MetAP-like Ebp1 occupies the human ribosomal tunnel exit and recruits flexible rRNA expansion segments

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Human Ebp1 is a member of the proliferation-associated 2G4 (PA2G4) family and plays an important role in cancer regulation. Ebp1 shares the methionine aminopeptidase (MetAP) fold and binds to mature 80S ribosomes for translational control. Here, we present a cryo-EM single particle analysis reconstruction of Ebp1 bound to non-translating human 80S ribosomes at a resolution range from 3.3 to ~8 Å. Ebp1 blocks the tunnel exit with major interactions to the general uL23/uL29 docking site for nascent chain-associated factors complemented by eukaryote-specific eL19 and rRNA helix H59. H59 is defined as dynamic adaptor undergoing significant remodeling upon Ebp1 binding. Ebp1 recruits rRNA expansion segment ES27L to the tunnel exit via specific interactions with rRNA consensus sequences. The Ebp1-ribosome complex serves as a template for MetAP binding and provides insights into the structural principles for spatial coordination of co-translational events and molecular triage at the ribosomal tunnel exit.
The ErbB3 receptor-binding protein (Ebp1, 394 residues) is a highly conserved, widely expressed, and multifunctional eukaryotic protein. It has attracted considerable attention due to its regulatory role in cancer progression, although the question of being "friend or foe" is still unanswered. This uncertainty is based on the contradictory functions of the two splice variants p42 and p48, with p42 (lacking 54 N-terminal residues) acting as tumor suppressor and p48 promoting cell proliferation. First described as part of the ErbB3 receptor pathway, it has been further on identified as an IRES-trans-acting factor (ITAF45) initiating viral mRNA translation. Moreover, Ebp1 binds to ribosomes and was also shown to inhibit phosphorylation of initiation factor eIF2α. Ebp1 itself is phosphorylated on multiple Ser/Thr sites, which promotes interactions with ErbB3 and Akt kinase involved in apoptosis (Ser360 phosphorylation) (reviewed in ref. 1). Further kinases described to interact with Ebp1 are protein kinase C (PKCα), dsRNA-activated kinase (PKR), p21-activated serine–threonine kinase (PAK1), and cyclin-dependent kinase (CDK2).

Intermediate resolution cryo-electron microscopy (cryo-EM) exclusively bind to 60S ribosomal subunits during maturation. While Ebp1 is known to originate from ribosomal intersubunit rotation, which we confirmed the predicted methionine aminopeptidase (MetAP) fold, also described as pita-bread fold, with a deep pocket on its concave surface accommodating the active site in MetAPs. MetAPs are metalloproteases essential for all kingdoms of life that cleave-off the first methionine from the growing polypeptide chain as soon as a length of 40 amino acids is reached. Together with the ribosome-associated chaperones RAC and Ssb, they belong to the first interaction partners of nascent chains (NCs) emerging from the ribosome. MetAPs are classified into two types, with MetAP-2 including a helical subdomain (insert domain) of ~60 residues (Supplementary Fig. 1). Ebp1 is MetAP-2 like and mainly distinguished from the MetAPs by the missing catalytic activity, a shorter N-terminus (~150 residues missing) and a C-terminal extension of about 50 residues harboring a highly positive charged patch including six consecutive lysine residues (Supplementary Fig. 1). The lysine cluster has been shown to act as major nucleolar localization signal and to be involved in phosphoinositide binding. Positive surface charges of Ebp1, indicative for the confirmed dsRNA-binding properties of Ebp1, cluster on the convex side and include a charged surface loop (residues 62–72) and the C-terminal region, although the lysine cluster was disordered and not part of the X-ray models (missing 30 C-terminal residues).

Ebp1 has been further on assigned as the human homolog of the 60S pre-ribosomal nuclear export factor Arxl from Saccharomyces cerevisiae, but any direct evidence for such functional homology is still missing. In particular, Ebp1 lacks structural features that are centrally involved in ribosome biogenesis factor interaction in yeast Arxl and it does not bind to nucleoporins, as would be required for a pre-60S nuclear export factor. Furthermore, Ebp1 stably binds to mature 80S ribosomes in vivo as shown in this study, while Arxl is known to exclusively bind to 60S ribosomal subunits during maturation. Intermediate resolution cryo-electron microscopy (cryo-EM) structures of Arxl bound to the yeast 60S (pre-)ribosome were reported previously, and finally refined to 3.4 Å resolution. These cryo-EM reconstructions confirmed the structural homology to the MetAPs and visualized Arxl in the same binding site on the ribosomal tunnel exit that was observed for a bacterial MetAP–ribosome complex in a low-resolution structure of >10 Å.

Recently, MetAPs enzymatic activity was shown in yeast to depend on its interaction with rRNA expansion segment ES27L, one of the longest tentative–like dsRNA insertions (714 nucleotides in human 285 rRNA) typical for eukaryotic ribosomes. Unexpectedly, this interaction was at the same time found to control the accuracy of ribosomal mRNA decoding. The mechanism is elusive, and sequence conservation in ESs is generally low and varies within species and even tissues. Deletion of ES27L is lethal in the ciliate Tetrahymena thermophila, and its close proximity to the tunnel exit perfectly positions it for the coordination of co-translationally interacting partners. The exact role of ESs in translational control, the precision of protein biosynthesis, and maturation and folding is not understood.

To shed light on the role of Ebp1 in translational regulation and molecular mechanisms underlying its multiple cellular functions, we wanted to address its structure in the context of the translation machinery using cryo-EM single-particle analysis. Here, we present the cryo-EM reconstruction of Ebp1 bound to the human 80S ribosome at a resolution ranging from 3.3 to ~8 Å. Ebp1 binds on top of the tunnel exit by several distinct interaction sites, with a remodeled tRNA helix H59 and ES27L presenting the most prominent contacts. The structure provides a generalized view of MetAP-fold recognition by the ribosome, and reveals structural principles for spatial coordination of co-translational events at the ribosomal tunnel exit.

Results

Cryo-EM structure of Ebp1 bound to the human 80S ribosome. Binding of factors to the ribosomal surface is often governed by considerable flexibility and conformational heterogeneity that can only be addressed by a hybrid structural biology approach integrating state-of-the-art cryo-EM analysis and high-resolution X-ray structures. The 1.6 Å X-ray structure of Ebp1 was solved in our lab previously. To gain insights into Ebp1 binding to the human 80S ribosome, we used cryo-EM single-particle analysis, and determined the structure of full-length Ebp1 (p48 isoform) in complex with puromycin-treated 80S ribosomes purified from HeLa cells (Supplementary Fig. 2, Supplementary Table 1). Interestingly, ~25% of the purified ribosomes were already decorated with endogenous Ebp1 (dataset 1), and this fraction could be raised up to ~75% upon addition of recombinant Ebp1 (dataset 2). The in vivo pulled-out and in vitro-reconstituted Ebp1–ribosome complexes were virtually identical, indicating that no significant conformational changes or rearrangements were introduced by reconstitution of the complex with the purified ribosomes. Therefore, the two datasets were merged to obtain a single higher resolution structure (Fig. 1a). In brief, ribosomal particles were automatically located and subjected to several consecutive rounds of in silico sorting. Initial refinement of the 34,467 particles retained after sorting resulted in a reconstruction at 3.3 Å global resolution (Supplementary Fig. 3a). Lower local resolution for the 40S ribosomal subunit (Supplementary Fig. 3d) indicated conformational heterogeneity originating from ribosomal intersubunit rotation, which we compensated for by separating the Ebp1–ribosome complex into two independently refined segments (“2-body” approach), comprising the 40S ribosomal subunit and the 60S ribosomal subunit plus Ebp1 and tRNA ES27L, respectively. This approach resulted in improved local and global resolution (Supplementary Fig. 3b, e) and density quality for the two ribosomal subunits and directly ribosome-associated Ebp1 segments. This set of cryo-EM densities thus allowed us to analyze the interface between Ebp1 and the ribosomal tunnel exit at a resolution of better than 4 Å (Supplementary Fig. 3e, g), with amino acid side chains and tRNA bases clearly resolved for the entire interface. In contrast, lower local resolution for more peripheral regions of Ebp1 and ES27L hindered interpretation of the Ebp1–ES27L interface, and suggested additional conformational mobility dependent from the ribosomal 60S subunit for these two components.
Ebp1 occupies the ribosomal tunnel exit and recruits ES27L. The MetAP fold of Ebp1 as determined by X-ray structure analysis binds to the 80S ribosome as a rigid body (root-mean-square deviation (rmsd) for 352 Ca-α-atoms of 1.0 Å) and thus the model could be directly fitted into the cryo-EM density (real-space correlation coefficient (cc) of 64%). In particular, the relative orientation of the insert domain in respect to the protein core remains unchanged. Ebp1 binds with its concave surface, harboring the active site in MetAPs, directly on top of the ribosomal 40S/60S subunit interface (“L1-position”; “ES27L-L1”) or projects toward the ribosomal tunnel exit (“exit-position”; “ES27L-exit”) (Supplementary Fig. 6). The two conformations were suggested to play a role in coordinating access of non-ribosomal factors to the ribosomal tunnel exit.

In the human Ebp1–ribosome complex, ES27L is resolved in the exit position and the ES27L-B-arm (370 nucleotides, 2912 Å²) reaches over the 60S tunnel exit (Fig. 1a, c) as previously only observed for 80S ribosomes of yeast and fungi (159 nts in baker’s yeast) to metazoans (714 nts in humans) for so far unknown reasons. Combining already available structural information from the Arx1–ES27L interaction in yeast with our cryo-EM reconstruction of the human Ebp1–ribosome complex, we could build a model for the corresponding regions of human ES27L, including 100 nts of ES27L-B reaching over the tunnel exit and parts of ES27L-C (30 nts). Although the base pairs are not resolved due to the extensive conformational plasticity of the central ES27L-B region (Supplementary Movie 1), the regular spacing of the A-form RNA helix emanating from the well-defined ES27L-A stem and resolved base pair mismatches.

Ebp1–ES27L interaction. MetAP binding to ES27L has been recently identified in yeast as an important principle increasing enzymatic activity and controlling translation fidelity, but the underlying principles are unclear. ES27L sequences are generally not conserved in eukaryotes: they vary from an AU-rich base content (57% GC) up to a single rich GC-content in yeast (57% GC) and extreme GC-rich version in Homo sapiens (89%) and ES27L length has been more than quadrupled from fungi (159 nts in baker’s yeast) to metazoans (714 nts in humans) for so far unknown reasons. Combining already available structural information from the Arx1–ES27L interaction in yeast with our cryo-EM reconstruction of the human Ebp1–ribosome complex, we could build a model for the corresponding regions of human ES27L, including 100 nts of ES27L-B reaching over the tunnel exit and parts of ES27L-C (30 nts). Although the base pairs are not resolved due to the extensive conformational plasticity of the central ES27L-B region (Supplementary Movie 1), the regular spacing of the A-form RNA helix emanating from the well-defined ES27L-A stem and resolved base pair mismatches.
allow for unambiguous extension of ES27L-B from the ribosomal core to Ebp1. The ES27L-B model allows for the definition of three specific Ebp1–ES27L contacts (Fig. 2a). Two of them involve N-terminal helices that are part of the conserved MetAP fold, while the last one is mediated by the Ebp1-specific C-terminal extension. On the RNA side, two consensus sequences are involved that are conserved from yeast to metazoans (Fig. 2b).

Consensus sequence 1 (cs1) contains two base mismatches, namely a purine–purine and a GU-wobble base pair, and is recognized by N-terminal regions of Ebp1 (Fig. 2a, b). The sterically demanding purine–purine interaction (G2947-A3247) pushes the phosphoribosyl backbone around A3247 into a positively charged loop (K65IFKEKEMKK, interacting residues are in italics) on the convex side of Ebp1 (Fig. 2c). The “exposed” phosphate group of A3247 is accommodated in a “P-loop” (phosphate-binding loop)-like structure typical for the binding of the active site (phosphate-binding loop)-like structure typical for the binding of the β-phosphate moiety of NTPs (N: any nucleotide) within the large superfamily of P-loop-containing NTPases (Pfam clan CL0023). In Ebp1, the phosphate approaches four main chain nitrogens oriented toward the phosphate group. The neighboring 5'-ribose of the conserved guanosine (G3246) is recognized by Thr19 on the N-terminus of helix α1 (Fig. 2d). Interactions around the GU wobble are completed by Ebp1 residues exposed by neighboring turns of helix α1 (Asp15, Lys22). Both mismatch recognitions within cs1 are conserved in yeast for the Arx1–ES27L interaction, as observed upon in-depth analysis of the original cryo-EM density by building the respective model for yeast ES27L (Supplementary Fig. 7a, b). However, ES27L in yeast has a slightly different orientation relative to Arx1 (rotational tilt), which results in an interaction of the neighboring GU wobble of cs1 (U1997-G2024) with Arx1 (Fig. 2b; Supplementary Fig. 7b).

The adjacent consensus sequence 2 (cs2) is characterized by the accumulation of mismatches rather than sequence homology (Fig. 2b), which reflects the different readout principles mediated by the C-terminal Ebp1- and Arx1-specific regions. Human cs2 is dominated by purine–purine mismatches, which overall result in an extensive widening of the major groove (by more than 75%) (Fig. 2a, e; Supplementary Fig. 7c). This typically is narrow and deep in A-RNA and not accessible for protein interactions. While the AG/GA tandem mismatch stretches the width of the RNA helix, it is constrained at the site of the GG mismatch (G2957/G3237) and best fitted by a cross-strand purine stack. The X-ray models of Ebp15,6 lack the most C-terminal 33 amino acid residues directly following the important phosphorylation site

![Fig. 2 Conserved structural features of ES27L are instrumental in Ebp1 binding.](https://www.nature.com/naturecommunications)

**Fig. 2 Conserved structural features of ES27L are instrumental in Ebp1 binding.** a Three distinct interaction sites between Ebp1 and the consensus sequences cs1 and cs2 mediate ES27L binding. The atomic models for Ebp1 and ES27L are superposed to the cryo-EM density after 3-body multibody refinement. Density was faded out toward the Ebp1–ribosome contact, which is better resolved in the reconstruction from 2-body multibody refinement. View is the same as in Fig. 1a left panel and as indicated by the small representation in the corner. b Consensus sequences (cs) of ES27L involved in Ebp1 binding. Conserved mismatches within cs1 are highlighted. c, d Structural details of ES27L interaction of the GA mismatch at cs1 with the Ebp1 P-loop structure (δ*: partial positive charge) following helix α2 (c), and of the GU wobble with Ebp1 helix α1 (d). Putative protein–RNA interactions are indicated by arrows. e Interactions at cs2 with the lysine-rich motif (KRM) within the Ebp1-specific C-terminal helix α C. The putative GG cross-strand purine stack is indicated by parallel lines.
Ser360 at the end of helix a10. In our cryo-EM reconstruction, the C-terminus of Ebp1 is resolved (Fig. 2a) and projects toward the widened major groove, where it forms a helix (aC) with the highly basic lysine cluster (S362RKTQKKKKKASKTAENA381, helical region in italics, side chains not traceable) that entirely fills the groove (Fig. 2c). This kind of major groove readout by positively charged a helices is well known from the so-called ARMs (arginine-rich motifs) as found i.e., in viral transactivation (HIV-1 Rev-peptide/Rev-response element RRE)26 and later on in the signal recognition particle (SRP68/SRP RNA)27. Accordingly, we define this motif as "KRM", with the arginine (R) being replaced for lysine (K). In Arx114, only one single turn of helix aC is formed, and the chain does not penetrate the major groove as the RNA continues in a regular A-form and yeast ES27L-B is terminated. The interaction of this single aC turn including two positive charges with the most distant ES27L-B GU wobble next to the tetranucleotide loop is reminiscent of the Ebp1–ES27L contact, but the overall architecture of the interaction site is different. All MetAPs are missing a corresponding positively charged C-terminal region (~50 residues less) and thus, this ES27L contact at c52 is specific for the PA2G4 family like Ebp1 and Arx1.

The general docking site for NC-associated factors revisited. The second half of the Ebp1–ribosome interface is formed by the inner ring of the ribosomal tunnel exit. The tunnel exit is a hot spot in cellular activity and a plethora of NC-associated factors compete for similar binding sites (reviewed in refs. 9,28). Most importantly, uL23 has been described as a general docking site for NC-interacting chaperones (RAC, NAC, ERj1p, Trigger factor), protein targeting factors plus their associated membrane insertion machineries (SecA and SRP/translocon, Get pathway, Oxa1), and NC-modifying enzymes like the MetAPs.

In the Ebp1–ribosome complex, docking of the insert domain of Ebp1 to uL23 constitutes the largest interface to the inner tunnel exit ring (500 Å²) (Fig. 3a). The interface includes four a helices (a6 and a8 of Ebp1) and has a methionine-rich hydrophobic core that is surrounded by hydrophilic and charged contacts. Most strikingly, the very C-terminus of uL23 forms a salt bridge with Arg243 of Ebp1 at the start of the insert domain (Fig. 3a). The insert domain also contacts ribosomal elements around uL23, and thus this general docking site is rather extended (1200 Å²). Overall, the extended docking site confers to 80% of the Ebp1 interaction with the inner exit ring (1500 Å²). All minor interactions that complete the overall triangular docking of Ebp1 to the exit, form small “lysine-trident” contacts with either the phosphoribose backbone of 5.8S rRNA helix 24 (Fig. 3a), or with the connection of uL24/28S rRNA helix 24 (not shown).

This extended general docking site is unique among the so far described tunnel exit interactions. Still, the mode of central uL23 recognition is very similar as found for the bacterial Trigger factor29. The N-terminal domain of Trigger factor, a co-translational chaperone that crowches over the tunnel exit30, also forms hydrophobic interactions and binds the very C-terminus of bacterial uL23 (Fig. 3b). However, this contact constitutes the major interaction in the Trigger factor–ribosome complex, and binding of the chaperone to the exit is known to be transient11.

Also typical for many exit tunnel interactions, docking occurs in tandem with the uL23-neighboring protein uL29. In the Ebp1–ribosome complex, the contact with uL29 is small and appears rather flexible as it involves van der Waals interactions of small residues (glycines and alanines) (Fig. 3a). In comparison, SRP establishes a major interaction with both uL23 and uL29 when binding to the ribosome for targeting of nascent secretory and membrane proteins to the ER membrane32–34 (Fig. 3c). Interestingly, the insert domain of Ebp1 does not sterically exclude binding of the SRP GTPase SRP54, which is centered more toward uL29. However, the flexibly linked signal sequence-binding domain of SRP54 (SRP54M) cannot be positioned over the tunnel exit in the presence of Ebp1 (or MetAP-2). Similarly, the protein conducting channel of the ER membrane (Sec61αβγ) strongly binds to the same site35,36. While Sec61y binds to uL23, Sec61αα binds to uL29 and H50 of 28S rRNA at the base of the tunnel exit (Fig. 3d). Finally, cryo-EM reconstructions of the co-translational eukaryotic chaperones NAC (nascent polypeptide-associated complex)37 and the ER membrane protein ERj1p (Hsp40-type co-chaperone of ER-lumenal Hsp70 BiP)38 also revealed binding to the general docking site, however, due to limited resolution atomic models thereof could not be built and further data are necessary for detailed descriptions. The general physiological necessity for a triage between protein folding and co-translational targeting, depending on NC-associated factors binding to the general docking site, has been remarked and described earlier9,28.

H59 forms a dynamic adaptor to the insert domain of Ebp1. A striking feature of the Ebp1–ribosome interaction is the extension of the Ebp1-binding site beyond the general docking site toward
eL19 and helix H59 of 28S rRNA (Fig. 4). This contact is even larger (700 Å²) than the uL23/uL29 interface, and unique among all other ribosomal complexes with available structural information (except of its yeast homolog Arx111). Both eL19 and H59 (also termed ES24L; 21 nts in human 28S rRNA, 2698–2718) are eukaryote specific. In general, the globular N-terminal domain of eL19, which is essential for cell viability and ribosome biogenesis12, binds to H59 at its closing loop (G2705GUUCCG2711 in human 28S rRNA). In all mammalian ribosome structures solved so far, guanine G2711 is bulged out and anchors H59 on the ribosomal surface by interactions with H53 and the H58-59 connection (defined here as “locked position”) (Supplementary Fig. 8a). In the Ebp1–ribosome complex, the loop is completely remodeled and bridges over toward the highly basic helix α6 of the Ebp1 insert domain (Fig. 4a; Supplementary Fig. 8a, b). This remodeling coincides with a lateral displacement of H59 by up to 10 Å (at G2711), which releases the guanine anchor G2711 and rotates H59 further onto eL19 (“locked position”). Thus, H59 serves as a conformationally dynamic determinant for Ebp1 recruitment to the tunnel exit (Supplementary Movie 2). It is also unlikely that the observed remodeling of H59 also occurs in yeast, because the bulged-out nucleotide is not present and in all yeast ribosome structures H59 is found in the “Ebp1-like” docked position.

The cryo-EM density around the interaction site approximates the overall resolution of 3.3 Å, which allows for the precise fitting of the interacting partners and especially the region around Ebp1 helix α6 (Fig. 4b) as a main docking partner within the insert domain (Supplementary Fig. 1). Overall, the interaction is dominated by π–π and π–cation stackings and two Watson–Crick-like base readouts (U2708 and G2711) with the protein main chain (Fig. 4c). Uridine U2708, which points into the H59 RNA loop in structures of vacant ribosomes (Supplementary Movie 2), is bulged out and accommodated in a binding pocket created by residues of Ebp1 helix α6 and its N-terminal loop (Fig. 4d; Supplementary Fig. 8b). While the uridine base is sandwiched between Asn254 and Phe266, its Watson–Crick edge is perfectly read out by the protein main chain (Tyr255 and Leu257). The interaction is completed by Ser267 hydrogen bonding to the ribose moiety. An almost identical interaction has been observed as a major determinant in the Arx1–pre-60S interaction14, although the base-specific readout was not described (Supplementary Fig. 8c).

As this specific readout is mediated by the main chain, the conservation of the binding pocket is not obvious in respect to the MetAP-2 family; however, the overall features of this docking region are preserved (Supplementary Fig. 1). The two subsequent nucleotides of H59 (C2709 and C2710) are stacked on top of the loop and held in place by the capping π–cation stacking with Arg271 (Fig. 4c).

Upon H59 remodeling, nucleotide G2711 undergoes the largest movements, and like U2708, it is fixed in a base-specific binding pocket at the N-terminal end of Ebp1 helix α6 in the docked position (Fig. 4e). Unlike the binding site for U2708, G2711 is accommodated in a composite pocket formed by Ebp1 and helix α3 of eL19. Here, the base is stacked between Arg263 of Ebp1 and Glu40 of eL19, and the Watson–Crick readout is mediated by the Ebp1 main chain (Leu257 and Lys258). The guanine base is further hydrogen bonded to side chains within helix α3 of eL19 (Ser37 and Gln39), which itself also contacts Ebp1 directly.
The structure of the Ebp1–ribosome complex revealed the importance of plastic rRNA expansion segments in specific ligand recruitment (or vice versa) at the ribosomal tunnel exit. Besides helix H59, the tentacle-like ES27L was identified in its ES27L–exit conformation to serve as a major rRNA docking site for Ebp1. In a most recent cryo-EM reconstruction of the yeast ribosome–NatA complex, the trimeric NatA complex (Naa10/Naa15/Naa50) was also shown to recruit ES27L in the exit position by binding to the closing tetraloop23 adjacent to the cs2 region. NatA was described with a unique mode of ribosome interaction, contacting ESs in three out of four binding patches. Interestingly, the NatA-binding site is directly adjacent (shifted toward ES7a and ES39a) to the Ebp1/Arx1-binding site, with only the PA2G4–specific C-terminal extension of the MetAP-fold clashing (Fig. 5a). Thus, while according to structural comparison, Ebp1 and NatA binding are likely mutually exclusive, concomitant MetAP/NatA binding would be possible23 (Fig. 5b). Moreover, the suggested minimal NC length of 50 amino acids in order to reach the Naa10 catalytic site25 is only ten residues longer as found for the MetAPs, strongly indicating a direct handover mechanism. The distal end of ES27L–B undergoes a conformational transition from the Arx1–MetAP–) to the NatA-bound state with a 30° bending (translation of 30 Å of the tetraloop) (Fig. 5a). Therefore, the MetAP/ES27L–B contact is likely to be disrupted upon NatA binding, which potentially prepares subsequent dissociation of MetAP.

The structural homology of the PA2G4 member Ebp1 with the MetAP family and the resulting competition for ribosome binding raises questions regarding physiological readout (schematized in Fig. 6) and potential pathological implications. Evidently, binding of Ebp1 and MetAPs or Nats is mutually exclusive, and persistent Ebp1 binding to the ribosomal tunnel exit would consequently prevent any co-translational protein modifications by either the MetAPs or the NATs with severe effects on the cellular proteome. Whether also protein synthesis per se is arrested upon Ebp1 binding is more challenging to address on a structural basis. The plurality of ribosomal protein and rRNA-binding events within the Ebp1–ribosome complex, in particular the arrest of conformational dynamics of ES27L, might induce conformational changes in the ribosome that permit long-scale signal transfer toward the peptidyl transferase center (PTC) and thus impact on translational kinetics. However, our cryo-EM reconstruction does not indicate such a conformational signal transfer upon Ebp1 binding, as the conformation of ribosomal proteins and rRNA in the PTC remains unaltered compared with the vacant mammalian 80S ribosome. As observed upon focused 3D classification, the same holds true for conformational dynamics of the ribosomal small subunit, which recapitulates the canonical ratcheting and rolling motions of the translational elongation cycle also after Ebp1 binding, as well as for translational elongation factor binding, which seems not to be affected (Supplementary Fig. 9). Therefore, our cryo-EM data do not per se support a consequent mechanistic translation inhibition upon Ebp1 binding. However, during elongation, any NC would be sterically hindered in exiting the ribosome by the blockade via (or potential binding to) Ebp1 and the conformational fixation of ES27L in the exit position, which collectively might affect translation in an indirect way. Whether such stalled Ebp1–ribosome–NC complexes exist and if the Ebp1–ribosome interaction may alter in the presence of a NC remains to be addressed.

Ebp1 inhibits phosphorylation of initiation factor eIF2α4, and phosphorylation is likely to be regulated by the phosphorylation status of Ebp1 itself. As estimated from computational particle sorting during cryo-EM data processing (Supplementary Fig. 2),
functions with respective molecular mechanisms will be the next challenge in Ebp1-related research.

Methods
Sample preparation. The total human tRNA was extracted from HEK293-S3 cells using the Direct-soil RNA Miniprep kit (Goyo Research) and reverse transcribed into cDNA utilizing the Maxima first strand cDNA synthesis kit with dsDNAse (Thermo Scientific). The coding sequence of the Ebp1 gene was amplified by PCR using the HEK cDNA as a template and the primers Ebp1Ncol_Fwd (CATGCGATGCGTCGGCAGGAGGGGCAAC) and Ebp1BamHI_Rev (GGCGAGATCCGATCACCCAGCTGACATTTCC). The ~1.2 kb PCR product was digested with Ncol/BamHI restriction nucleases (NEB) and ligated into the pET24d-His6-linker-TEV (tevagocil edge virus) resulting in the pET24d-His6-linker-TEV-Ebp1 plasmid.

Ebp1 was expressed from the pET24d-His6-linker-TEV-Ebp1 plasmid in E. coli Rosetta 2 cells (Novagen) using autoinduction medium. Cells were cultured at 37 °C until reaching OD600 0.6–0.8, upon which the temperature was shifted to 21 °C and expression was continued for 16 h. Cells were harvested and resuspended in lysis buffer (40 mM HEPES KOH pH 7.5, 1 M NaCl, 10 mM MgCl2, 10 mM KCl, 40 mM Imidazole, 0.02% 1-thioglycerol) supplemented with protease inhibitor cocktail (Roche) at 1× final concentration. Resuspended cells were lysed utilizing a microfluidizer (Microfluidics Corp.), and the lysate was cleared via centrifugation for 20 min at 200,000 x g and 4 °C. The cleared lysate was applied to Ni-IMAC (immobilized metal affinity chromatography) (GE Healthcare) and thoroughly washed with lysis buffer. Ebp1 was eluted in 20 CV using elution buffer (40 mM HEPES KOH pH 7.5, 500 mM NaCl, 5 mM MgCl2, 5 mM KCl, 5 mM 2-mercaptoethanol) at 4 °C. TEV digested Ebp1 was isolated via reverse Ni-IMAC and in a final step purified via Superdex 75 (GE Healthcare) size-exclusion chromatography (SEC) equilibrated in SEC Buffer (20 mM HEPES KOH pH 7.5, 5 mM Mg(OAc)2, 175 mM KOAc, 1 mM tris(2-carboxyethyl)phosphine). Ebp1 containing fractions were pooled, snap frozen in liquid nitrogen, and stored at −80 °C until further use.

Human non-translating 60S ribosomes were isolated from HeLa cells in a protocol as described previously43 that we adapted from a large-scale setup44. Briefly, HeLa cells were grown in suspension cultures and harvested cells (1 × 10^8 cells per 100 ml) were lysed with detergent. After clearing of the lysate from debris and membranes, ribosomes were purified via centrifugation through a sucrose cushion. The pellet was resuspended, treated with puromycin and the monosomes further purified in a sucrose gradient. The monosome peak was collected and concentrated to 1 mg/ml in the same physiological buffer as Ebp1 (SEC buffer), aliquoted and snap frozen in liquid nitrogen and stored at −80 °C. The typical yield is 1 mg per 1 × 10^8 cells.

Immunoblot analysis. HEK293 and HeLa cell lysates were separated on a 12.5% SDS-PAGE gel and transferred onto a Protran nitrocellulose membrane (Amersham). The membrane was stained with Ponceau-red to detect the total protein and probed with antibodies against Ebp1 (N-terminus, ABE43, 1:5000, Jackson ImmunoResearch) and visualized with the ECL Western Lightning Ultra (Perkin Elmer), according to the manufacturer’s protocol.

Grid preparation. Two batches of EM grids were prepared. The first batch used for collection of dataset 1 contained the in vivo-pulled Ebp1–ribosome complex without addition of recombinant Ebp1. The second batch used for collection of dataset 2 contained the same purified ribosomes, but supplemented with recombinant Ebp1 (p48 isoform) to increase Ebp1 occupancy on the ribosomes. Right before freezing, Quantifoil Multi A holey carbon supported grids (Quantifoil, Multi A, 400 mesh) were glow-discharged for 10 s in oxygen atmosphere using a Solarus plasma cleaner (Gatan, Inc.). In total, 3 μL of freshly prepared samples (100 nM ribosomes without/with an eightfold excess of recombinant Ebp1) were directly applied to glow-discharged grids. Under a blot force of 0 at 100% humidity, the grids were blotted for 3 s with Whatman #1 filter papers using a Vitrobot Mark IV (FEI Company) operated at room temperature, and then immediately plunge-frozen in liquid ethane cooled with liquid nitrogen.

Data collection. Cryo-EM data were acquired on a Titan Krios transmission electron microscope (Thermo Fisher/FEI Company) operated at an acceleration voltage of 300 kV. Two data sets were collected. The first dataset (dataset 1) was acquired on a K2 Summit direct electron detector (Gatan, Inc.) at an object pixel size of 1.07 Å in Latitude (Gatan Company) with a target defocus range of −1.5 to −3.0 μm. Micrographs were acquired using dose fractionation to record 40 frames per exposure with a dose rate of 0.9 electrons per Å² per frame, resulting in a total dose of 39 electrons per Å² per micrograph. The second dataset (dataset 2) was acquired on a K3 direct electron detector (Gatan, Inc.) at an object pixel size of 1.07 Å. Data were collected using the SerialEM software package with a target...
defocus range of –0.5 to –2.0 μm. Micrographs were acquired using dose fractionation to record 20 frames per exposure with a dose rate of 1.85 electrons per Å² per frame, resulting in a total dose of 37 electrons per Å² per micrograph.

**Image processing.** All steps of image processing are summarized in a visual flow chart (Supplementary Fig. 2). Both data sets were initially processed separately, but following the same workflow. All processing steps were performed with the RELION 3.0-beta software package, unless stated otherwise45. Movie stacks were motion-corrected using MotionCor2 with the number of patches set to 5 × 546. Estimation of contrast transfer function (CTF) parameters was performed using Gctf on the motion-corrected micrographs47. To generate reference templates for auto-picking, ~500 ribosomal particles were manually selected from the micrographs and subjected to unsupervised two-dimensional (2D) classification into ten classes. Classes depicting 80S ribosomes were used for auto-picking, resulting in 162,886 particles from dataset 1 and 41,482 particles from dataset 2. The particles were extracted at a pixel size of 4.28 Å in boxes of 128 × 128 pixels and subjected to 3D classification using a human 80S ribosome filtered to 40 Å resolution as an initial reference. Only ribosomal classes depicting high-resolution structural features were retained for further processing, yielding 70,414 and 73,759 particles from dataset 1 and 2, respectively. A second round of 3D classification was focused on the polypeptide exit tunnel to enrich ribosomal particles with clear density for Ebp1 and ES27L yielding 21,743 and 53,177 particles from the first and second dataset, respectively. For this purpose, we generated a binary mask encompassing only Ebp1 and ES27L from one of the 3D classes via manual segmentation in UCSF Chimera48 and used it as a reference mask during 3D classification. Sampling was switched off during this second round of 3D classification, and optimal translations and rotation from the first round of 3D classification were used for each particle. The retained particles were re-centered, extracted at a pixel size of 1.52 Å in boxes of 360 × 360 pixels and subjected to 3D auto-refinement. After 3D auto-refinement, particles were subjected to a refinement using standard parameters (plus dataset-wise beam tilt estimation) and Bayesian particle polishing49 using a training set of 5000 particles.

Features were retained for further processing, yielding 70,414 and 73,759 particles after post-processing using the subunit masks also used for multibody refinement. A refinement criterion of independently moving segments (60S), and 5.7 Å global resolution (Ebp1 – 60S) were selected and subjected to a multibody refinement run. Three out of five local resolution maps (FSC 0.143) within RELION. Local resolution and validation was performed with the PHENIX suite51,52.

**Data availability.** The following cryo-EM densities for the Ebp1–ribosome complex have been deposited in the EMD database. Ebp1: 60S segment at full spatial resolution for 3-body multibody refinement (EMD-10344); Ebp1–ES27L segment from 3-body multibody refinement (EMD-10609); Ebp1–60S segment from 2-body multibody refinement after sorting for ES27L conformation (EMD-10608). The atomic coordinates for Ebp1 and interacting ribosomal components have been deposited in the RCSB with accession ID 6X5O. For visualization of the model in context of the entire human 80S ribosome, we recommend to superpose our atomic coordinates to the structure of the human ribosome solved at 2.9 Å resolution (PDB ID 6EKO)53, which is virtually identical except for the Ebp1–interacting region. Other data are available from the corresponding authors upon reasonable request.

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