Unorthodox Mechanisms to Initiate Translation Open Novel Paths for Gene Expression

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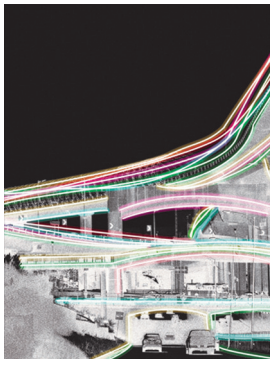

***Legend:** Decades of research on translation in eukaryotes have established the crucial role of the scaffold protein eIF4G as a center-stage player during the initiation step. The recent discovery of various alternative mechanisms that use scaffold proteins phylogenetically unrelated to eIF4G or translation initiation complexes lacking an eIF4G-like central multipurpose ribosome adaptor has stirred the orthodox view of how eukaryotes perform protein synthesis. Thus, a new picture is emerging in which the unorthodox translation initiation complexes open novel paths for gene expression in eukaryotes. The cover refers to the Featured Perspective article by G. Hernández et al. "Unorthodox mechanisms to initiate translation open novel paths for gene expression" published in this JMB issue. The illustration depicts an artistic representation of the novel, different avenues of gene expression.*

Abstract

Translation in eukaryotes is dependent on the activity of translation initiation factor (eIF) 4G family of proteins, a scaffold protein that, during the initiation step, coordinates the activity of other eIFs to recruit the 40S ribosomal subunit to the mRNA. Three decades of research on protein synthesis and its regulation has provided a wealth of evidence supporting the crucial role of cap-dependent translation initiation, which involves eIF4G. However, the recent discovery of a surprising variety of alternative mechanisms to initiate translation in the absence of eIF4G has stirred the orthodox view of how protein synthesis is performed. These mechanisms involve novel interactions among known eIFs, or between known eIFs and other proteins not previously linked to translation. Thus, a new picture is emerging in which the unorthodox translation initiation complexes contribute to the diversity of mechanisms that regulate gene expression in eukaryotes.

The multipurpose ribosome adapter

Translation is a fundamental process for all organisms and is mostly regulated at the initiation step.[1,2] In eukaryotes, this step consists of recruitment of the 40S ribosome subunit to the 5′-untranslated region (UTR) of an mRNA through the action of translation initiation factors (eIFs). At

the core of this process is the scaffold protein eIF4G (formerly p220 or eIF-4γ), that interacts with and coordinates other eIFs to achieve ribosomal recruitment.[3–5] eIF4G is, as M. Hentze first defined it, a multipurpose ribosome adapter.[6]

In the early 1970s, the question of how ribosomes are recruited to the mRNA in eukaryotes remained open.[7,8] In 1975, the cap structure (m^7GpppN, where N is any nucleotide) at the 5′ end of mRNAs was discovered and found to strongly promote translation.[9,10] Soon, experimental evidence led to elaboration of the scanning mechanism, which posits that the 40S ribosome subunit binds the 5′ end of a messenger and subsequently scans the UTR until it encounters the first translation initiation site.[8] In 1978, a 24 kDa polypeptide that specifically binds to the mRNA cap was purified to homogeneity and termed later eIF4E.[11,12]

It was shown later that mammalian eIF4E associates with eIF4G and eIF4A, to form a complex called eIF4F that can revert the rapid and profound inhibition of protein synthesis caused in cells by poliovirus infection.[13] Indeed, biochemical analyses demonstrated that infected cells were defective in the activity of only eIF4F, caused by the cleavage of its eIF4G subunit.[14,15] The subsequent purification and biochemical characterization of other initiation factors led to the establishment of the canonical pathway of translation initiation in which eIF4G serves as a scaffold or a multipurpose adapter that brings together in functional association eIF4E, PABP (poly(A) binding protein), the 40S-associated eIF3 and eIF4A to recruit ribosomal 43S pre-initiation complex (PIC). This network of interactions turns eIF4G a center-stage player during translation initiation.[6,15] The cloning of eIF4G from many species showed that eIF4G possesses a modular structure with binding sites for different initiation factors that is highly conserved across eukaryotes, in particular its central region and the eIF4E-binding site.[15,16]

Here, we describe the recent discovery of functional analogs of eIF4G, phylogenetically unrelated to it, and translation initiation complexes lacking an eIF4G-like central multipurpose ribosome adaptor that are found in different species. These findings pave the way toward a deeper understanding of translation initiation, and open novel paths for gene expression in eukaryotes.

The canonical model of translation initiation in eukaryotes

For many, if not most, eukaryotic mRNAs, translation initiation is performed by the canonical cap-dependent mechanism (Figure 1). It begins when the cap structure of the mRNA is recognized by eIF4E. During this process, the scaffold protein eIF4G binds both eIF4E and the DEAD box ATP-dependent RNA-helicase eIF4A to form the eIF4F complex. The eIF4G-PABP interaction promotes mRNA circularization that causes a crosstalk between both the 5′-UTR and 3′-UTR ends of mRNA (Figure 1(a)). This architecture of the mRNA is called the "closed-loop model". In the meantime, a free 40S ribosomal subunit interacts with eIF1, eIF1A, eIF3, eIF5 and a ternary complex (TC, consisting of eIF2 bound to an initiator Met-$tRNA_i^{Met}$ and GTP) to form a 43S PIC (Figure 1(b)). eIF4G also performs interactions with PABP and the ribosome-bound eIF3, to drive recruitment of the 43S PIC to the mRNA 5'-UTR.[4,5,13,17] Thus, eIF4G's role is at the core of translation, as it coordinates the activity of the factors involved in ribosome recruitment.[6,15] In the closed-loop model of mRNA, the two ends of mRNA communicate by physical 5′–3′ proximity caused by the cap-eIF4E-eIF4G-PABP-poly(A) interaction network.[18] A functional crosstalk between the two mRNA ends might also be achieved by different interactions among several factors or even by intrinsic features of mRNA.[18] However, mRNA circularization might not be a universal rule to initiate translation, as actively translated mRNAs can be found with a relaxed 5′–3′ proximity *in vivo*.[18]

After recruitment, the 48S PIC scans base-by-base the mRNA 5′-UTR to reach the authentic AUG start codon (Figure 1(c)). During this process the helicase eIF4A unwinds secondary structures present along the mRNA 5′-UTR, an activity assisted by eIF4B. eIF1, eIF1A, eIF2, 3, and 5 drive faithful selection of the start codon (most often an AUG) by the ribosomal complex. In a complex, concerted action these factors arrest mRNA scanning, and catalyze tight and stable positioning of Met-$tRNA_i^{Met}$ within the peptidyl (P) site of the ribosome with the anticodon making perfect Watson-Crick base-pairing with AUG codon. AUG recognition triggers irreversible GTP hydrolysis bound to eIF2, resulting in the formation of a stable 48S PIC and the release of the eIF2-GDP-eIF5 complex, free Pi, and eIF1 from the PIC. eIF5B-GTP promotes 60S ribosomal subunit joining, dissociation of eIF5B-GDP and the assembly of an 80S initiation complex.[4,5,17]

Alternative eIF4G-independent complexes for translation initiation

Functional convergence with eIF4G in *Drosophila* germline development

We have described a functional, non-phylogenetically related analog of eIF4G termed Mextli (Mxt).[19] Early work had shown that Mxt is a component of cap-binding complexes during *Drosophila* embryogenesis.[20] We further showed that Mxt possesses a modular structure with a region resembling the MIF4G (middle domain of eIF4G) only at the secondary structure level but not

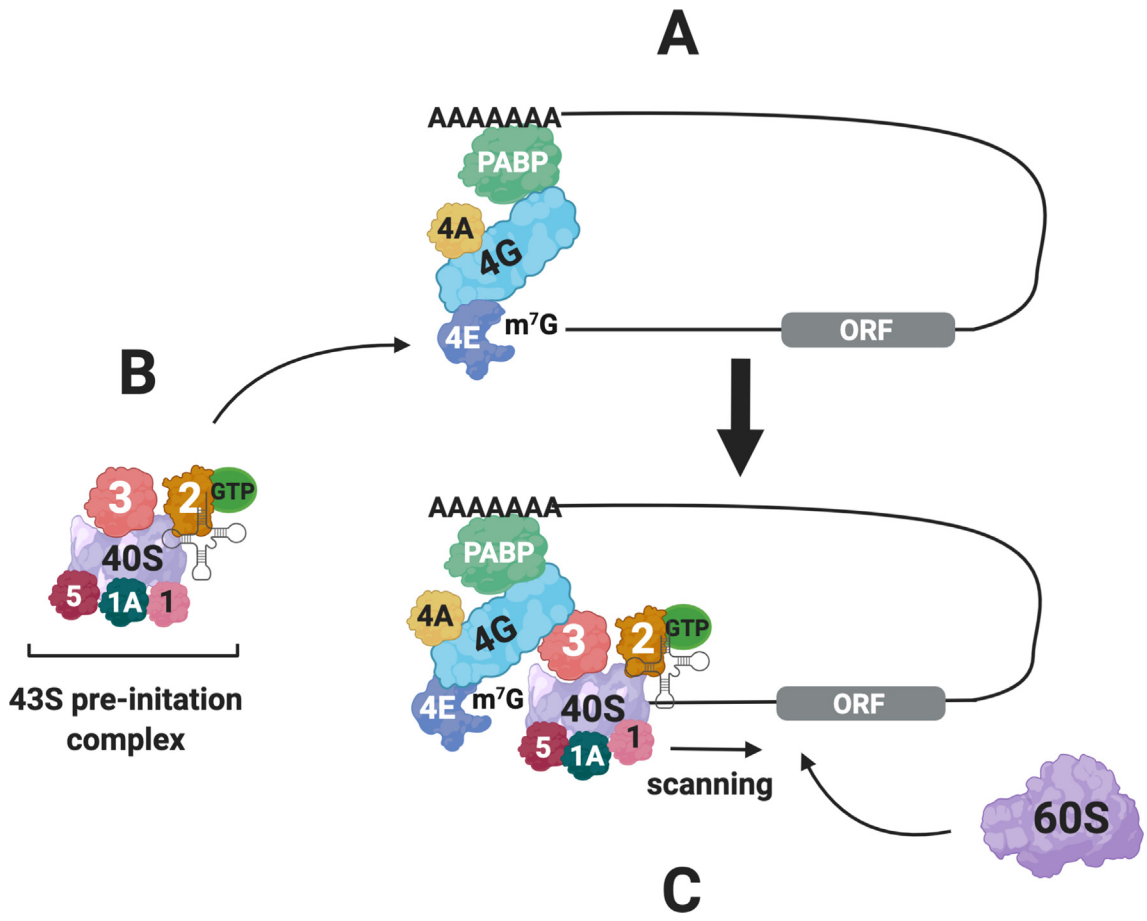

Figure 1. The canonical translation initiation in eukaryotes. **(a)** A circularized, competent for translation mRNA is established by its interaction with translation initiation factors. The cap structure (m^7G) at the 5′ end of an mRNA is recognized by eIF4E (*4E*). The scaffold protein eIF4G (*4G*) simultaneously binds to the cap-bound eIF4E, the RNA helicase eIF4A (*4A*) and to PABP which is bound to the poly(A) tail of mRNA, to form the mRNA-eIF4F complex. **(b)** A 40S ribosomal subunit interacts with eIF3 (*3*), eIF1 (*1*), eIF1A (*1A*), eIF5 (*5*) and a ternary complex consisting of eIF2-GTP-Met-tRNA$_i^{Met}$, forming a 43S pre-initiation complex (PIC) which is recruited to the 5′-end of the mRNA in the mRNA-eIF4F complex. **(c)** The 43S PIC scans the mRNA 5′-UTR to reach the translation initiation site, ultimately leading to the joining of a 60S ribosomal subunit to the mRNA—ribosome complex. Created with BioRender.com.

sequence similarity which interacts with eIF3, a mini-KH domain for RNA-binding, and a canonical eIF4E-binding site that allows it to interact with most of the eIF4E paralogs that exist in this species.[19] Further crystallography studies showed that Mxt uses both a canonical eIF4E-binding motif and a novel tripartite, non-canonical interphase to form eIF4E complexes.[21] Unlike eIF4G, Mextli does not associate with PABP nor eIF4A, but it interacts with the *Drosophila* ortholog of human DHX35 (DEAH-box helicase 35), encoded by the gene *CG3225* (Figure 2(b)). Unexpectedly, we found that Mxt promotes translation *in vitro* by serving as a novel type of multipurpose adaptor for the 43S PIC recruitment.[19] Thus, Mxt represented the first example of functional convergence of eIF4G in evolution.

The discovery of Mxt was followed by growing evidence that, compared to differentiated cells, translation and the translational machinery in pluripotent cells appear to have a stronger preference for non-canonical components.[22] Mextli plays a role in oogenesis, early embryogenesis, and functions in germline stem cell (GSC) maintenance. Mxt forms a complex with the RNA helicase DHX35, but not with the canonical RNA helicase eIF4A.[19] So far, no functional specificity for the Mextli-eIF4F complexes containing any of the different eIF4E isoforms has been determined. Other alternative factors in *Drosophila* are eIF4E3 and eIF4G2[23–26] that play critical roles during spermatogenesis, and the variant ribosomal proteins RpS5b, RpS10a, RpS19b, and RpL22-like, expressed in ovary and embryo GSCs, and in primordial germ cells (PGCs).[22,27] RpS5b, but not RpS5a, is required for oogenesis and the synthesis of mitochondrial electron transport and cellular metabolic proteins.[28] Thus, gene expression regulation in GSCs and PGCs differs from that in somatic cells, which is reflected in the translational machinery itself.

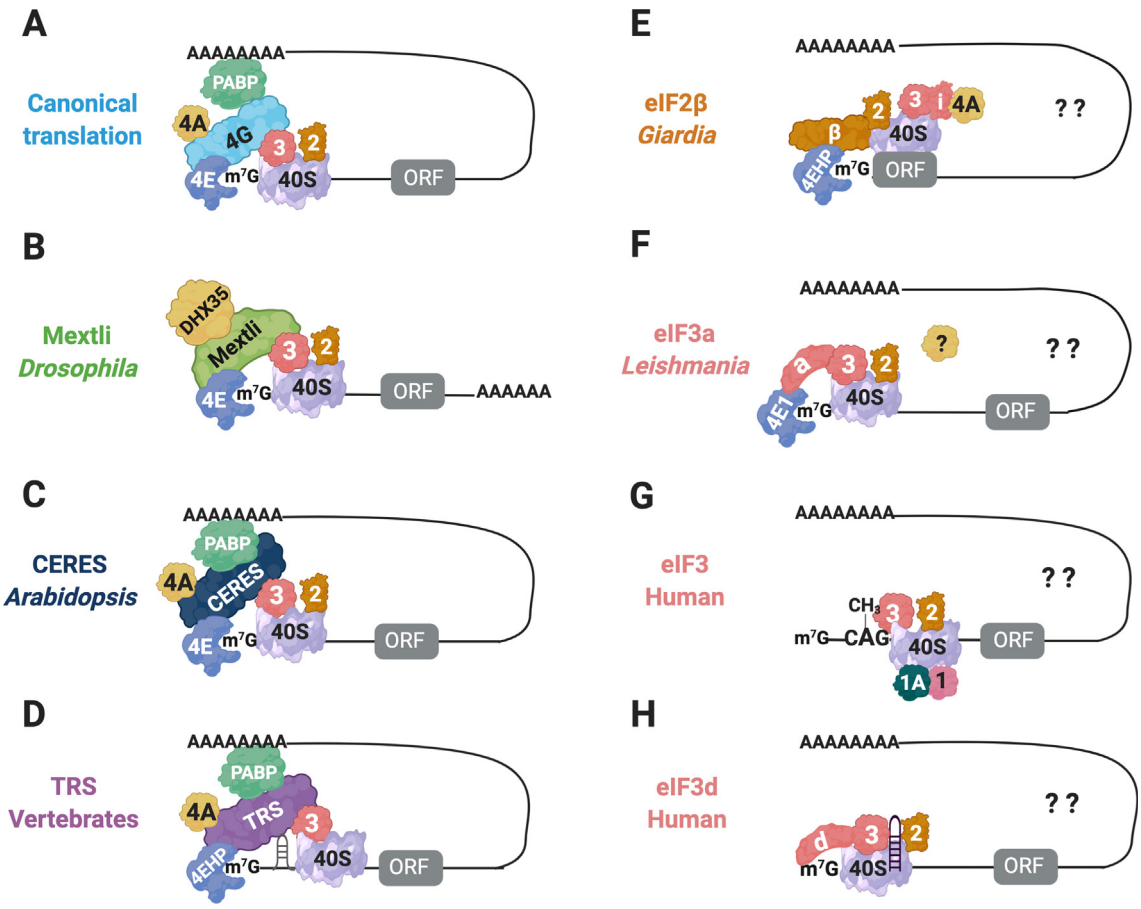

Figure 2. eIF4G-independent translation initiation. Models of unorthodox protein complexes for 43S PIC recruitment in the absence of eIF4G from different species are depicted. For comparison, **(a)** the formation of 48S pre-initiation complex in the canonical, eIF4G-dependent translation initiation is illustrated. Only some eIFs are shown. **(b–d)** eIF4G-independent translation initiation using unorthodox, phylogenetically unrelated to eIF4G, eIF4E-interacting proteins serving as central scaffold. **(b)** *Drosophila melanogaster* Mextli,[19] **(c)** *Arabidopsis thaliana* CERES,[33] and **(d)** vertebrates threonyl-tRNA synthetase (TRS)[36] functionally replace eIF4G as multipurpose ribosome adapters. **(e)** *Giardia lamblia* eIF2β,[41] and **(f)** *Leishmania major* eIF3a[43] directly bind eIF4E to promote translation. ***G*-(h)** Human alternative protein complexes are depicted for both eIF4E- and eIF4G-independent translation initiation using eIF3 as a scaffold. **(g)** eIF3 binds N^6-methyladenosine (m^6A) residues within the 5′-UTR to selectively promote cap-independent translation of heat shock protein 70 (*Hsp70*) mRNA.[46] **(h)** Human eIF3d[48] binds the mRNA cap and promotes translation of *c-Jun* mRNA in the absence of eIF4E and eIF4G. (*??*) indicates that a closed-loop model for the mRNA is possible.[18] Created with BioRender.com.

Functional convergence with eIF4G in Arabidopsis light and carbohydrates metabolism

Perhaps the most conspicuous feature of the plant translational machinery is the absence of eIF4E-binding proteins (4E-BPs) orthologs,[29] which in animals inhibit the eIF4E-eIF4G interaction by sequestering eIF4E under certain conditions. Besides 4E-BPs, in animals and yeast, at least a dozen of other proteins also associates with eIF4E to negatively control either global translation or the expression of specific mRNAs.[29–31] In contrast, only one protein that binds to eIF4E *in vivo* had been described in plants, namely Conserved Binding of eIF4E 1 (CBE1).[32] However, a role in translation of CBE1 has not been proved.[32]

Recently, clear evidence of another eIF4E-interacting protein, called CERES that is able to regulate translation was reported in the plant *Arabidopsis thaliana*.[33] CERES associates with eIF4E and eIF(iso)4E via a canonical eIF4E-binding site. The CERES-eIF4E and CERES-eIF(iso)4E complexes do not include eIF4G, but they form part of cap-binding complexes, interact with eIF4A, PABP and eIF3, co-sediment with translation initiation complexes *in vivo* and promote translation *in vitro* and general translation *in vivo* (Figure 2(c)).[33] *CERES* encodes a 66 kDa protein containing 15 LRR repetitions with no orthologues

outside the Plant kingdom. Surprisingly, besides a canonical eIF4E-binding site, CERES does not share domain or local similarity either with eIF4G or Mextli. CERES modulates the translation of specific mRNAs related to light and carbohydrate change responses.[33] Thus, CERES is a second non-canonical translation initiation factor that functionally converged with eIF4G in evolution.

Functional convergence with eIF4G during vertebrate development

Aminoacyl tRNA synthetases (aaRSs) are essential molecules for translation elongation, as they catalyze the specific aminoacylation reaction of tRNAs for protein synthesis. Several aaRSs are versatile molecules that possess 'moonlighting activities' beyond translation, and are involved in stress response, embryogenesis, cell death, cell signaling, immune responses, transcriptional regulation, or RNA splicing. These non-canonical functions often involve the transient association of aaRSs with various molecules.[34,35]

It was recently discovered that vertebrate threonyl-tRNA synthetase (TRS) also performs moonlighting activities within the translation process itself. Mass spectrometry analyses and yeast two-hybrid assays led to the discovery that human TRS interacts with 4EHP via a canonical eIF4E-binding site present in the N-terminal region of TRS.[36] This interaction is conserved in mouse, zebrafish, and *Drosophila*. It was further determined that vertebrates TRS interacts with eIF4A and PABP, and may drive efficient translation initiation by substituting eIF4G as a scaffold protein (Figure 2(d)).[36] RNA immunoprecipitation and sequencing (RIP-seq) experiments allowed to determ that most mRNAs regulated by TRS participate in the nerves, skeleton, and circulatory system development. Using the vascular endothelial growth factor (VEGF) mRNA, it was also observed that TRS binds to the 5′-UTR through an anticodon-like loop structure.[36]

The translation initiation mechanism performed by TRS might have arisen as a single gain-of-function event in the vertebrate lineage, and represents another example of evolutionary convergence of eIF4G function.[36]

Divergence in *Giardia* and *Leishmania* initiation complexes

Protists are the biggest and most diverse group among eukaryotes, yet they are the least well understood at the morphological, cellular, and molecular levels.[37] In terms of their protein synthesis apparatus, only a few groups have been studied, including the deep branching parasite *Giardia*[38,39] and some trypanosomatids.[40] The *Giardia lamblia* translation machinery has diverged, possessing very atypical features, not found in other eukaryotes. It contains the 70S rather than 80S ribosomes, a few subunits of eIF3, and *Giardia* mRNAs have extremely short 5′-UTRs of 0–14 nucleotides.[38,39] It is intriguing that orthologs of eIF4E and eIF4A have been identified in the *Giardia* genome but not orthologs of eIF4G. This raised the fundamental question of how the ribosomal PIC is recruited to the 5′ end of mRNAs.[41] Recently, it was discovered that eIF4E2 interacts with the β subunit of eIF2, which is part of the PIC.[41] As well, eIF4A interacts with eIF3i, also a part of the PIC. Both interactions are thought to facilitate translation initiation in the absence of eIF4G[41] (Figure 2(e)).

The trypanosomatid *Leishmania major* is a parasite that cycles between invertebrate vectors and mammalian hosts, with extreme temperature changes during its life cycle. It possesses an unusual genome organization and RNA metabolism, which are adaptations to its parasitic life.[42] It also contains several paralogs of eIF4E and eIF4G with different functions.[40] In biochemical assays, it was observed that eIF4G3 associates with both the cap-bound eIF4E4 and eIF3 for ribosomal recruitment. After prolonged exposure to mammalian-like temperatures, eIF4E4 loses its ability to bind both the cap-structure and eIF4G3. Under these conditions, eIF4E1 is still able to bind m^7GTP and associates with eIF3a to assemble an alternative initiation complex that lacks any eIF4G paralog. Thus, it is proposed that eIF4E1 promotes translation during prolonged stresses[43] (Figure 2(f)).

Alternative eIF4G- and eIF4E-independent complexes for translation initiation

Stress- and cell proliferation-related mRNAs in humans

Methylation of the N^6 position of adenosine (N^6-methyladenosine, m^6A) is the most frequent internal posttranscriptional modification in eukaryotic mRNAs,[44] with a frequency in mammalian cells of ~1 m^6A modification per 2000 nucleotides, or 1–3 m^6A modification per average mRNA transcript.[45] The m^6A modification supports a dynamic process that involves methyltransferases, demethylases, and an m^6A-specific protein.[44] Upon heat shock, an m^6A within the 5′-UTR of the human heat shock protein 70 (*Hsp70*) mRNA can selectively promote translation initiation independent of cap and the eIF4F complex[46] (Figure 2(g)). Using both crosslinking and toeprinting assays, it was demonstrated that eIF3 binds directly to m^6A residues in the 5′-UTR to promote translation and that the formation of the 48S complex in methylated mRNAs only requires the factors eIF1, eIF1A, eIF2, eIF3, and the 40S ribosomal subunit. It was also shown that m^6A must have a specific

context (GAC) to efficiently promote translation. Accordingly, silencing of the m^{6}A methyltransferase METTL3 resulted in a reduction of translation of mRNAs containing m^{6}A in their 5′-UTR *in vivo*. This mechanism to initiate translation could play a key role in the regulation of gene expression of different stress-induced mRNAs.[46]

Recently, it was discovered that direct interactions between the cap structure and eIF3 promote translation of the transcription factor *c-Jun* mRNA, which is a key regulator of cell proliferation.[47] Its translation is dependent on the cap and a stem-loop structure within the 5′-UTR, and is not affected by eIF4E inactivation.[48] In *in vitro* translation assays using 293 T cells and the *c-Jun* mRNA, 48S complexes were isolated and found to contain eIF3 and the 40S ribosomal subunit, but to lack the eIF4F complex. Mass spectrometry and crystal structure analyses revealed that the alpha-helices 5 and 11 of carboxy-terminal of the d subunit of eIF3 makes contacts with the cap in a similar way as endonucleases involved in RNA turnover (Figure 2(h)). eIF3d forms a cup-shaped architecture with a positively charged central tunnel that is negatively charged at its base for cap-binding. The *c-Jun* mRNA forms an inhibitory RNA structure that blocks eIF4E, eIF4G, and eIF4A recruitment, thus enforcing alternative cap recognition by eIF3d.[48] This translation pathway might allow cells to control specific gene expression in cellular environments where eIF4E is disabled. However, this possible function has not been demonstrated.[48]

Novel paths in gene expression

Some components of the translation machinery[49–51] and the signaling pathways regulating translation,[52] have diverged throughout eukaryotic evolution. While the fundamental process of translation remained well conserved across all forms of life, the initiation step underwent a substantial increase in complexity.[16,49,51,53] Moreover, different stresses, including apoptosis, viral infection, heat shock, starvation, or hypoxia, may trigger a diversity of noncanonical translation initiation mechanisms that depend on mRNA elements and proteins such as internal ribosome entry sites (IRESs), ribosome shunting, cap-binding complex (CBC), cap-independent translation elements (CITEs), Death Associated protein 5 (DAP5)/p97 and potyviral proteins that substitute for the mRNA m^{7}G cap. Except for the Cricket Paralysis and the Hepatitis C viruses IRESs, these mechanisms use eIF4G or a protein phylogenetically related to it as a central scaffold.[54,55] Here, we have reviewed recent evidence showing that, besides, different kinds of mechanisms have emerged in disparate species that perform 43S PIC recruitment either in the absence of eIF4G or of both eIF4G and eIF4E.

eIF4F-like complexes in the 48S PIC

The structure of a reconstituted human 48S PIC was recently resolved by cryo-electron microscopy.[56] It is most consistent with mRNA being recruited to the 40S subunit by a slotting mechanism, placing eIF4F upstream (to the 5′ end) of the 43S PIC at the mRNA exit channel via a set of coordinated interactions between eIF4F with eIF3. The complex eIF4G-eIF4A binds to a solvent pocket formed by eIF3k, -l, and -e near the ribosomal RNA expansion segment ES6^S. This pocket may also contain eIF4E possibly interacting with eIF3k, and -l. Moreover, eIF3d potentially interacts with eIF4F and eIF3e, and eIF3g maybe interacts with the mRNA backbone at the mRNA entry site.[56] These observations agree with previous biochemical evidence.[57–60]

eIF3 can adopt different order states when in complex with human of yeast PIC 48S during both cap- or IRES-dependent translation initiation.[56,61–64] Thus, upon spatial changes in eIF3 conformation, the solvent pocket formed by eIF3k, -l, and -e near the mRNA exit channel could allow its assembly with eIF4F-like complexes (i.e., containing Mextli, CERES, or TRS) during mRNA recruitment. Because human eIF4F interacts with the 43S PIC through eIF3e, -k, and -l, that are not present in yeast, Brito Querido *et al*. (2020) suggested that “the interactions that eIF4F makes with different components of the 48S PIC are likely to differ greatly between species”.[56] This suggestion agrees with early evidence indicating that eIF4G might be dispensable for 43S PIC recruitment in the yeast *Saccharomyces cerevisiae*. Jivotovskaya *et al*. reported that depleting eIF2 and eIF3 impaired mRNA binding to free 40S subunits, but depleting eIF4G led to an accumulation of mRNA on 40S subunits, suggesting that eIF3 and eIF2 are more critically required than eIF4G for binding of at least some mRNAs to native PIC.[65] In concordance, an interaction between eIF4G and eIF3 has not been detected in *S. cerevisiae*, and the eIF3-binding domain described in mammalian eIF4G does not appear to exist in the two yeast eIF4Gs.[58–60,66]. Thus, Jivotovskaya *et al*. suggested that at least for some transcripts, the ribosome-bound eIF3 might stimulate mRNA recruitment by directly interacting with it and that eIF4G might have a rate-limiting function at a step downstream of 48S PIC assembly *in vivo*.[65] In this model, eIF4G does not associate with eIF3 to bridge the 40S ribosomal subunit, as happens in mammals

A novel landscape of gene expression in eukaryotes

Mextli extended the concept of the scaffold to proteins non-phylogenetically related to eIF4G for translation initiation. Its discovery argues for

functional convergence of factors for ribosome recruitment during evolution. Subsequently, further variations to the theme of ribosome recruitment by the mRNAs via different protein complexes have been found in different processes, i.e., germline development in *Drosophila*, during *Arabidopsis* responses to light and carbohydrate changes, throughout vertebrate development, in some protists, and during cell proliferation and stress conditions. These findings illustrate the versatility of the translation machinery for ribosomal recruitment in different species, and open the possibility that a wide variety of other non-canonical mechanisms exist, in particular among the scarcely explored protists. Thus, the newly discovered, unorthodox mechanisms for ribosome recruiting during translation initiation open a whole novel landscape of avenues of gene expression in eukaryotes.

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Author contributions

G.H. conceived the manuscript, gathered information and wrote the paper. A.G assembled all figures, gathered information and wrote part of the paper. N.S. gathered information and wrote part of the paper. P.L. gathered information and wrote part of the paper.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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