).

30. Kabsch, W. *Xsd*. *Acta Crystallogr. D* **66**, 125–132 (2010).

31. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D* **66**, 213–221 (2010).

32. Terwilliger, T. C. Reciprocal-space solvent flattening. *Acta Crystallogr. D* **55**, 1863–1871 (1999).

33. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D* **60**, 2126–2132 (2004).

34. Karplus, P. A. & Diederichs, K. Linking crystallographic model and data quality. *Science* **336**, 1030–1033 (2012).
Extended Data Figure 1 | c-Jun expression is unaffected by 4E-BP1 activation. Representative western blot of 293T cells after 24 h treatment with mTOR inhibitor INK128. The results are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 2 | Mapping of a C-terminal region of eIF3d that protects the c-Jun 5′ cap structure. a, Validation of eIF3d subunit identification. eIF3d-cap crosslinking was validated by immunoprecipitation of eIF3d after crosslinking and denaturing the eIF3 complex by boiling in SDS. The result is representative of biological replicates. b, Limited proteolysis of eIF3 crosslinked to 32P-cap-labelled c-Jun 5′ UTR RNA. Full-length and proteolysis fragments of eIF3d are indicated by black and maroon arrows, respectively, on the phosphorimage and Coomassie-stained SDS gels. c, Mass spectrometry identification of trypsinized peptides from limited proteolysis of cap-crosslinked eIF3d. Identified peptides are highlighted in blue. The results in b and c are representative of three independent experiments.
Extended Data Figure 3 | Purification of eIF3d cap-binding domain. a, Alignment is coloured by phylogenetic conservation of amino acid physiochemical property similarity and a cartoon schematic of the eIF3d secondary structure is depicted below the sequences. Colouring begins at 30% conservation (lightest blue).

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Extended Data Figure 4 | Structure-based alignment of eIF3d cap-binding domain and DXO cap-endonuclease domain sequences.

Structure-based alignment of eIF3d and DXO sequences according to superposition of eIF3d and DXO (PDB 4J7L) structures\(^\text{11}\). Alignment is coloured by phylogenetic conservation as in Extended Data Fig. 2, and cartoon schematics of the secondary structures are depicted below the sequences. eIF3d is coloured in blue and magenta as in Fig. 2, and DXO is coloured in green and magenta.
Extended Data Figure 5 | Structural details of eIF3d 'RNA gate' stabilizing interactions. a–c, Structural overview of eIF3d with cut-away sections highlighting charged interactions stabilizing the closed 'RNA gate' conformation. No significant van der Waals interactions stabilize the closed gate conformation, supporting likely repositioning of the RNA gate before 5′ mRNA cap recognition. Charged interactions occur in three areas: a, at the beginning of the gate insertion sequence (gate beginning); b, at the tip of the unstructured loop (gate tip); and c, at an 'arginine anchor' point stabilizing the return of the loop insertion sequence to the α-helix shared with DXO family endonucleases. Residues are numbered according to the human eIF3d sequence, and all positions are conserved between human and N. vitripennis except S292N. eIF3d RNA gate residues are displayed with blue side chains and the residues making stabilizing contacts are coloured in green. 2Fo − Fc map regions are shown at 1.5σ.
Extended Data Figure 6 | Packing interactions observed in alternative eIF3d crystal forms. Cartoon representation of crystallographic packing in eIF3d crystal form 1, 2 and 3 (a, b and c). Crystal forms 1 and 3 have two copies of eIF3d in the asymmetric unit coloured in blue/magenta and green/magenta, respectively; crystal form 2 has only one copy of eIF3d per asymmetric unit. Symmetry-related molecules are depicted in grey. Cut-away zoom illustrates position of the eIF3d RNA gate (red) relative to the nearest symmetry-related molecule. In crystal form 1, the RNA gate is packed against a neighbouring symmetry molecule, but in crystal forms 2 and 3, the RNA gate is positioned towards a major solvent channel. Relative conformation of the RNA gate remains unchanged in either eIF3d crystal form.
Extended Data Figure 7 | eIF3d cap-binding activity requires the eIF3-recruitment stem–loop RNA. Phosphorimage of SDS gel resolving RNase-protected \(^{32}\)P-cap-labelled c-Jun stem–loop RNA crosslinked to eIF3 subunits. The result is representative of three independent experiments.
Extended Data Figure 8 | Incorporation of HA epitope-tagged eIF3d into translation initiation complexes. 

a, Coomassie blue-stained SDS gel of recombinant eIF3 containing wild-type or helix α5- or α11-mutated eIF3d. 
b, Representative native agarose gel electrophoresis of recombinant wild-type and mutant eIF3 complexes bound to the c-Jun stem–loop. 
c, Polysome profiles of untransfected 293T cells, plotted as relative absorbance at 254 nm versus elution fractions. 
d, Western blot analysis of eIF3d and the small (rpS19) and large (rpLP0) ribosomal subunits. The results in b–d are representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 9 | eIF4E recognizes the 5′ end of the c-Jun mRNA less efficiently than ACTB mRNA. a, Coomassie-blue-stained SDS gel of recombinant human eIF4E expressed in E. coli. b, Phosphorimage of SDS gel resolving RNase-protected 32P-cap-labelled ACTB or c-Jun 5′ UTR RNA crosslinked to eIF4E. The result is representative of three independent experiments. For gel source data, see Supplementary Fig. 1.
### Extended Data Table 1  
**Summary of data collection, phasing and refinement statistics**

|                        | **eIF3d** | **eIF3d** | **eIF3d** | **eIF3d** |
|------------------------|-----------|-----------|-----------|-----------|
|                        | Crystal Form 1  | Crystal Form 2  | Crystal Form 3  | (S-SAD)  |
| **Data collection**    |            |            |            |           |
| Space group            | P 2₁2₁2₁   | P 2₁2₁2₁   | P 2₁       | P 2₁2₁2₁  |
| Cell dimensions        |            |            |            |           |
| a, b, c (Å)            | 61.90, 62.93, 192.24 | 49.01, 61.84, 138.31 | 49.97, 144.32, 55.30 | 61.66, 62.72, 193.28 |
| α, β, γ (°)            | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 109.12, 90.0 | 90.0, 90.0, 90.0 |
| Wavelength             | 1.11586    | 1.11586    | 1.11587    | 1.71371   |
| Resolution (Å)         | 48.06–1.40 (1.42–1.40) | 46.20–1.70 (1.73–1.70) | 49.13–2.00 (2.05–2.00) | 38.66–1.92 (1.96–1.92) |
| R_pим                   | 2.6 (45.3) | 5.0 (45.0) | 8.6 (52.3) | 0.9 (8.1) |
| I/σ(I)                 | 12.9 (1.5) | 9.1 (1.6)  | 6.5 (1.4)  | 65.2 (6.7) |
| CC_{1/2}               | 99.9 (57.9) | 99.6 (63.9) | 98.8 (55.6) | 100 (94.7) |
| Completeness (%)       | 100 (99.2) | 99.4 (89.4) | 99.8 (98.3) | 98.5 (86.1) |
| Redundancy             | 15.8 (10.1)| 4.1 (3.6)  | 3.0 (2.7)  | 129.0 (46.6) |
| **Refinement**         |            |            |            |           |
| Resolution (Å)         | 48.06–1.40 | 46.20–1.70 | 49.13–2.00 |           |
| No. reflections        |            |            |            |           |
| Total                  | 2,349,428  | 193,627    | 147,613    |           |
| Unique                 | 148,455    | 47,123     | 49,757     |           |
| Free (%)               | 2          | 5          | 5          |           |
| R_work / R_free        | 17.5 / 19.3| 16.2 / 19.6| 17.7 / 20.8|           |
| No. atoms              |            |            |            |           |
| Protein                | 5837       | 2953       | 5686       |           |
| Ligand/ion             | 3 (Cl)     | 18 (glycerol) | -          |           |
| Water                  | 910        | 385        | 741        |           |
| B factors              |            |            |            |           |
| Protein                | 21.7       | 24.2       | 26.6       |           |
| Ligand/ion             | 31.7       | 29.1       | -          |           |
| Water                  | 33.1       | 36.7       | 34.3       |           |
| r.m.s. deviations      |            |            |            |           |
| Bond lengths (Å)       | 0.007      | 0.012      | 0.004      |           |
| Bond angles (°)        | 1.167      | 1.359      | 0.638      |           |

Single crystals were used to collect data for each structure.

*Values in parentheses are for highest-resolution shell.*