The structure of a human translation initiation complex reveals two independent roles for the helicase eIF4A

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Abstract
Canonical initiation of translation in eukaryotes involves recruitment of the 43S pre-initiation complex to the 5′ end of mRNA by the cap-binding complex eIF4F to form the 48S initiation complex (48S), followed by scanning along the mRNA until the start codon is selected. We had previously shown that eIF4F binds near the mRNA channel exit site of the 43S. Here we describe a human 48S positioned at the start codon which reveals a second molecule of eIF4A at the mRNA entry site and provides a mechanism for how it facilitates scanning of the 5′ UTR of mRNA. The significantly improved resolution for eIF4F reveals universally conserved interactions with mRNA and the 43S, shedding light on its role in recruitment and scanning.

Introduction

Initiation of translation in eukaryotes involves over 20 different initiation factors (eIFs). The process starts with the assembly of the 43S pre-initiation complex (43S) consisting of the 40S ribosomal subunit bound to initiation factors eIF1, eIF1A, eIF3 and eIF5 and a ternary complex (TC) of eIF2, guanosine 5′-triphosphate (GTP), and methionyl initiator transfer RNA ($tRNA_i^{Met}$). In parallel, the cap-binding complex eIF4F bound to the 5′ end of mRNA recruits the 43S to form the 48S translation initiation complex, which then scans along the mRNA until it encounters a start codon (*1–8*).

The cap-binding complex eIF4F consists of a scaffold protein eIF4G, an m^7G cap-binding protein eIF4E, and a DEAD-box helicase eIF4A (*9*). Additionally, eIF4G also interacts with poly(A)-binding protein PABP located at the 3′ untranslated region (UTR) of mRNA, which further brings the 3′ and 5′ ends close together. In metazoans, the attachment of the 43S to the mRNA–eIF4F–PABP complex is mediated by a direct interaction between eIF3 and eIF4G (*3–5*, *8*, *10*, *11*). Recently, we determined the structure of a human 48S complex during scanning, which revealed the binding site of eIF4F (*4*). eIF4F interacts with eIF3e and eIF3k/l located upstream of the 43S, near the mRNA channel exit site. Nevertheless, the fact that eIF3k/l are dispensable in some eukaryotic cells (*12*, *13*) and moreover, *Saccharomyces cerevisiae* eIF3 lacks those subunits, suggests that other elements of the 43S likely make additional interactions with the cap-binding complex eIF4F. Whether eIF4F can interact directly with the 40S during initiation remained

unknown. The low local resolution in eIF4F in the previous structure (*4*) did not allow us to describe its interaction with the 43S further.

Additionally, eIF4B or its homolog eIF4H enhance the helicase activity of eIF4A (*14–16*), raising the question of how the function of eIF4F is coordinated with that of an eIF4A–eIF4B binary complex. Moreover, yeast eIF4B may induce conformational changes in the 40S small ribosomal subunit and facilitate the attachment of the 43S to mRNA (*14*). In yeast, the ATPase activity of eIF4A does not require eIF4G and eIF4E (*17*), whereas in mammals, an early study indicated that the eIF4A–eIF4B activity during recruitment and scanning requires the cap-binding complex eIF4F (*15*). RNA proximity labeling (APEX-Seq) data place eIF4A at the entry site (*18*), on the opposite side of the 43S from where we had previously located eIF4F (*4*), which raises the issue of how these two findings could be reconciled.

In our prior structure, we used an mRNA which lacked AUG codon and which turned out to be shorter than the footprint of the 48S predicted by the structure (*4*). Here we capture a later stage initiation complex of the 48S complex positioned over a start codon, using a longer more physiological mRNA with a long and structured 5′ UTR, AUG codon, and a 3′ UTR ending in a poly(A) tail. The structure shows some striking additional features compared to our previous structure, and reconciles disparate biochemical data as well as shed new light on the role during initiation of the 48S complex, in particular a second molecule of the helicase eIF4A at the entry channel side of the 40S ribosomal subunit relative to that of eIF4F.

Results and discussion

Structure a human 48S translation initiation complex

To reconstitute as complete and physiological a 48S complex as possible for cryo-EM analysis, we use a capped mRNA containing a long 5′ UTR (105 nucleotides), an AUG codon, followed by a short CDS (9 nucleotides) and a poly(A) tail (~90 nucleotides) (fig. S1). To promote the proposed interaction of the poly(A) tail with the 5′ end of mRNA during complex formation, we included PABP in addition to a full complement of initiation factors, including full-length eIF4G1. Inclusion of a start codon enables us to generate a late-stage initiation intermediate representing the 48S with the start codon in the P site. To stabilize the complex further, we used Rocaglamide A (RocA), which clamps eIF4A onto polypurine sequences (*19–22*), while also including three polypurine motifs $(GA)_6$ in the 5′ UTR of the mRNA.

Single-particle reconstruction using cryo-EM reveals density corresponding to the cap-binding complex eIF4F in the same location as seen in our previous structure (Fig. 1, fig. S2) (*4*). Masked classification on the mRNA channel entry site followed by additional masked classification on the region we previously saw for eIF4F yielded a cryo-EM map at 3.1 Å resolution (fig. S3A). Further masked classification on TC yielded a map with a reduced overall resolution of 3.7 Å (fig. S3B) but improved the density for TC.

The local resolution of 5.9 Å for the cap-binding complex eIF4F (fig. S3C and d) is greatly improved compared to our previous structure (*4*), and allows us to see direct interactions not only to additional subunits of eIF3 but unexpectedly to the 40S small ribosomal subunit. The improved density shows secondary structure elements and enables us to accurately model the middle domain

of eIF4G (HEAT-1 domain) and eIF4A (Fig. 1). Although the mRNA has an m^7G cap and a poly(A) tail, we did not see any additional density that could be assigned to eIF4E or PABP.

Importantly, we observe additional density extending out from the eIF3bgi module at the mRNA channel entry site (Fig. 1). This density was not present in any previous structures of 48S complexes. The local resolution and detailed shape indicate that this additional density is due to a second eIF4A molecule bound to mRNA, in addition to the eIF4A that is part of eIF4F on the other side of the 40S subunit (Fig. 1). This second location is consistent with previous biochemical, cross-link mass spectrometry and proximity labeling (*4*, *17*, *18*). To test whether the presence of this second, entry-site eIF4A is a result of its being trapped by RocA, we determined the structure of a complex without RocA and found that both eIF4F and the second eIF4A are still present (fig. S4). To assemble this complex, we used a capped mRNA with the 5′ UTR of human b-globin (55 nucleotides) followed by a CDS containing a GC-rich region located 27 nucleotides downstream of the AUG. This GC-rich region forms a downstream loop (DLP) in the mRNA and has been used to trap eIF4A in the 48S (*23*).

The overall conformation of the structure is a post-scanning intermediate – the initiator tRNA is inserted completely into the P site (a P_{in} state) where it bases pairs with the start codon, and the 40S subunit mRNA binding channel is in the closed conformation. Additionally, the overall resolution allowed us to identify density corresponding to the 40S, eIF1A, eIF2a, eIF2b, eIF2g, $tRNA_i^{Met}$, the octameric structural core of eIF3 as well as its peripheral subunits (b, d, g, i) (Fig. 1, table S1).

Upon start-codon recognition, human eIF5-NTD occupies the site vacated by eIF1

Around the site normally occupied by eIF1, there is additional density which cannot be accounted for by the factor (Figs. 1, S5). Both previous biochemical data and a structure of a yeast initiation complex showed that the yeast N-terminal domain of eIF5 (eIF5-NTD) replaces eIF1 upon start codon recognition (*24*, *25*). Rigid-body fitting of the known structure of human eIF5-NTD (PDB: 2E9H) accounted for the entire density present at the platform of the 40S (fig. S5). Moreover, we could fit the zinc-binding domain (ZBD) of eIF5-NTD into the density (fig. S5). This domain is not present in eIF1, confirming that this density arises from bound eIF5-NTD that has displaced eIF1 upon start-codon recognition just as was previously proposed for the yeast system (*24*, *25*).

Thus, there is an evolutionarily conserved set of events that occur on start-codon recognition, whose fidelity is enhanced by the factors eIF1, eIF1A and eIF5 (*26*). First, eIF1A and eIF1 bind to the 40S platform near the A and P sites, respectively, and promote the open conformation of the 40S that facilitates the binding to the $tRNA_i^{Met}$. During scanning, the $tRNA_i^{Met}$ is not fully inserted into the P site of the ribosome (*4*), which allows codon-anticodon sampling until recognition of the AUG start codon (*4*, *27*). After start-codon recognition (Fig. 2A), the $tRNA_i^{Met}$ is inserted fully into the P site of the ribosome (*27–33*), resulting in a clash with eIF1. Thus, upon start-codon recognition, eIF1 dissociates from the complex and is replaced by eIF5-NTD (Fig. 2). Like eIF1, eIF5-NTD monitors the codon-anticodon interaction (Fig. 2B-C). However, while eIF1 residue Asn39 clashes with the anticodon stem-loop, the eIF5-NTD residue Asn30 adopts a different conformation (Fig. 2D-E), which allows it to monitor codon-anticodon interaction without clashing with the $tRNA_i^{Met}$ (Fig. 2C).

During scanning, eIF1 Loop 2 interacts with the $tRNA_i^{Met}$ D loop, which prevents the accommodation of the $tRNAi^{Met}$ into the P_{IN} state. Upon start codon selection, Loop 2 undergoes conformational changes to allow the accommodation of the $tRNA_i^{Met}$ in the P_{IN} state (*27*, *34*). The destabilization of eIF1 and its replacement with eIF5-NTD avoids a clash with the $tRNA_i^{Met}$ and stabilizes it in the P_{IN} state because eIF5-NTD Loop 2 is both shorter and adopts a different conformation than that of eIF1 (Fig. 2D).

eIF4F interacts with eIF3 and ribosomal protein eS7

The recruitment of the 43S to the 5′ UTR of mRNA is a critical step in the translation of eukaryotic mRNAs. Our previous structure of a human 48S complex allowed us to identify the location of eIF4F (*4*), but the low local resolution of the eIF4F and the adjacent highly flexible region of the 43S meant that we could only infer interactions with non-core subunits of eIF3 (eIF3e, -k, -l). Since these subunits do not exist in *S. cerevisiae*, it was not clear how eIF4F could interact with the yeast 43S complex. The improved local resolution in this region allows us not only to build eIF4F more accurately but also identify its interactions with the 43S in greater detail, including additional ones that are likely universally conserved.

The density shows almost the entire secondary structure of the middle domain of eIF4G (HEAT-1) and eIF4A (Fig. 3A-C). Rigid-body fitting of a crystal structure of a human eIF4A (PDB:5ZC9) (*19*) allowed us to assign its corresponding density (Fig. 3D-F). A model of the middle domain of eIF4G based on an AlphaFold prediction (*35*) was used for rigid-body fitting into the density (Fig. 3D-F). The predicted model agreed well with the density, allowing us to locate the domain of eIF4G in the structure.

eIF4F is positioned on the 5′ side of mRNA relative to the 40S subunit, near the mRNA channel exit site. Unlike in previous structures, mRNA interacting with eIF4A is clearly visible (Fig. 3A-C), and eIF4A here adopts a structure intermediate between the extended conformation, found in a crystal structure of a yeast eIF4A-eIF4G complex without mRNA in which the two RecA domains are rotated away from each other (*36*), and the closed conformation of an eIF4A-mRNA dimeric complex (*19*). This conformation allows the two RecA domains to interact with the mRNA while maintaining their interactions with eIF4G (Fig. 3A-F and fig. S6).

In metazoans, eIF3 plays a critical role during the recruitment of 43S to the eIF4F–mRNA (*3–5*, *8*, *10*). The structure reveals a large interaction network between eIF3 and eIF4F (Fig. 3G-I). Here, we describe the interaction between eIF3l and eIF4F further. Previously, because of high flexibility, the structure of eIF3l-NTD remained elusive. The improved local resolution reveals a near-complete structure of eIF3l-NTD and its interaction with eIF4G. Interestingly, the interaction between eIF3l-NTD and eIF4G occurs through a domain of eIF4G located between residues Met775 and Ala787, which was unpredicted (Fig. 3G). Additionally, the cryo-EM map reveals an unassigned density connecting eIF4G with eIF3c and -h (Fig. 3). Although the local resolution does not allow us to assign this density unambiguously, it likely belongs to the C-terminal tail of eIF3c (Fig. 3C). The interaction between eIF3c and eIF3-binding domain of eIF4G was previously predicted by site-specific cross-linking (*3*). The structure shows that this interaction occurs through the same unpredicted eIF3-binding domain of eIF4G as is involved in the interaction with eIF3l (Fig. 3 C and G).

Previously, eIF4F was observed to interact with the 43S complex through the eIF3 subunits -e, -k and -l (*4*). However, these subunits are not present in *S. cerevisiae* eIF3, and eIF3k and -l are dispensable in *Neurospora crassa* and *Caenorhabditis elegans* (*12*, *13*). Thus, these previously identified interactions could not occur in all eukaryotes, raising the question of how eIF4F makes an interaction with the 43S in those species. In this higher-resolution structure, we see several additional interactions between eIF4F and the 43S complex that are likely to be universally conserved. One of these could be the eIF4G-eIF3c interaction, given that eIF3c is conserved among all eukaryotes. Consistent with this idea, the eIF3c C-terminal tail includes residues highly conserved among eukaryotes, including in *S. cerevisiae* (fig. S7), which also explain why a knockdown of eIF3c reduces the recruitment of the 43S (*37*).

eIF4A binds to a pocket formed by eIF3c, -e, -h, and -l (Fig. 3). We previously described interactions between eIF4A and eIF3e, and eIF3k/l (*4*). The improved local resolution of this post-scanning complex allowed us to further characterize the interaction between eIF4A and the octameric structural core of eIF3. Of the two RecA domains of eIF4A, the C-terminal domain (CTD) is in close proximity to eIF3l, while the NTD makes contact with eIF3c, -e, and -h.

The interactions between eIF4A and eIF3h raises many questions. eIF3h is known to interact with METTL3 and promote mRNA circularization during m^6A dependent translation (*38*). The two helices from eIF3h extend toward the solvent site of the 43S, but the role of eIF3h in initiation was not clear. The core of the mammalian eIF3 octameric structural core is formed by a 7-helix bundle consisting of two helices from eIF3h and one helix from -c, -e, -f, -l and -k (*4*, *29*, *32*, *39*, *40*). Here, we see a direct interaction between eIF3h and eIF4A-NTD (Fig. 3i and fig. S8) through which eIF3h may play a regulatory role in initiation (Fig. 3). Consistent with this idea, phosphorylation at residue Ser183 of eIF3h, located just above the domain that interacts with eIF4A (fig. S8), has been implicated in the process of reinitiation in plants (*41*). In humans, the same phosphorylation has been associated with a possible oncogenic role of eIF3h (*42*). It is possible that this post-translational modification of eIF3h affects its interaction with eIF4F. Furthermore, this domain of eIF3h also interacts with mRNA (fig. S8).

The structure also reveals a direct interaction between eIF4A-NTD and ribosomal protein eS7 (Fig. 3J). The interaction of ribosomal proteins with the cap-binding complex was completely unexpected. eS7 is known to play a critical regulatory role in translation. Indeed, the structure rationalizes previous studies on translational control. eS7 is monoubiquitinated by the human E3 ubiquitin ligase CNOT4 or its yeast ortholog Not4 (*43–47*). Deubiquitination is required to allow the cap-binding complex eIF4F to bind to the 43S (*45*, *48*). Yet, how ubiquitination of eS7 can impair the binding of eIF4F (*45*) was unknown. Here, we see a direct interaction between eIF4A-NTD and the domain of eS7 ubiquitinated by E3 ubiquitin ligase (Fig. 3J-K). Superimposing our structure with a structure of ubiquitin (Ub) (*49*) at the predicted binding site in eS7 (*48*) places the Ub in a position where it could clash with eIF4A (Fig. 3K), thereby preventing the binding of the cap-binding complex eIF4F, showing why deubiquitination of eS7 is required for translation initiation (*45*, *48*).

A second molecule of eIF4A in the 48S

Compared to our previous 48S structure, we observed additional density at the entry site of the mRNA channel (Fig. 4A-B). Much of this density can be accounted for by eIF4A, which after rigid-body fitting of the known crystal structure of human eIF4A (*19*) shows close agreement with the density, especially the eIF4A-CTD, for which almost the entire secondary structure was resolved (Fig. 4B). eIF4A binds to a pocket formed by eIF3bgi, eIF3a-CTD and rRNA h16 (Fig. 4C-D). This position of eIF4A agrees well with previous biochemical, cross-link mass spectrometry, and proximity labelling data (*4*, *17*, *18*). Moreover, recent single-molecule data revealed FRET between eIF4A and ribosomal uS19 (*50*); the latter is located within FRET distance of this second eIF4A in our structure.

Compared to the intermediate conformation eIF4A adopts as part of eIF4F upstream of the 43S (Fig. 3), the two RecA domains of this entry site eIF4A adopt a more open conformation (fig. S9). However, this open conformation differs from that described for the yeast eIF4A-eIF4G complex (*36*) (fig. S9). Interestingly, the conformation of this entry site eIF4A resembles that of the crystallographic structure of the eIF4A-Pdcd4 (Programmed cell death 4) complex (*51*) (fig. S9). This conformation allows the NTD and CTD to contact the mRNA while the nucleotide-binding domain adopts a more open conformation. The structure shows that eIF4A can remain bound to mRNA as it cycles through the open and closed conformations during scanning, which can explain the factor-dependent processivity of human eIF4A described previously (*16*).

Unlike previous 48S structures in which mRNA outside the 40S subunit was not seen, there is clear and continuous density for the mRNA which allowed us to trace its path from the cap-binding complex eIF4F upstream of the 43S, through the 40S subunit mRNA channel all the way to the second molecule of eIF4A downstream of the 43S (~40 nt upstream and ~20 nt downstream of the AUG; Fig. 4A-B). This allows us to clearly see the nature of the interactions of mRNA with the two eIF4A molecules on opposite sides of the 40S subunit.

It has been shown that yeast 43S stimulates the ATPase activity of eIF4A (*17*). Interestingly, this activity of yeast eIF4A does not require eIF4G or eIF4E (*17*). Instead, eIF3, especially eIF3i and eIF3g, are required to stimulate the ATPase activity of eIF4A. Because the cap-binding complex eIF4F binds upstream of the 43S (Fig. 3), it was unclear how eIF3 subunits downstream of the 43S could affect the activity of eIF4A. This apparent discrepancy is clarified by the direct interaction observed between the second molecule of eIF4A and the eIF3bgi module (Fig. 4). Indeed, the structure reveals that eIF3i is the main binding partner of this second eIF4A.

eIF3a binds to the solvent exposed site of the 40S, near the mRNA channel exit site. However, its C-terminal domain extends toward the solvent side of the 40S, where it interacts with eIF3bgi at the mRNA channel entry site (*52*). Our previous cross-linking mass spectrometry indicated that eIF3a-CTD (Lys632) is in close proximity to eIF4A-CTD (Lys291) (*4*). Consistent with this observation, the structure shows a close proximity between $eIF3a_{630-646}$, a disordered region in the eIF3a-CTD and Lys291 of eIF4A-CTD (Fig. 4C-D). Indeed, the structure also explains prior biochemical data indicating that eIF3a-CTD and eIF3bgi play important role during recruitment and scanning (*53*).

Mammalian eIF4G has two eIF4A-binding domains, one located in the middle domain (HEAT-1) and a second one located at the C-terminal (HEAT-2) (*54*, *55*). The eIF4G HEAT-1 is highly conserved amongst eukaryotes and is the core of the eIF4F complex, while the eIF4G HEAT-2, not present in *S. cerevisiae*, is poorly conserved and is not essential (*54*, *55*). It has been proposed that HEAT-2 plays a stimulatory role (*54*). To test whether HEAT-2 is required for recruitment of the second molecule of eIF4A, we analyzed a 48S complex with a truncated eIF4G lacking HEAT-2. The second molecule of eIF4A is still present in this complex (Fig. S10), suggesting that its binding at the mRNA entry site does not require eIF4G, consistent with previous biochemical data showing that *S. cerevisiae* 43S stimulates the ATPase activity of eIF4A in the absence of eIF4G (*17*).

A previous biochemical study proposed that eIF4F binds at the mRNA entry site (*8*). However, at the entry site, the part of eIF4A that interacts with eIF4G in the eIF4F complex is involved in interactions with eIF3i (Fig. 5A-B). The structure suggests that the position of eIF4A at the mRNA channel entry site is incompatible with its interaction with the eIF4G HEAT-1 seen in the cap-binding complex eIF4F (Fig. 5A-B). We therefore propose that this second molecule of eIF4A acts separately from eIF4F.

A possible location for eIF4B

By itself, eIF4A has low helicase activity. Even though its activity at the mRNA channel entry site is modulated by the 43S (*17*), the helicase activity of eIF4A in scanning the 5′ UTR of mRNA with stable secondary structure is enhanced by the cofactor eIF4B or its homolog eIF4H (*14*, *15*). The precise binding site of eIF4B remains elusive. Our cryo-EM map contains an unassigned density adjacent to the eIF4A-NTD, in close contact with eIF3g-RNA recognition motif (RRM) and ribosomal protein uS10 (Fig 5C-D). Although the resolution of the additional density is low, its size and shape are consistent with that eIF4B-RRM (Fig. 5C-D). Consistent with this interpretation, cryo-EM maps of complexes formed without eIF4B does not contain this additional density (fig. S11). This location of eIF4B agrees well with previous biochemical data indicating that eIF4B or eIF4H interact with the same region of the eIF4A-NTD (*56*). Furthermore, previous yeast two-hybrid analysis identifying the ribosomal protein uS10, located in close proximity to the unassigned density, to be the main interaction partner of eIF4B (*14*). This possible location, which suggests a direct interaction between eIF4B and the entry-site eIF4A, agrees well with the role of eIF4B in increasing the directionality of eIF4A translocation (*16*).

In addition to its role as a subunit of eIF4F, a second molecule of eIF4A at the mRNA channel entry site likely promotes mRNA recruitment and scanning. A second eIF4A in the 48S is consistent with the finding that eIF4A is in excess over other components of eIF4F (*57*, *58*). However, even a second eIF4A in the 48S does not account for the substantial excess eIF4A in the cell (*57*, *58*). Thus, it possible that eIF4A could have additional functions apart from its role in the 48S complex. Consistent with this idea, the eIF4A-mRNA interaction has a long lifetime (*59*), and eIF4A is known to be able to melt RNA secondary structure in the absence of the 43S (*57*).

Molecular mechanism for translational regulation by eIF4A and eIF4F

In our structure, the cap-binding complex eIF4F binds upstream of the 43S complex, while a second molecule of eIF4A binds downstream, at the mRNA channel entry site. These locations

suggest a model where independently of its role as part of eIF4F, eIF4A also plays an eIF4F-independent role during mRNA recruitment (*17*) and scanning (Fig. 6A-C).

The cap-binding complex eIF4F at the 5′ end of mRNA plays a critical role in recruiting the 43S downstream of it, in a process that involves the mRNA being slotted into its channel in the 40S subunit (Fig. 6C). In the previous study (*4*), the proposed slotted mechanism raised questions since *S. cerevisiae* lacks the subunits of eIF3 involved in the interaction with eIF4F. However, as we show here, eIF4F makes additional interactions including with eIF3c and ribosomal protein eS7 – both universally conserved among eukaryotes.

Canonical eIF4F-dependent initiation is enhanced by a topologically closed loop of the mRNA, often mediated by an interaction between eIF4G at the 5′ end and PABP at the 3′ end (*60*, *61*). Such a topology would require a slotting mechanism for recruitment. Similarly, during m^6A-dependent translation, it has been shown that the mRNA is circularized through an interaction between METTL3 located at the 3′ of mRNA and eIF3h (*38*). Other modes of initiation also require the slotting mechanism, including that via internal ribosome entry sites (IRES), eIF3d-dependent or circular mRNA. However, some mRNAs with unusually short 5′ UTR (less than 40 nucleotides in mammals) may use alternative recruitment pathways. A recent study proposed that these mRNAs may use the slotting mechanism as well (*62*). However, this would require a backward movement of the 43S (3′ to 5′) rather than the small oscillations that that are known to exist (*50*). Thus, it is more likely that those transcripts use alternative recruitment pathway.

In addition to eIF4F, a second molecule of eIF4A at the entry site (Fig. 6C) may promote the accommodation of the mRNA into the channel, especially when secondary structure is present in the 5′ UTR (*17*, *63*). Consistent with this idea, the cryo-EM 3D classification reveals a class of particles with eIF4A bound to the 43S but without the mRNA accommodated into the channel at the entry site (fig. S12), suggesting that the binding of the second eIF4A to the 43S precedes the binding of the mRNA. Consistent with this idea, *S. cerevisiae* eIF3bgi stimulates the eIF4G–eIF4E-independent ATPase activity of eIF4A during initiation (*17*). Moreover, given the possible location of the eIF4B-RRM, we cannot rule out additional roles of eIF4B in this process (*64*).

The position of the second molecule of eIF4A at the mRNA channel entry site revealed by our structure provides strong evidence that this conserved RNA helicase likely functions to unwind the mRNA secondary structure during scanning. Thus, the structure suggests a model whereby after recruitment, both eIF4F and eIF4A may work in concert to ensure the directionality of scanning (Fig. 6D). In this model, eIF4F would prevent the reverse movement of the 43S complex (*4*) while the second molecule of eIF4A at the leading edge of the ribosome would use the energy from ATP hydrolysis to unwind downstream RNA secondary structure and translocate along the 5′ UTR of mRNA during scanning (Fig. 6D). Moreover, it is possible that the ATP hydrolysis by eIF4F would further help the movement of the 43S and help overcome energy barriers encountered by the eIF4A.

Compared to many other helicases, eIF4A has low helicase activity. Even though its ATPase activity is stimulated by the 43S and its co-factor eIF4B, scanning of 5′ UTR of mRNA with highly stable secondary structure may require additional helicases, such as mammalian DHX29, which has been seen to bind at the mRNA channel entry site (*39*, *65*). In our structure, eIF4A–eIF4B

interacts with the 43S at the exact location that DHX29 does (*39*, *65*) (fig. S13). Thus, it is possible that for initiation on specific mRNAs with high secondary structure, other helicases such as DHX29 are recruited to the 43S complex instead of a second eIF4A molecule. Such a method would allow additional control of translation initiation of such mRNAs.

eIF4E stabilizes the binding of mRNA to eIF4G-eIF4A (*59*) and stimulates the helicase activity of eIF4A (*66*). Moreover, selective 40S ribosome profiling indicated that scanning is cap-tethered in most human cells (*67*) (Fig. 6E). In the prior structure of 48S there is a low-resolution density that we tentatively assigned to eIF4E (*4*). That structure used a very short mRNA so no significant scanning had occurred. In the structure here, scanning has spanned 105 nucleotides until the start codon was reached, thus representing a later stage of initiation. Although local resolution was significantly improved, we did not see such additional density that we could assign to eIF4E, suggesting that it, along with the 5′ cap, are released from eIF4F and the rest of the 48S during scanning (Fig. 6F). This is not consistent with biochemical experiments suggesting tethering of the cap to the 48S (*67*), and the reasons for the discrepancy are not clear.

Conclusion

This work reveals the cap-binding complex eIF4F at a much better resolution which sheds light on several critical and unpredicted interactions with the 43S, including that with the ribosomal protein eS7. It also reveals a second molecule of eIF4A and a possible location for eIF4B at the mRNA entry site, on the opposite side of the 43S from eIF4F. The presence of two molecules of eIF4A in the 48S, one part of eIF4F and the other acting with eIF4B but independently of eIF4F, suggests a model for how scanning occurs on structured mRNAs and resolves several discrepancies in prior studies.

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Supplementary Materials:

Materials and Methods

Figures S1-S13

Tables S1

References (*68-84*)

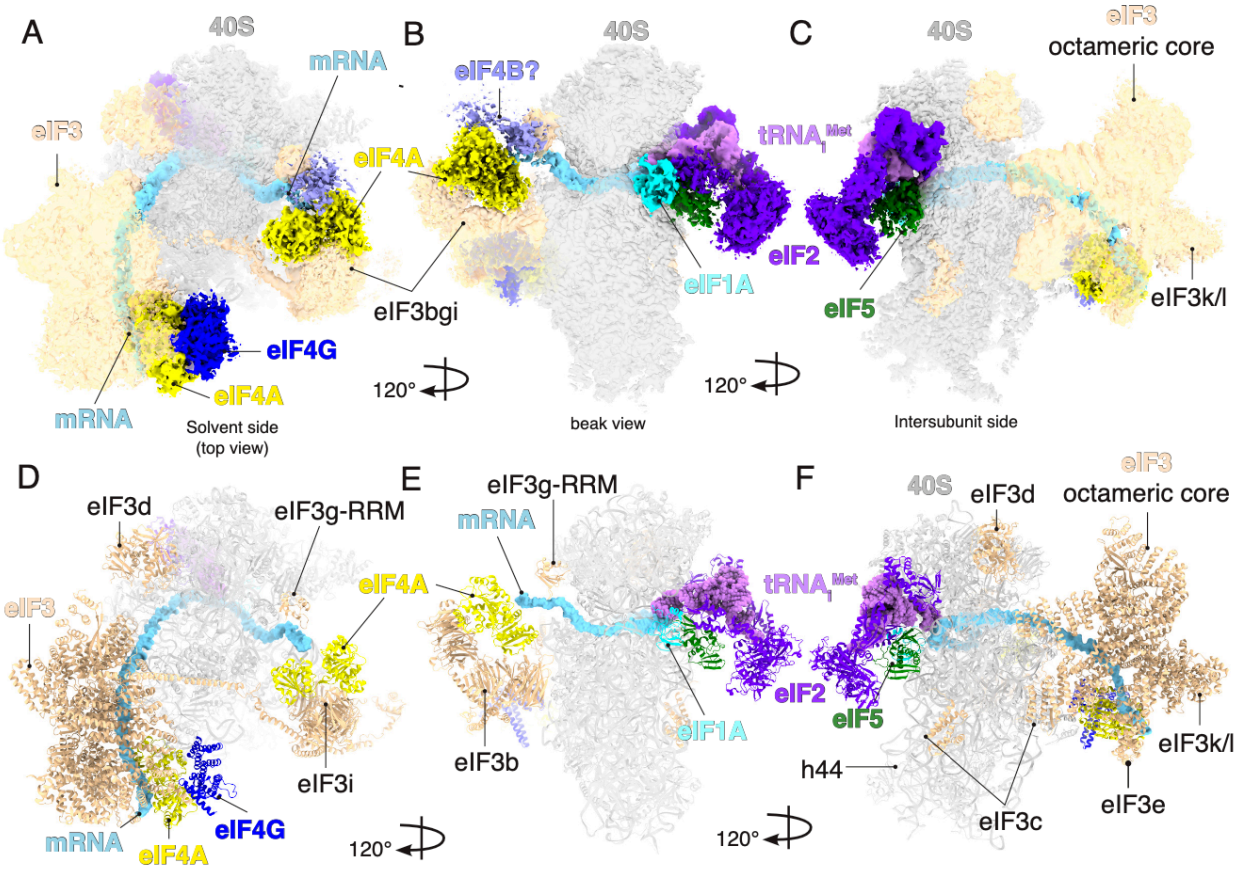

Fig. 1. Cryo-EM 3D reconstruction and the structure of human 48S complex. Overview of the cryo- EM density map (**A** to **C**) and molecular model of human 48S (**D**-**F**) shown in different orientations. The map contains densities for 40S small ribosomal subunit, eIF3, eIF1A, eIF5-NTD, eIF2, tRNA$_i^{Met}$, mRNA, eIF4F (eIF4G and eIF4A), and the second molecule of eIF4A bound to eIF3 at the mRNA channel entry site. The map also contains a possible density for eIF4B in contact with the second molecule of eIF4A at the entry site.

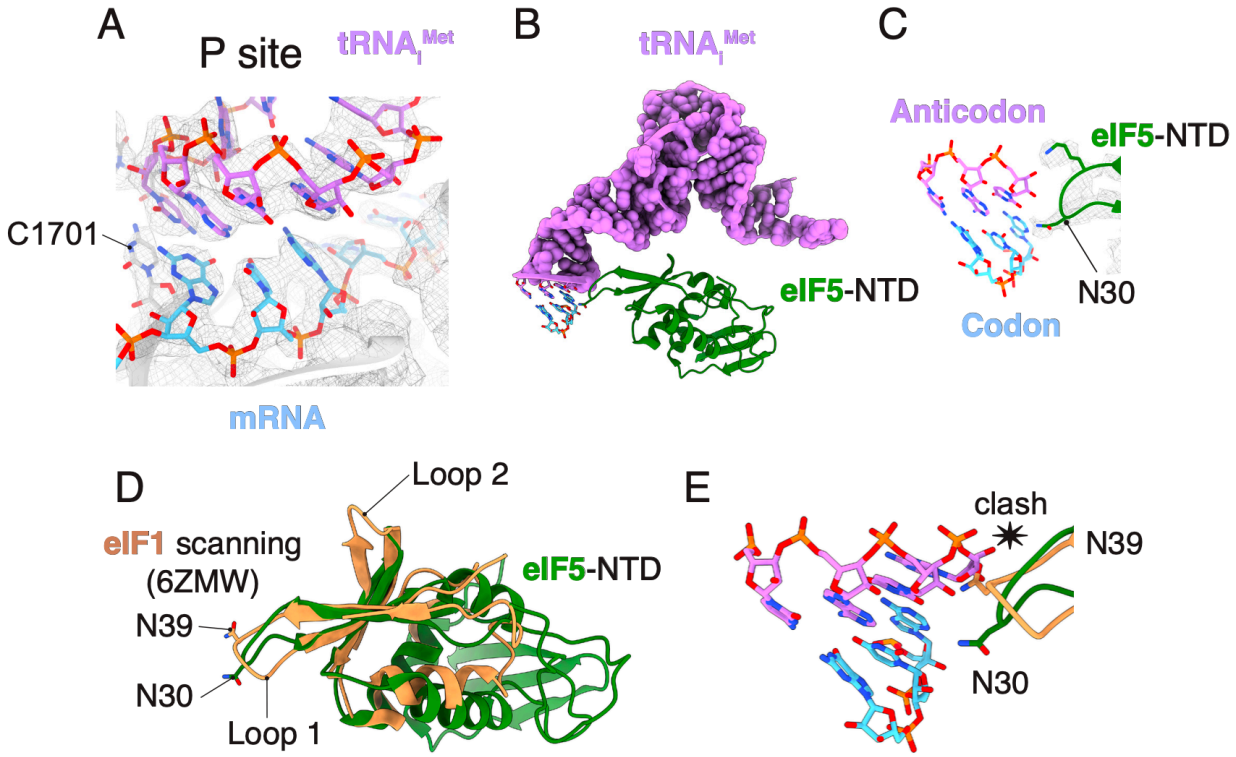

Fig. 2. Codon-anticodon base pairing in the P site. (A) Close-up of the P site to highlight the codon-anticodon base pairing. (**A** to **C**) eIF5-NTD replaces eIF1 after AUG start codon recognition. (**D** to **E**) Superposition of eIF5-NTD with the structure of human eIF1 during scanning (PDB:6ZMW) *(4)*. The conformation of eIF1 during scanning would clash with the anticodon stem loop of the $tRNA_i^{Met}$ after start codon recognition.

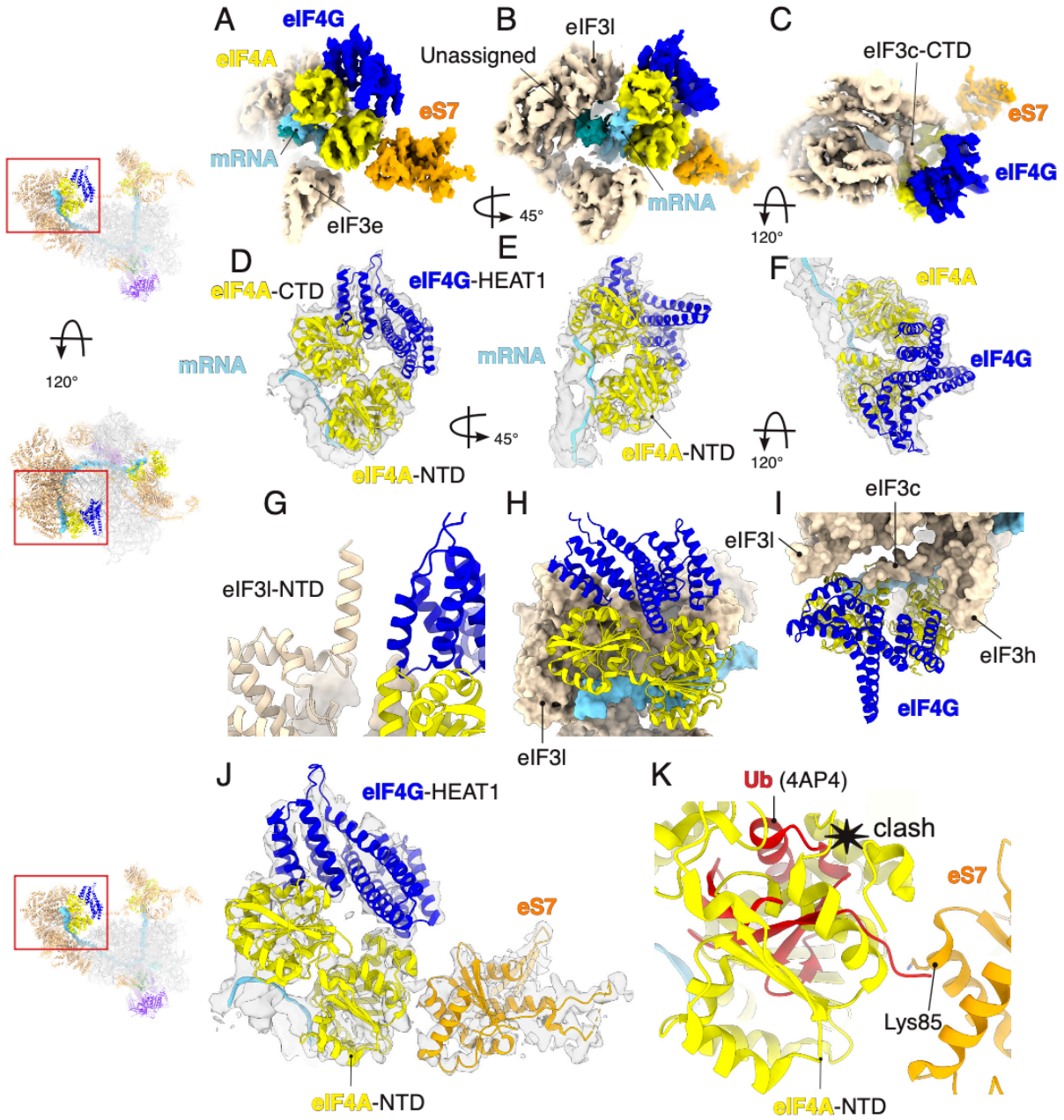

Fig. 3. Structure of eIF4F and its interactions with eIF3 and ribosomal protein eS7. (**A** to **C**) Cryo-EM map to highlight eIF4F and its interaction network. (**D** to **F)** Atomic model of human eIF4A and eIF4G fitted into the cryo-EM map. (**G** to **I)** The insets highlight the interaction network of eIF4F with eIF3. (**J**) Close up to highlight the interaction between eIF4F and ribosomal protein eS7. (**K**) Superposition of ubiquitin (PDB:4AP4) *(49)* with the structure of human 48S to highlight the possible clash between eIF4A and ubiquitin at its predicted binding site (Lys85).

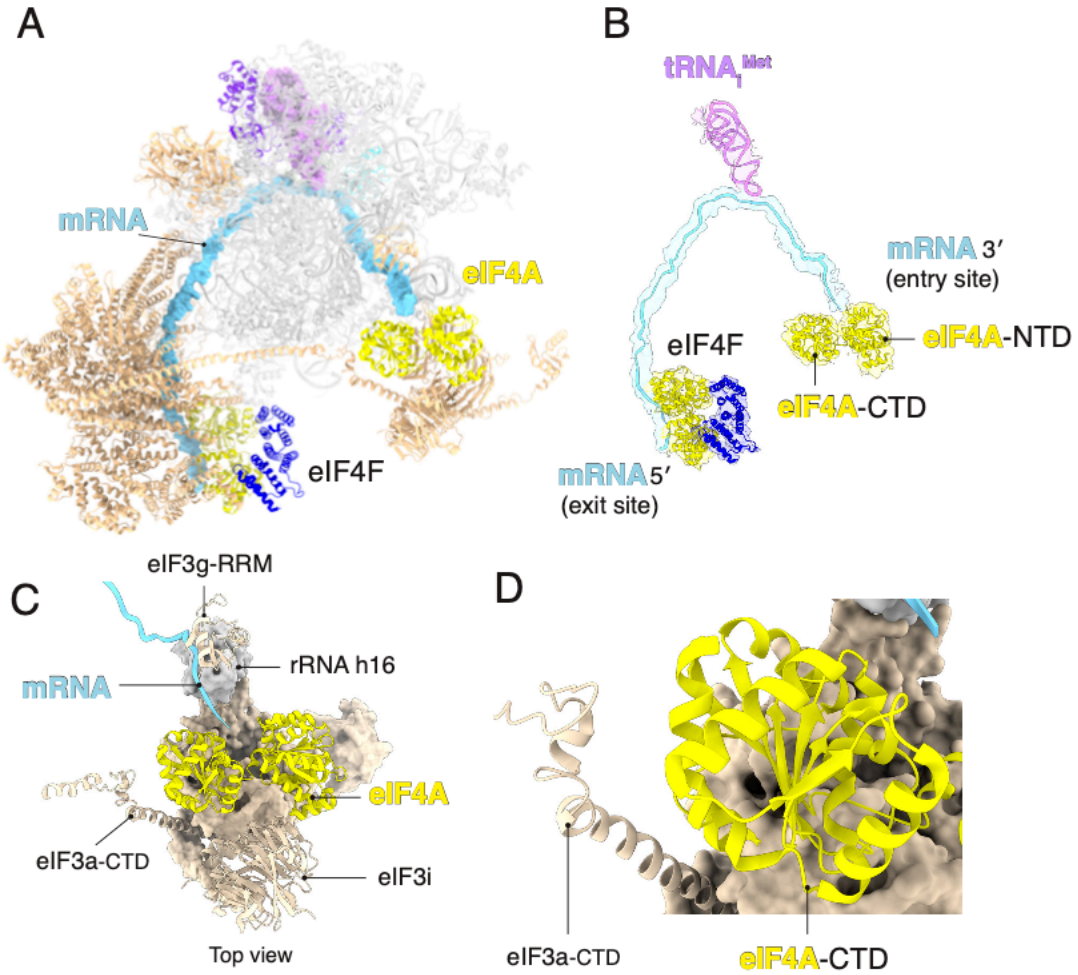

Fig. 4. Structure of human 48S reveals a second molecule of eIF4A bound at the mRNA channel entry site. (**A**-**B**) Structure of human 48S revealing the path of the mRNA from eIF4F towards the second molecule of eIF4A at the entry site. (**B**) Atomic model of eIF4A, eIF4F, mRNA, and tRNA$_i^{Met}$ fitted into the cryo-EM map. (**C**) Interactions of eIF4A at the entry site. (**D**) The interface between eIF4A-CTD and eIF3a- CTD.

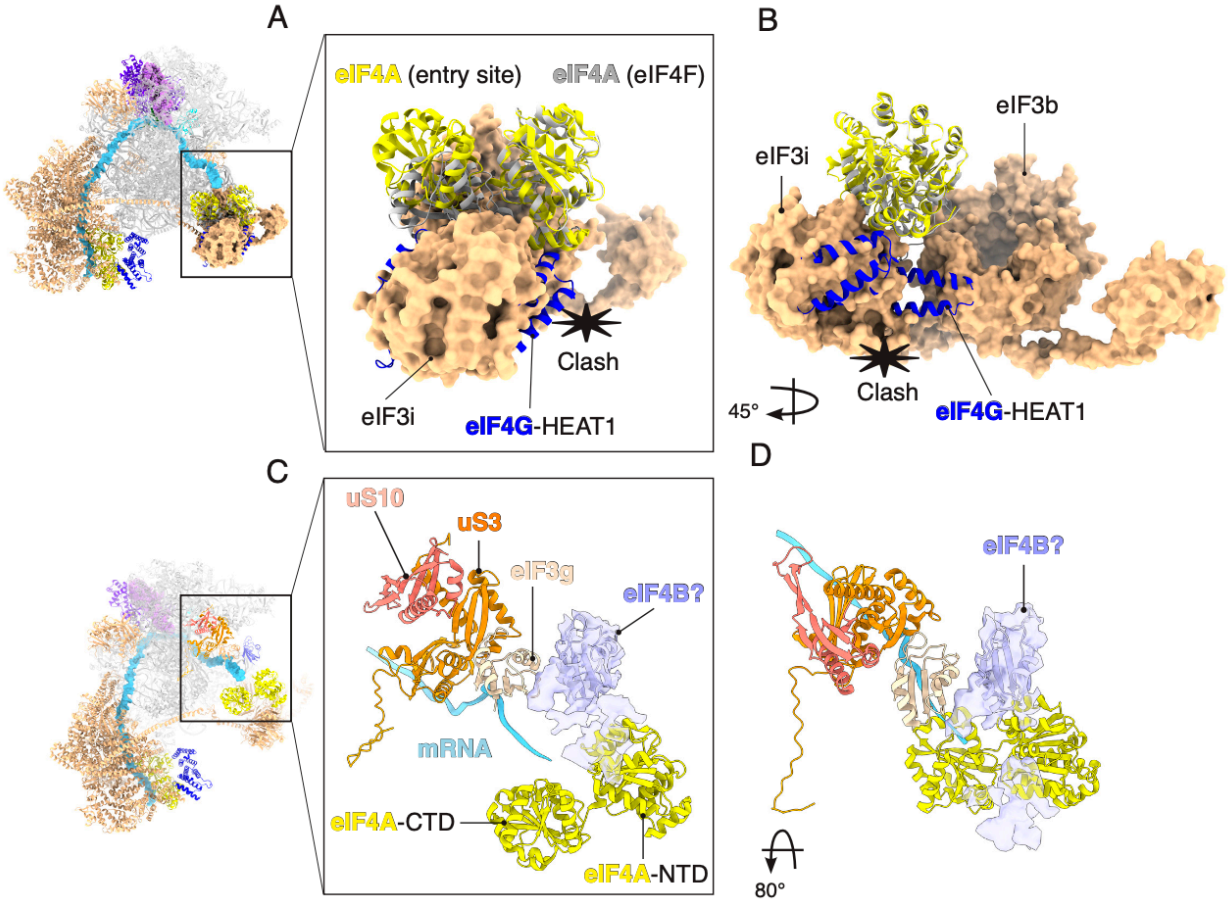

Fig. 5. Conformational differences between the two molecules of eIF4A and a possible location of eIF4B in the 48S. (**A** to **B**) Superposition of eIF4F with entry site eIF4A to highlight conformational differences. eIF4G-HEAT1 would clash with eIF3i and -b. (**C** to **D**) Atomic model of human eIF4B- RRM (AlphaFold prediction) fitted into the unassigned cryo-EM density map at the entry site. The possible location of eIF4B places it in close proximity to eIF4A-NTD, eIF3g-RRM, uS3, and uS10.

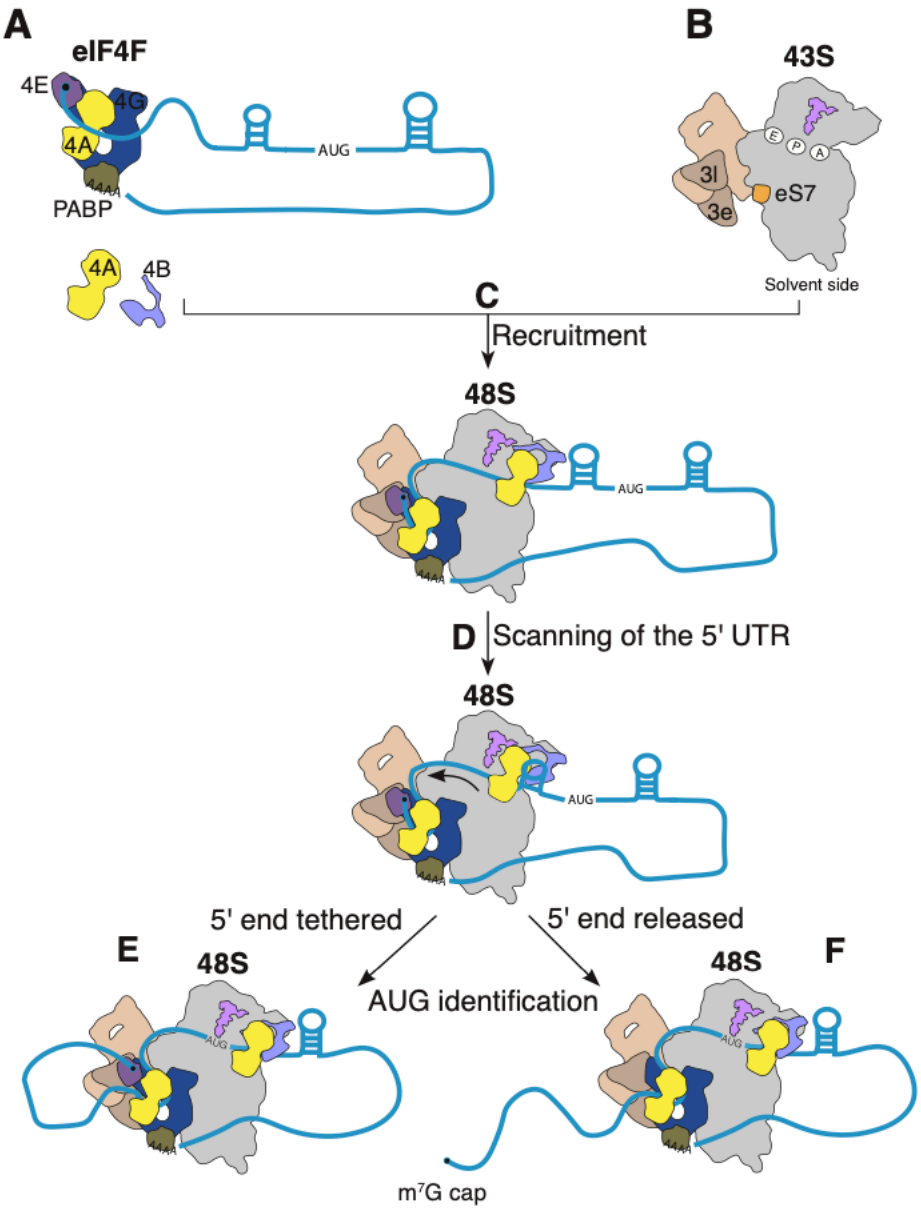

Fig. 6. Model for translational regulation by eIF4F and eIF4A. (**A** to **C**) The cap-binding complex eIF4F (**A**) binds at the 5′ end of mRNA and recruits the 43S (**B**) to form the 48S complex (**C**). eIF4F binds to eIF3 and ribosomal protein eS7 located upstream of the 43S. The position of eIF4F is compatible with a model whereby the mRNA is slotted into the mRNA channel in the 40S small ribosomal subunit. In addition to eIF4F, a second molecule of eIF4A at the mRNA entry site is likely to play a role in this process by facilitating the accommodation of the mRNA into the channel. The ATPase activity of eIF4A is stimulated by eIF4B and eIF3bgi module *(17)* located at the entry site. Thus, eIF4A at the entry site is likely to use the energy from ATP hydrolysis to unwind the RNA secondary structure downstream of the 43S (**D**). It remains unclear whether eIF4E remains attached to the rest of eIF4F (**E**) or is released (**F**) during the scanning process, but the structure suggests the latter.