POST-TRANSCRIPTIONAL CONTROL

The mechanism of eukaryotic translation initiation and principles of its regulation

Richard J. Jackson, Christopher U. T. Hellen‡ and Tatyana V. Pestova‡*

Abstract | Protein synthesis is principally regulated at the initiation stage (rather than during elongation or termination), allowing rapid, reversible and spatial control of gene expression. Progress over recent years in determining the structures and activities of initiation factors, and in mapping their interactions in ribosomal initiation complexes, have advanced our understanding of the complex translation initiation process. These developments have provided a solid foundation for studying the regulation of translation initiation by mechanisms that include the modulation of initiation factor activity (which affects almost all scanning-dependent initiation) and through sequence-specific RNA-binding proteins and microRNAs (which affect individual mRNAs).

Met-tRNA$^{Met}_i$
The unique initiator tRNA, aminoacylated with methionine, that is used to initiate protein synthesis. Its anticodon is complementary to the AUG initiation codon; it forms a specific ternary complex with eIF2 and GTP and it binds to the ribosomal P-site.

P-site
The site on a ribosome that holds the tRNA that is linked to the growing peptide chain (peptidyl-tRNA).

**Department of Biochemistry, University of Cambridge, Cambridge, CB2 1GA, UK. ‡Department of Cell Biology, State University of New York Downstate Medical Center, Brooklyn, New York 11203, USA.*
e-mails: rjj@mole.bio.cam.ac.uk; christopher.hellen@downstate.edu; tatyana.pestova@downstate.edu
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Translation initiation is the process of assembly of elongation-competent 80S ribosomes, in which the initiation codon is base-paired with the anticodon loop of initiator tRNA (Met-tRNA$^{Met}_i$) in the ribosomal P-site[1]. It requires at least nine eukaryotic initiation factors (eIFs; TABLE 1) and comprises two steps: the formation of 48S initiation complexes with established codon–anticodon base-pairing in the P-site of the 40S ribosomal subunits, and the joining of 48S complexes with 60S subunits. On most mRNAs, 48S complexes form by a 'scanning' mechanism, whereby a 43S preinitiation complex (comprising a 40S subunit, the eIF2–GTP–Met-tRNA$^{Met}_i$ ternary complex (eIF2 TC), eIF3, eIF1, eIF1A and probably eIF5) attaches to the capped 5′ proximal region of mRNAs in a step that involves the unwinding of the mRNA's 5′ terminal secondary structure by eIF4A, eIF4B and eIF4F. The 43S complex then scans the 5′ untranslated region (5′ UTR) in the 5′ to 3′ direction to the initiation codon (FIG. 1). After initiation codon recognition and 48S complex formation, eIF5 and eIF5B promote the hydrolysis of eIF2-bound GTP, the displacement of eIFs and the joining of a 60S subunit. Although most mRNAs use the scanning mechanism, initiation on a few mRNAs is mediated by internal ribosome entry sites (IRESs; BOX 1).

Here, we summarize the current state of knowledge concerning the mechanism of translation initiation in vertebrates and discuss the principles underlying its regulation. We focus on examples in which the regulatory mechanism is well understood and/or the biological significance is particularly high, and we include evidence from lower eukaryotes only when it enhances our understanding of the mechanisms in vertebrates.

Mechanism of 5′ end-dependent initiation

The canonical mechanism of translation initiation can be divided into several stages (FIG. 1), as described below.

Formation of 43S preinitiation complexes. Translation initiation requires a pool of separated ribosomal subunits. Translation is a cyclical process, and ribosomal subunits that participate in initiation derive from the recycling of post-termination ribosomal complexes (post-TCs), which comprise an 80S ribosome still bound to mRNA, P-site deacylated tRNA and at least one release factor, eukaryotic release factor 1 (eRF1). Post-TCs are recycled by releasing these ligands and dissociating ribosomes into subunits. At a low free (nucleotide-unbound) Mg^{2+} concentration (1 mM), recycling can be mediated by eIFs[2]. eIF3, in cooperation with its loosely associated eIF3j subunit, eIF1 and eIF1A, dissociates post-TCs into free 60S subunits and mRNA- and tRNA-bound 40S subunits. Subsequently, eIF1 promotes release of the tRNA, after which eIF3j, which binds 40S subunits with negative cooperativity with mRNA[3,4], mediates mRNA dissociation. eIF3, and probably eIF1 and eIF1A, remain associated with recycled 40S subunits, preventing their re-association with 60S subunits. Recycling at even slightly elevated Mg^{2+} concentrations (which stabilize ribosomal subunit association) also

Internal ribosome entry site
A structure that is located in the 5′ UTR or ORF of some mRNAs of cellular or viral origin. It mediates translation initiation independently of the 5′ end of mRNA by recruiting the ribosome directly to an internal position on the mRNA.

requires ATP-binding cassette subfamily E member 1 (ABCE1) (A.V. Pisarev, M.A. Skabkin, V.P. Pisareva, O.V. Skabkina, A. Rakotondrafara, M.W. Hentze, C.U.T H. and T.V.P., unpublished observations), an essential member of the ATP-binding cassette (ABC) family of proteins[5]. ABCE1 splits post-TCs into free 60S subunits and tRNA- and mRNA-bound 40S subunits, and subsequent release of P-site tRNA and mRNA from these 40S subunits also requires eIF3, eIF1 and eIF1A. Thus, eIF3, eIF1 and eIF1A are recruited to 40S subunits during recycling, whereas eIF2–GTP–Met-tRNA$^{Met}_{i}$ subsequently attaches to recycled 40S subunits, bound simultaneously to eIF3, eIF1 and eIF1A, to form 43S complexes. Another protein that can prevent ribosomal subunit re-association, in this case by binding to 60S subunits, is eIF6, but its status as an initiation factor is uncertain (see Supplementary information S1 (box)).

Recent studies have yielded insights into the architecture of 43S complexes. Eukaryotic and prokaryotic small ribosomal subunits share a common structural core that includes the decoding centre, whereas additional eukaryotic ribosomal proteins (rps) and 18S rRNA expansion segments (rapidly evolving regions interspersed throughout the conserved rRNA core that might

Table 1 | **Eukaryotic initiation factors**

Name	Number of subunits and their molecular mass (kDa)	Function
Core initiation factors		
eIF2	3 (36.1, 38.4 and 51.1)	Forms an eIF2–GTP–Met-tRNA$_i$ ternary complex that binds to the 40S subunit, thus mediating ribosomal recruitment of Met-tRNA$_i$
eIF3	13 (800 total)	Binds 40S subunits, eIF1, eIF4G and eIF5; stimulates binding of eIF2–GTP–Met-tRNA$_i$ to 40S subunits; promotes attachment of 43S complexes to mRNA and subsequent scanning; and possesses ribosome dissociation and anti-association activities, preventing joining of 40S and 60S subunits
eIF1	1 (12.7)	Ensures the fidelity of initiation codon selection; promotes ribosomal scanning; stimulates binding of eIF2–GTP–Met-tRNA$_i$ to 40S subunits; and prevents premature eIF5-induced hydrolysis of eIF2-bound GTP and P_i release
eIF1A	1 (16.5)	Stimulates binding of eIF2–GTP–Met-tRNA$_i$ to 40S subunits and cooperates with eIF1 in promoting ribosomal scanning and initiation codon selection
eIF4E	1 (24.5)	Binds to the m^{7}GpppG 5′ terminal 'cap' structure of mRNA
eIF4A*	1 (46.1)	DEAD-box ATPase and ATP-dependent RNA helicase
eIF4G‡	1 (175.5)	Binds eIF4E, eIF4A, eIF3, PABP, SLIP1 and mRNA (see FIG. 3a) and enhances the helicase activity of eIF4A
eIF4F	3 (246.1 total)	A cap-binding complex, comprising eIF4E, eIF4A and eIF4G; unwinds the 5′ proximal region of mRNA and mediates the attachment of 43S complexes to it; and assists ribosomal complexes during scanning
eIF4B	1 (69.3)	An RNA-binding protein that enhances the helicase activity of eIF4A
eIF4H	1 (27.4)	An RNA-binding protein that enhances the helicase activity of eIF4A and is homologous to a fragment of eIF4B
eIF5	1 (49.2)	A GTPase-activating protein, specific for GTP-bound eIF2, that induces hydrolysis of eIF2-bound GTP on recognition of the initiation codon
eIF5B	1 (138.9)	A ribosome-dependent GTPase that mediates ribosomal subunit joining
eIF2B	5 (33.7, 39.0, 50.2, 59.7 and 80.3)	A guanosine nucleotide exchange factor that promotes GDP–GTP exchange on eIF2
Auxiliary factors		
DHX29	1 (155.3)	A DExH box-containing protein that binds 40S subunit and promotes ribosomal scanning on mRNAs with long, highly structured 5′ UTRs
Ded1	1 (65.6)	A DEAD box-containing NTPase and RNA helicase that potentially promotes scanning in *Saccharomyces cerevisiae*
eIF6	1 (26.6)	An anti-association factor that binds 60S subunits and prevents them from joining to 40S subunits
p97	1 (102.4)	Closely related to the carboxy-terminal two-thirds of eIF4G; binds eIF4A and eIF3; and promotes initiation in a potentially mRNA-specific manner
PABP	1 (70.7)	Binds to the 3′ poly(A) tail of mRNA, eIF4G and eRF3; enhances binding of eIF4F to the cap; and might facilitate recruitment of recycled post-termination 40S subunits back to the 5′ end of mRNA

Ded1, DEAD box helicase 1; DHX29, DExH box protein 29; eIF, eukaryotic initiation factor; PABP, poly(A)-binding protein. *Two paralogues (eIF4AI and eIF4AII), encoded by different genes, are functionally indistinguishable, but eIF4AIII has no activity as an eIF. ‡Two paralogues (eIF4GI and eIF4GII), encoded by different genes, are functionally similar but show some selectivity towards different mRNAs. eIF4GI is generally the more abundant.

function in eukaryote-specific aspects of translation) are situated peripherally[6]. The highest-resolution structure of eukaryotic ribosomes (7.3Å) has been determined by cryoelectron microscopy[7], but the structural homology between eukaryotic and prokaryotic ribosomes allows the use of high-resolution crystal structures of prokaryotic ribosomes to model 40S–eIF interactions, based on biochemical data. The 40S subunit consists of a head, a platform and a body, with the mRNA-binding channel wrapping around the neck (FIG.2). The bulk of the five-lobed eIF3 molecule binds to the 40S subunit side facing the solvent[8] (FIG.2a), whereas the eIF3j carboxy-terminal domain localizes in the mRNA-binding channel, in the A-site area on the intersubunit side[4]. Although the structure of eIF2 is available[9], the position of eIF2–GTP–Met-tRNA$^{Met}_{i}$ on 40S subunits has not been determined. However, in 43S complexes, the Met-tRNA$^{Met}_{i}$ anticodon loop is probably not inserted as deeply into the P-site as in ribosomal complexes with established codon–anticodon base pairing (as shown in FIG.2b), and its acceptor end, to which Met is linked, might be rotated towards the E-site[10–12]. eIF1 binds to the interface between the platform and Met-tRNA$^{Met}_{i}$ (REF. 10). eIF1A's structured domain resides in the A-site, forming a bridge over the mRNA channel, whereas its N- and C-terminal tails extend into the P-site[13] (FIG.2b). Importantly, binding of eIF1 and eIF1A to 40S subunits induces conformational changes[14], which involve the opening of the mRNA entry channel 'latch' (formed by helix 18 (h18) in the body and h34 and rpS3 in the neck) and the establishment of a new head–body connection on the solvent side between h16 and rpS3 (FIG.2c).

Attachment of 43S complexes to mRNA. Although 43S complexes are intrinsically capable of 5′ end-dependent attachment to model mRNAs with completely unstructured 5′ UTRs[15], natural 5′ UTRs possess sufficient secondary structure for loading of 43S complexes onto them to require the cooperative action of eIF4F and eIF4B or eIF4H, which unwind the 5′ cap-proximal region of mRNA to prepare it for ribosomal attachment. eIF4F comprises the cap-binding protein eIF4E, the DEAD-box RNA helicase eIF4A and eIF4G, which functions as a 'scaffold' that binds eIF4E, eIF4A, poly(A)-binding protein (PABP) and eIF3 (FIG.3a). eIF4B and eIF4H enhance eIF4A's helicase activity, contain RRM domains and are homologous over the entire length of eIF4H[1]. The cap stacks between two Trp residues on eIF4E's concave surface and additional contacts with the cap-proximal nucleotide stabilize eIF4E's binding to capped mRNA[16]. A segment of eIF4G wraps around eIF4E's N terminus, inducing structural changes that enhance eIF4E's affinity for the cap[17,18]. eIF4A has two domains and alternates between an inactive 'open' and an active 'closed' conformation, in which both domains form a contiguous RNA-binding surface and the ATP-binding site is at the domain interface[19]. The low individual helicase activity of eIF4A is strongly enhanced by eIF4G and eIF4B (or eIF4H)[20]. eIF4G's N-terminal HEAT domain, HEAT1, stimulates eIF4A's helicase activity by aligning the DEAD-box motifs in both domains in a productive conformation, whereas HEAT2, which also binds eIF4A, plays a modulatory role[21,22]. The topology of eIF4A–eIF4G–eIF4H complexes (FIG.3b) indicates that eIF4H binds the single-stranded mRNA behind eIF4A (relative to the direction of helicase translocation), suggesting that eIF4H (or eIF4B) could stimulate eIF4A's helicase activity by preventing mRNA re-annealing and promoting processive unidirectional eIF4A movement[22]. As suggested[22], because of the high, but nevertheless limited, processivity of eIF4F–eIF4B (or eIF4F–eIF4H) complexes, eIF4A eventually dissociates from mRNA but, being anchored to its 5′ end by the eIF4E–cap interaction, these complexes resume another cycle of unwinding, thereby keeping the 5′ proximal region constantly prepared for ribosomal attachment that is probably facilitated by the eIF3–eIF4G interaction[23]. Thus, recruitment of 43S complexes is ultimately achieved by the cap–eIF4E–eIF4G–eIF3–40S chain of interactions. The 'open latch' conformation of 40S subunits[14], induced by eIF1 and eIF1A, is likely to be strongly conducive for attachment.

Despite these advances, the position of eIF4E in ribosomal complexes and the mechanistic aspects of how mRNA enters the mRNA-binding channel remain unknown. If the cap–eIF4E interaction persists during attachment, it is unlikely that eIF4E-bound mRNA could be threaded through the entire mRNA-binding channel, and loading of 43S complexes would therefore be more compatible with direct positioning of eIF4E–cap at the channel's E-site side. This would raise the question: from which nucleotide do 43S complexes begin inspecting the 5′ UTR during scanning? The efficiency of initiation on mRNAs with short 5′ UTRs has been investigated in a cell-free system[24]. However, the data obtained, which showed that about 50% of ribosomes bypass an AUG codon located within 12 nucleotides of the cap, could reflect the susceptibility of initiation complexes potentially forming close to the mRNA 5′ end to dissociation by eIF1, as discussed below[15], rather than by the inability of tRNA$^{Met}_{i}$ to inspect mRNA from certain positions.

Ribosome scanning of mRNA 5′ UTRs. After attachment, 43S complexes scan mRNAs downstream of the cap to the initiation codon. Scanning consists of two linked processes: unwinding of secondary structures in the 5′ UTR and ribosomal movement along it. 43S complexes can scan unstructured 5′ UTRs without factors associated with RNA unwinding and are thus intrinsically capable of movement along mRNA[15]. Omission of eIF1A substantially reduces this ability and lack of eIF1 almost abrogates it[15], indicating that movement of 43S complexes requires the scanning-competent conformation induced by eIF1 and eIF1A[14]. Although eIF3 is indispensable for 48S complex formation, it is difficult to separate its scanning role from functions such as ribosomal recruitment of eIF2–GTP–Met-tRNA$^{Met}_{i}$ and attachment of 43S complexes. However, eIF3 interacts with mRNA upstream of the E-site (FIG.2a), forming an extension of the mRNA-binding channel that might contribute to scanning[25]. Even the scanning of 5′ UTRs containing weak secondary structures requires ATP and eIF4A, eIF4G and eIF4B[15],

ATP-binding cassette (ABC) family
A family of proteins that typically contain two nucleotide-binding domains (NBDs), which form two composite nucleotide-binding sites. The transition between the closed ATP-bound and open ADP-bound states induces a tweezer-like powerstroke between NBDs, which causes conformational changes in associated domains and/or macromolecules.

18S rRNA
Ribosomal RNA of the 40S ribosomal subunit. It determines the overall shape of the 40S subunit and is the main component of its decoding centre. It is also involved in formation of the main contacts between 40S and 60S ribosomal subunits.

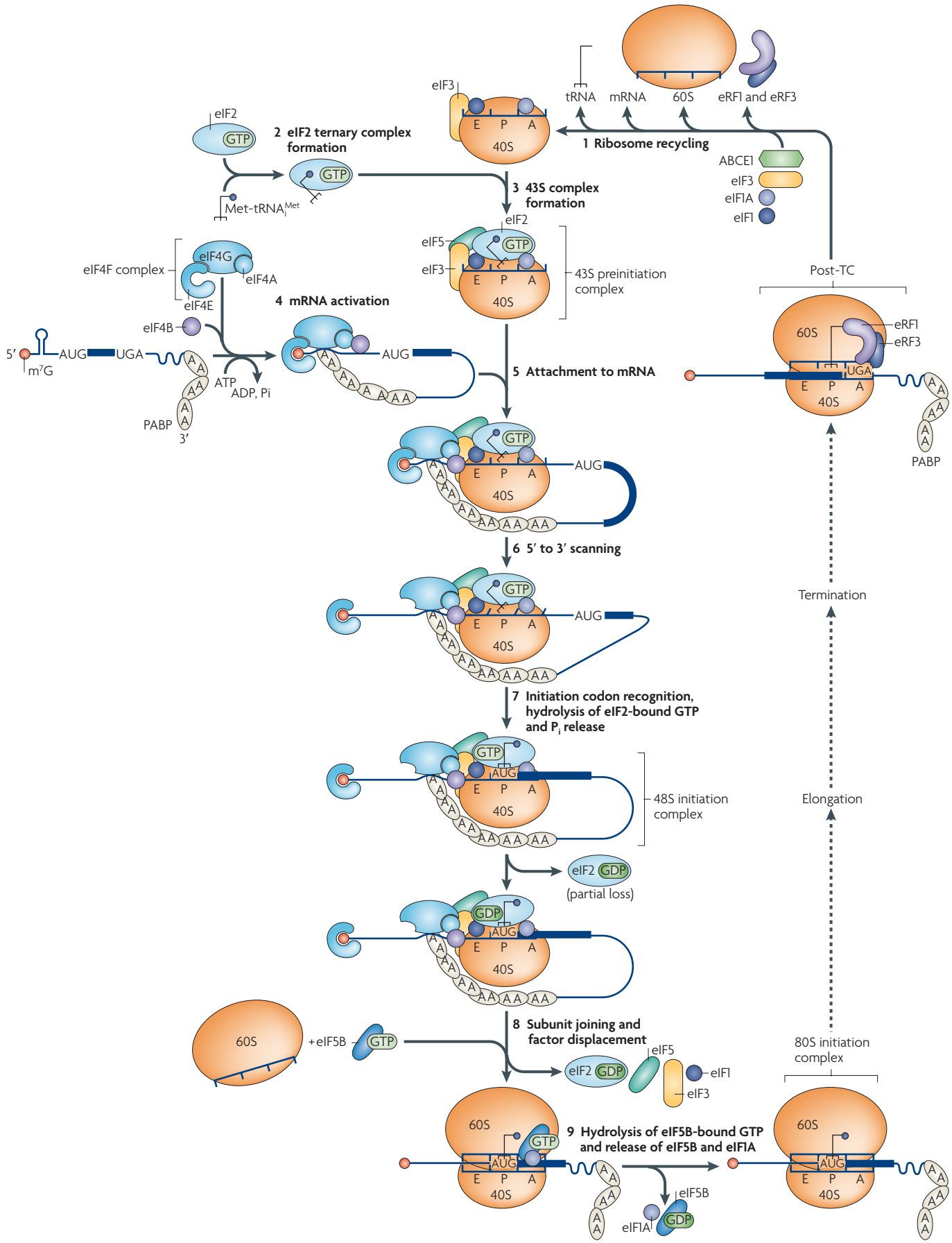
A-site
The site on a ribosome that holds the new, incoming aminoacyl-tRNA.

E-site
The site on a ribosome that accommodates deacylated tRNA before it is released from the ribosome.

DEAD-box RNA helicase
An RNA helicase that contains the DEAD (Asp-Glu-Ala-Asp) or DExD/H (Asp-Glu-X-Asp,His; where X represents any amino acid) motifs. These proteins use the energy of ATP hydrolysis to unwind RNA.

RRM domain
(RNA-recognition motif). A protein domain that contains two short consensus sequences embedded in a structurally conserved region of ~80 amino acids.

HEAT domain
A protein domain of 37–47 amino acids that consists of tandemly repeated pairs of antiparallel α-helices. HEAT domains have a superhelical structure and often function as protein–protein interaction surfaces.



◀ Figure 1 | **Model of the canonical pathway of eukaryotic translation initiation.** The canonical pathway of eukaryotic translation initiation is divided into eight stages (**2**–**9**). These stages follow the recycling of post-termination complexes (post-TCs; **1**) to yield separated 40S and 60S ribosomal subunits, and result in the formation of an 80S ribosomal initiation complex, in which Met-tRNA$^{Met}_{i}$ is base paired with the initiation codon in the ribosomal P-site and which is competent to start the translation elongation stage. These stages are: eukaryotic initiation factor 2 (eIF2)–GTP–Met-tRNA$^{Met}_{i}$ ternary complex formation (**2**); formation of a 43S preinitiation complex comprising a 40S subunit, eIF1, eIF1A, eIF3, eIF2–GTP–Met-tRNA$^{Met}_{i}$ and probably eIF5 (**3**); mRNA activation, during which the mRNA cap-proximal region is unwound in an ATP-dependent manner by eIF4F with eIF4B (**4**); attachment of the 43S complex to this mRNA region (**5**); scanning of the 5′ UTR in a 5′ to 3′ direction by 43S complexes (**6**); recognition of the initiation codon and 48S initiation complex formation, which switches the scanning complex to a 'closed' conformation and leads to displacement of eIF1 to allow eIF5-mediated hydrolysis of eIF2-bound GTP and P_i release (**7**); joining of 60S subunits to 48S complexes and concomitant displacement of eIF2–GDP and other factors (eIF1, eIF3, eIF4B, eIF4F and eIF5) mediated by eIF5B (**8**); and GTP hydrolysis by eIF5B and release of eIF1A and GDP-bound eIF5B from assembled elongation-competent 80S ribosomes (**9**). Translation is a cyclical process, in which termination follows elongation and leads to recycling (**1**), which generates separated ribosomal subunits. The model omits potential 'closed loop' interactions involving poly(A)-binding protein (PABP), eukaryotic release factor 3 (eRF3) and eIF4F during recycling (see Supplementary information S5 (box)), and the recycling of eIF2–GDP by eIF2B. Whether eRF3 is still present on ribosomes at the recycling stage is unknown.

and the requirement for ATP and eIF4A is proportional to the degree of secondary structure[26,27]. Thus, in addition to promoting attachment, eIF4A, eIF4G and eIF4B assist 43S complexes during scanning.

However, the mechanism by which these factors assist scanning remains unknown. Cryoelectron microscopy-based modelling placed eIF4G at the 40S subunit's trailing edge, near the E-site[8] (FIG.2a), which would be consistent with eIF4A, eIF4G and eIF4B acting by helicase-mediated 'ratcheting' of mRNA through the mRNA-binding channel, whereas mRNA secondary structure would be unwound by the 40S subunits themselves at their leading edge. However, an alternative model, in which eIF4A, eIF4G and eIF4B unwind mRNA before it enters this channel, has also been suggested[22] (FIG.3b).

Although ribosomal attachment is achieved by the cap–eIF4E–eIF4G–eIF3–40S chain of interactions, the fate of each link during the transition from attachment to scanning and during scanning *per se* is unclear. Their maintenance would cause 5′ UTRs to 'loop out', allowing only one 43S complex to scan at a time, whereas breaking of even one link in the chain would permit multiple complexes to scan simultaneously on a single 5′ UTR.

Another important question concerns the directionality of scanning. The fact that initiation frequency at the 5′ proximal AUG is reduced by the presence of a nearby downstream AUG[28] suggests that scanning may consist of forward (5′ to 3′) thrusts alternating with limited relaxation over distances of a few nucleotides in the reverse direction.

Importantly, recent data obtained using yeast and mammalian systems suggest that initiation involves other DEAD box family members in addition to eIF4A, and that eIF4A can act with p97, a distinct eIF4G-related protein. Mammalian DExH box protein 29 (DHX29) binds 40S subunits directly and is required for efficient scanning through highly structured 5′ UTRs *in vitro*[29]. *In vivo*, silencing DHX29 impairs translation, resulting in polysome disassembly and the accumulation of mRNA-free 80S monomers[30]. DHX29 has been suggested to increase scanning processivity by influencing the conformation of the mRNA-binding channel at its entrance[29].

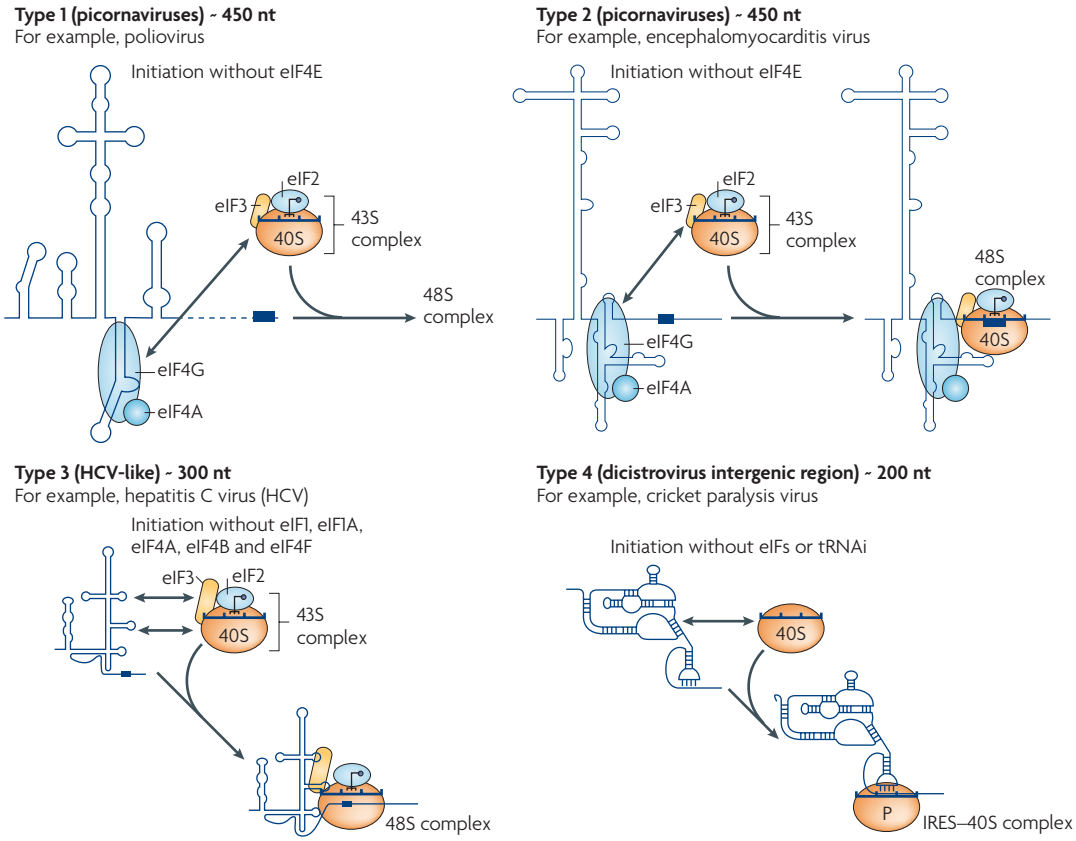
Yeast DEAD box helicase 1 (Ded1) has also been implicated in initiation. Ded1 is likely to be a more potent helicase than eIF4A and their functions are not redundant, suggesting that eIF4A promotes ribosomal attachment whereas Ded1 assists scanning, particularly on long 5′ UTRs[31–33]. The involvement in initiation of DEAD box protein 3 (DDX3), a mammalian Ded1 homologue, is more controversial, although some data suggest that DDX3 depletion specifically affects translation of mRNAs with long, highly structured 5′ UTRs[34].

p97, which is ubiquitously expressed in the tissues of mammals, birds and some insects, is homologous to the C-terminal two-thirds of eIF4G and binds eIF4A and eIF3, but lacks an eIF4E-binding region[35] (FIG.3a). p97 activates translation of uncapped mRNAs *in vitro*[36] and its role in translation of capped mRNAs is not fully redundant with that of eIF4G. Although depletion of eIF4GI and p97 individually impaired global translation by ~ 20–30% and co-depletion reduced it by ~ 60%, depletion of eIF4GI, but not of p97, selectively impaired translation of mRNAs containing upstream open reading frames (uORFs), suggesting that these factors promote initiation on different classes of mRNAs[37].

Initiation codon recognition. To ensure the fidelity of initiation, scanning complexes must have a discriminatory mechanism that prevents partial base pairing of triplets in the 5′ UTR with the Met-tRNA$^{Met}_{i}$ anticodon and promotes recognition of the correct initiation codon. This is usually the first AUG triplet in an optimum context — GCC(A/G)CCAUGG, with a purine at the −3 and a G at the +4 positions (relative to the A of the AUG codon, which is designated +1)[24]. eIF1 plays the key part in maintaining the fidelity of initiation. It enables 43S complexes to discriminate against non-AUG triplets and AUG triplets that have poor context or are located within 8 nucleotides of the mRNA 5′ end, and also dissociates the ribosomal complexes that aberrantly assemble at such triplets in its absence[15,38,39]. Genetic studies in yeast also identified eIF1 as a determinant of initiation codon recognition[40]. In a current model, eIF1 in cooperation with eIF1A promotes a scanning-competent 'open' conformation of the 43S complex[14] (FIG.2c), but to establish stable codon–anticodon base-pairing, ribosomal complexes must undergo conformational changes that are antagonized by eIF1. Establishment of codon–anticodon base pairing is accompanied by tightening of the eIF1A–40S interaction[41] and eIF1's displacement from near the P-site[3,10,42], which switches the complex to a 'closed' conformation that is locked onto the mRNA. Consistently, yeast eIF1 mutants that dissociate more rapidly from 48S subunits enhance initiation at non-AUG codons[43]. The eIF1A's N-terminal and C-terminal tails, which reach into the P-site[13] (FIG.2b), have opposite effects on start codon selection: the C-terminal tail increases its stringency and was proposed to promote

the open conformation of scanning complexes, whereas the N-terminal tail decreases the accuracy of initiation and promotes the closed conformation[44]. The purines at −3 and +4 positions probably affect initiation codon selection by stabilizing conformational changes that occur on codon–anticodon base pairing, by interacting with the eIF2α (sometimes designated eIF2S1) subunit of eIF2 and nucleotides $AA_{1818–1819}$ in helix 44 of 18S rRNA, which form part of the A-site, respectively[39]. In eIF1's absence, the stability of 48S complexes is not challenged, so that complexes with partial base pairing can form and participate in subsequent steps in translation. Such complexes cannot maintain their conformation on binding eIF1, and mispaired tRNA is probably ejected.

Box 1 | **IRES-mediated translation initiation**



Internal ribosome entry sites (IRESs) are RNA elements that mediate end-independent ribosomal recruitment to internal locations in mRNA. Structurally related viral IRESs use distinct mechanisms, based on non-canonical interactions with eukaryotic initiation factors (eIFs) and/or 40S subunits (see the figure). Initiation on type 1 and type 2 IRESs involves their specific binding to the p50 domain of eIF4G (FIG. 3a), which is enhanced by eIF4A[112–114], on type 3 IRESs involves their interaction with the eIF3 and 40S subunit components of 43S complexes[8,115] and on type 4 IRESs involves their binding to 40S subunits[7,116]. The eIF4G–eIF4A complex recruits 43S complexes to type 1 and type 2 IRESs without the involvement of eIF4E. Type 3 IRESs directly attach 43S complexes to the initiation codon independently of eIF4F, eIF4B, eIF1 and eIF1A, whereas type 4 IRESs initiate without eIFs or $tRNA_i^{Met}$ (the P-site of the 40S subunit is occupied by an IRES domain that mimics codon–anticodon base pairing). Hence, IRES-mediated initiation might be resistant to cellular regulatory mechanisms, such as eIF2 phosphorylation (type 4 IRESs) and/or eIF4E sequestration (all types of IRESs)[116]. Initiation on some IRESs also requires IRES *trans*-acting factors (ITAFs) — RNA-binding proteins that are thought to stabilize the optimal three-dimensional IRES conformation[117].

The list of cellular mRNAs that are thought to contain IRESs is growing and, although a recent stringent test has questioned some of these claims[118], it would be prudent to presume that many are still valid. Cellular IRESs show little structural relationship to each other and their underlying mechanism remains largely unknown but probably follows the picornavirus paradigm of binding the eIF4G–eIF4A complex. Importantly, cellular IRES-containing mRNAs can also be translated by the scanning mechanism, which raises the crucial question of what regulates the switch between these modes of initiation. One key parameter might be the intracellular concentration of eIF4G. The concentration of eIF4G (but not eIF4E) is highly elevated in many advanced breast cancers, and in inflammatory breast cancer this results in efficient IRES-dependent translation of p120 catenin and vascular endothelial growth factor (*VEGF*) mRNAs[119]. In other breast cancer cell lines with high eIF4G levels, overexpression of eIF4E-binding protein 1 (4E-BP1), to sequester eIF4E, coupled with hypoxia, activates VEGF and hypoxia-inducible factor 1α (HIF1A) IRESs[120]. Another parameter that may determine which mechanism predominates is the intracellular concentration of ITAFs.

GTPase-activating protein
A protein that stimulates the intrinsic ability of a GTPase to hydrolyse GTP to GDP.

Arginine finger
A catalytic residue that was first defined for RasGAPs, and that supplies a catalytic arginine residue into the active site of Ras to increase the reaction rate.

Commitment of ribosomes to a start codon. Initiation codon recognition is followed by a step during which the arrested ribosome becomes committed to initiation at that codon. The commitment step is mediated by eIF5, an eIF2-specific GTPase-activating protein (GAP)[1]. eIF5 binds to eIF2's β-subunit but induces the GTPase activity of eIF2's γ-subunit only in eIF2–GTP–Met-tRNA$^{Met}_i$ complexes that are bound to 40S subunits. eIF5 has been proposed to act as a classical GAP by providing an arginine finger[45]. An alternative hypothesis suggests that eIF5 derepresses eIF2γ's GTPase activity[46]. Premature hydrolysis of eIF2-bound GTP in 43S complexes, and particularly subsequent P_i release, are prevented by eIF1[3,47]. Establishment of codon–anticodon base pairing results in eIF1's displacement[42], which relieves repression of GTP hydrolysis and P_i release[3,47]. Thus, in addition to its role in initiation codon selection during 48S complex formation, eIF1 also maintains initiation fidelity at a later stage by linking hydrolysis of eIF2-bound GTP with the establishment of codon–anticodon base pairing. Importantly, in addition to eIF1, genetic suppressor studies in yeast also implicate eIF2 and eIF5 in ensuring the fidelity of initiation codon selection[40]. GTP hydrolysis reduces eIF2's affinity for Met-tRNA$^{Met}_i$, leading to partial dissociation of eIF2–GDP from 40S subunits[39,48]. eIF2B mediates guanine nucleotide exchange on eIF2, recycling it for the next initiation round[1].

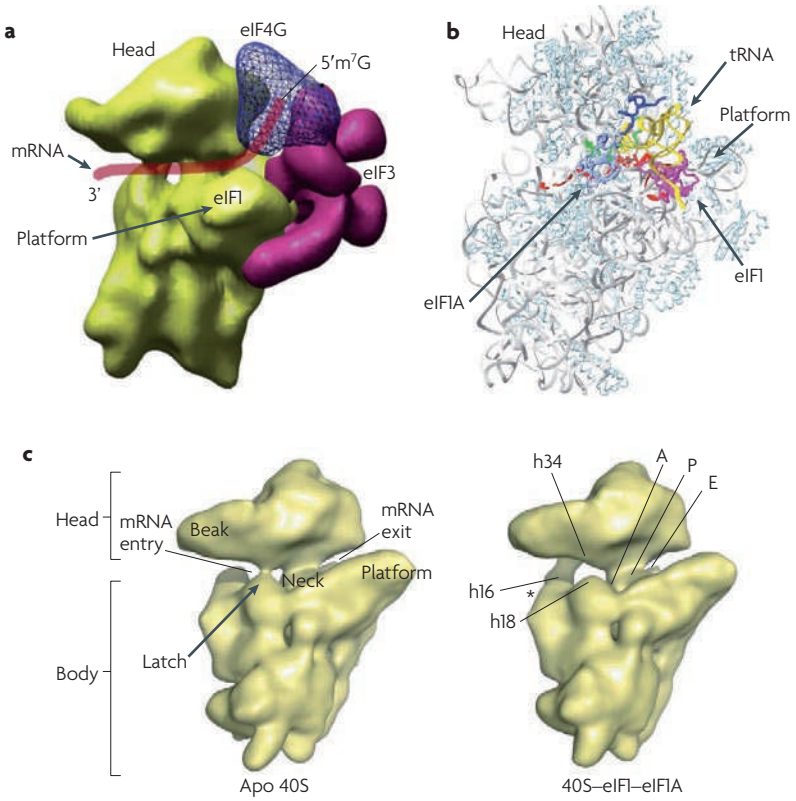


Figure 2 | **Architecture of ribosomal initiation complexes. a** | Model of a 40S subunit with eIF3 (magenta) on its exterior (solvent) surface and eIF4G (purple) bound to eIF3 near the E-site, based on cryoelectron microscopy analysis, and showing positions of mRNA (red line) and eIF1 (green) on the subunit interface. Binding of eIF3 to the solvent surface of the 40S subunit is compatible with its potential partial retention on ribosomes during translation of short upstream open reading frames (uORFs). **b** | Positions of eIF1 (magenta) and eIF1A (with its structured domain in light blue, its carboxy-terminal tail in dark blue and its amino-terminal tail in green) on the 40S subunit, relative to mRNA (red) and P-site tRNA (yellow), based on directed hydroxyl radical probing data[10,13] and modelled using *Thermus thermophilus* 30S subunit crystal structures (protein data bank codes 1JGO and 1JGP). **c** | Cryoelectron microscopy reconstructions of yeast apo 40S subunits (left panel) and 40S–eIF1–eIF1A complexes (right panel), labelled to indicate the A-site, P-site and E-site in the mRNA-binding channel and the positions of rRNA helices h16, h18 and h34, which are involved in forming the mRNA entry channel (h18–h34) and the eIF1- and eIF1A-induced head–shoulder connection (h16–ribosomal protein S3 (rpS3); indicated by an asterisk). Part **a** is adapted, with permission, from REF. 8 © American Association for the Advancement of Science (2005). Part **c** is adapted, with permission, from REF. 14 © Cell (2007).

Ribosomal subunit joining. Joining of 60S subunits and dissociation of eIF1, eIF1A, eIF3 and residual eIF2–GDP are mediated by eIF5B[3,49], a ribosome-dependent GTPase that is homologous to prokaryotic initiation factor IF2 (REF. 1) Hydrolysis of eIF5B-bound GTP is not required for subunit-joining, but is essential for eIF5B's own release from assembled 80S ribosomes[1]. eIF5B and IF2 occupy the same region in the intersubunit cleft[50], which was proposed to promote subunit joining by burying large solvent-accessible surfaces on both subunits[12]. eIF5B alone can partially displace eIF2–GDP from 40S subunits, whereas complete dissociation occurs only in the presence of 60S subunits during the actual subunit joining event[39]. Interaction of the C-terminal domain of eIF5B with the C-terminal tail of eIF1A[51,52], which probably becomes possible only after the displacement of eIF1A's C-terminal tail from the P-site (FIG.2b) on initiation codon recognition[13], is required for efficient subunit joining and GTP hydrolysis by eIF5B. This indicates that eIF1A remains associated with ribosomal complexes throughout the subunit joining process and dissociates from assembled ribosomes with eIF5B[53,54]. Although those eIFs that bind to the interface of the 40S subunit must be released before or at subunit joining, dissociation of eIF3 and eIF4G, which are largely bound to the solvent side (FIG.2a), may be delayed (as discussed below).

Reinitiation after a short upstream ORF. About 45–50% of mammalian genes (but only ~ 13% of yeast genes) encode mRNAs that have at least one short uORF (typically < 30 codons) upstream of the main protein coding ORF[55–57]. In these cases, some (usually < 50%) of the ribosomes that have translated the uORF resume scanning and reinitiate at downstream sites. Post-termination events at uORF stop codons probably proceed conventionally, with release of 60S subunits followed by deacylated tRNA, but some 40S subunits then remain on the mRNA and resume scanning. At this stage, such 40S subunits are incompetent for reinitiation because they lack an eIF2-TC, but this does not prevent scanning, during which a new eIF2-TC can be acquired. eIF2-TC availability determines how far 40S subunits migrate before acquiring one.

Rescanning and reinitiation efficiency decreases quite abruptly with increasing length of the uORF[58], or if the uORF includes stable RNA secondary structures

that cause pausing of elongation[59]. This suggests that it is the time taken to translate the uORF that is crucial, rather than the length *per se,* which leads to the idea that rescanning might depend on some of the eIF–ribosome interactions that promoted initiation at the uORF AUG persisting for the time taken to complete uORF translation. The indications are that the critical interactions are those involving eIF4G (and therefore also eIF3, which bridges eIF4G binding to the 40S subunit), because reinitiation is only seen if eIF4F and eIF4B, or at a minimum the eIF4G p50 fragment (FIG. 3a) plus eIF4A and eIF4B, actually participated in the primary initiation event at the uORF AUG[60]. Because eIF3 binds mainly to the solvent face of the 40S subunit[8] (FIG. 2a), not all of the eIF3–40S contacts need to be broken in order to allow subunit joining. eIF3 could therefore remain bound transiently to the 40S subunit in a metastable state, and if this and the eIF4G–eIF3 interaction were still in place by the time uORF translation had been completed, it could retain the post-termination 40S subunit on the mRNA and promote its rescanning.

As a general rule, the uORF sequence has little influence on reinitiation in mammalian systems, but there are exceptions, and the few well-characterised uORFs in yeast mRNAs are quite strongly sequence dependent (see Supplementary information S2 (box) for a possible explanation for these differences).

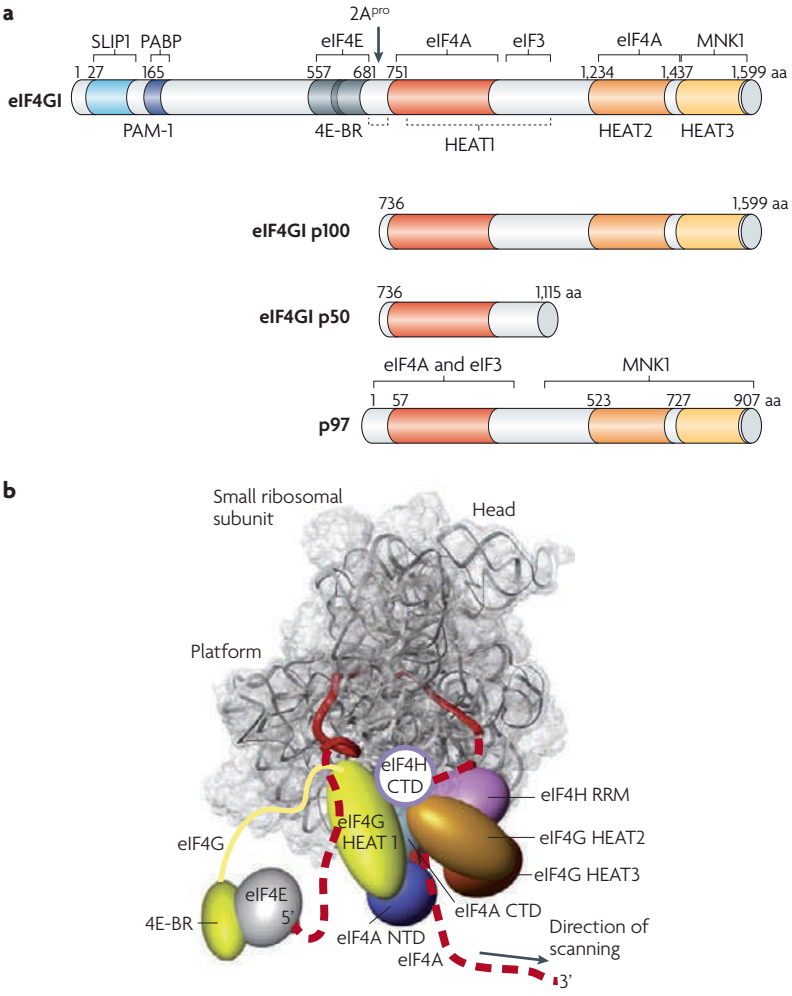


Figure 3 | **eIF4GI domain structure, interactions and position in a scanning 43S complex. a** | Schematic representation of the longest isoform of eIF4GI (Genbank accession NP_937884), of its p100 (carboxy-terminal two-thirds) and p50 (central one-third) fragments, and of p97, showing binding sites for SLBP-interacting protein 1 (SLIP1; sometimes designated MIF4GD), poly(A)-binding protein (PABP), eIF4E, eIF4A, eIF3 and MAP kinase interacting Ser/Thr kinase 1 (MNK1) or MNK2 and for RNA (dotted lines below eIF4GI). The interactions of eIF4GI with eIF4E and MNK1 are required for phosphorylation of eIF4E by MNK1. Interactions of eIF4GI with PABP and SLIP1 tether eIF4GI to the mRNA's 3′ end. The amino acid residues at the amino-termini of the PABP-binding domain (PAM1), eIF4E-binding domain (4E-BR) and HEAT1 (also known as MIF4G), HEAT2 (also known as MA3) and HEAT3 (also known as W2) domains are indicated, as is the cleavage site in eIF4GI for the picornavirus proteinase $2A^{pro}$ (see Supplementary information S3 (box)), which divides eIF4GI into an N-terminal domain that binds eIF4E and PABP, and a C-terminal domain that provides all functions of eIF4GI required for initiation on type 1 and type 2 internal ribosome entry sites (see BOX 1). This cleavage event contributes to the switch from host to viral translation during many picornavirus infections (see Supplementary Information S3 (box)). **b** | Hypothetical model of the scanning 43S preinitiation complex, viewed from the solvent face, showing associated factors and domains of factors, including eIF4E, the 4E-BR, HEAT1, HEAT2 and HEAT3 domains of eIF4G, the C-terminal and RRM domains of eIF4H and the N-terminal domain (NTD) and C-terminal domain (CTD) of eIF4A. The direction of scanning (5′ to 3′) is shown by an arrow and, in this model, eIF4A is on the leading (3′) side of the scanning complex. Figure adapted, with permission, from REF. 22 © Cell (2009).

Control of initiation factor activity

Mechanisms of regulating initiation fall into two broad categories: those that impact on the eIFs (or ribosomes), and therefore affect virtually all scanning-dependent initiation events; and those that impact on the mRNA itself, either through sequence-specific RNA-binding proteins or microRNAs (miRNAs), and are therefore potentially selective for certain mRNAs. The best-established examples of the first type are control of the availability of active eIF2 and eIF4F by reversible protein phosphorylation, but eIF4F's activity is also regulated by irreversible proteolysis of eIF4G (see Supplementary information S3 (box)).

There are four mammalian protein kinases that phosphorylate eIF2α on Ser51 (REF. 61): haem-regulated kinase (sometimes designated EIF2AK1), which is probably significant only in erythroid cells; PKR (sometimes designated EIF2AK2), which is activated by double-stranded RNAs of more than ~ 40 bp and is important in the antiviral response; PKR-like endoplasmic reticulum kinase (PERK; sometimes designated EIF2AK3), which is a transmembrane endoplasmic reticulum enzyme, with its kinase domain in the cytoplasm, that is activated by ER stress (due to misfolded proteins in the ER lumen); and a homologue (sometimes designated EIF2AK4) of the only eIF2 kinase in yeast, Gcn2, which is activated by starvation of certain amino acids. Phosphorylated eIF2 is fully capable of forming an initiation-competent eIF2-TC, but following its release, phosphorylated eIF2–GDP tightly binds to and sequesters the guanine nucleotide-exchange factor eIF2B, abrogating its activity. eIF2-TC levels consequently fall and most mRNA translation is reduced, but protein synthesis from certain mRNAs with at least two uORFs of appropriate type and position can actually be stimulated. The best-characterized mammalian examples are the transcription factors ATF4 and ATF5, the expression of which is increased ~ 5-fold by

MicroRNA
A small RNA of ~ 21 nucleotides that regulates the expression of mRNAs with which it is partially complementary in sequence.

activation of PERK[62,63]. As shown in FIG.4, this stimulation is explained by the particular uORF configuration shared by both mRNAs, with a very short upstream uORF1, and a longer uORF2 overlapping the *ATF4* (or *ATF5*) ORF. Yeast *Gcn4* mRNA translation is regulated in a superficially similar way, but with important differences (see Supplementary information S4 (box)).

Phosphorylation also affects the intracellular concentration of the eIF4F complex, but indirectly through eIF4E-binding proteins[64], of which there are three functionally equivalent homologues in mammals (4E-BP1, 4E-BP2 and 4E-BP3; sometimes designated EIF4EBP1–3). When hypophosphorylated, a 4E-BP binds eIF4E (in a binary complex), which prevents the eIF4E from associating with eIF4G, but phosphorylation of the 4E-BP on multiple sites, mainly by mTOR, releases eIF4E for assimilation into eIF4F.

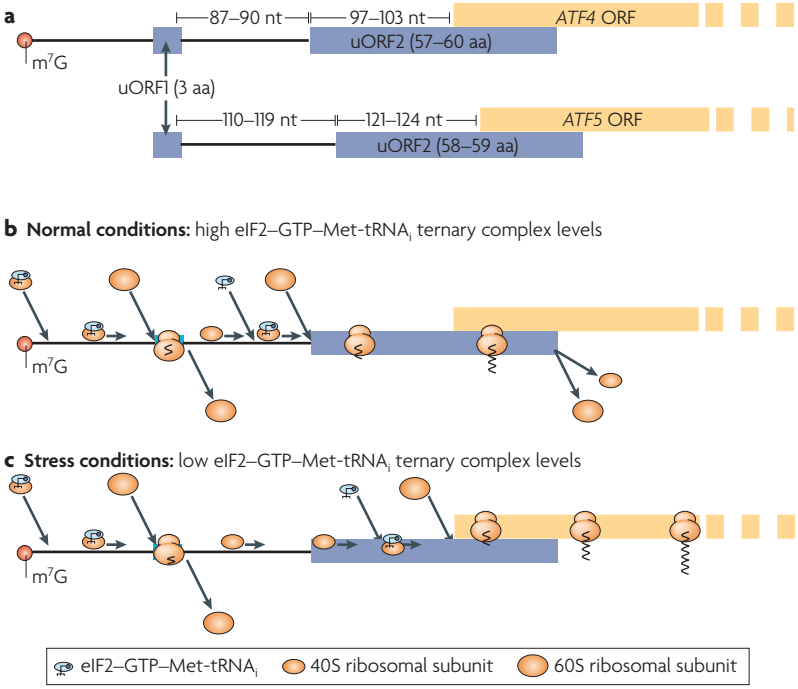


Figure 4 | **The mechanism of regulation of *ATF4* and *ATF5* mRNA translation.** **a** | Diagram showing the sizes, spacing and disposition of the two upstream open reading frames (uORFs) in human, mouse, rat, cow and chicken activating transcription factor 4 (*ATF4*) mRNAs and the four mammalian *ATF5* mRNAs[62,63]. **b** | The pattern of translation in control (unstressed) conditions, when eukaryotic initiation factor 2 (eIF2)–GTP–Met-tRNA$_i$ ternary complexes (eIF2-TCs) are abundant. Small (40S) ribosomal subunits, with associated eIF2-TCs (blue), scan the mRNA in the direction shown. Nascent protein chains are shown by the black zigzag line associated with the large (60S) ribosomal subunit. If eIF2-TCs are abundant, most of the 40S subunits that resume scanning after uORF1 translation will acquire a new eIF2-TC in time to initiate translation of uORF2, and ribosomes that translate this second uORF will be unable to initiate at the *ATF4 or ATF5* AUG because uORF2 is too long to allow rescanning, and because it would require backwards scanning, which doesn't seem to occur over long distances[59]. **c** | Pattern of translation in stressed conditions (for example, following thapsigargin treatment), when eIF2-TC availability is low owing to eIF2 phosphorylation by activated PKR-like endoplasmic reticulum kinase (PERK; sometimes designated EIF2AK3). Consequently, most of the 40S subunits that resume scanning after translating uORF1 acquire a new eIF2-TC only after they have migrated past the uORF2 initiation codon, but in time to initiate at the next AUG, which is at the start of the *ATF* ORF in both cases.

eIF4E itself is also subject to phosphorylation (on Ser209) by MAP kinase interacting Ser/Thr kinase 1 (MNK1) and MNK2, which bind eIF4G's C terminus (FIG.3a) and only phosphorylate eIF4E in *cis*; that is, if the eIF4E is bound to the same eIF4G. Although eIF4E phosphorylation appears to fluctuate in parallel with changes in translation efficiency, MNK1 and MNK2 double knockout mice show no eIF4E phosphorylation, yet exhibit no negative phenotype, showing that phosphorylation–dephosphorylation cycles cannot be essential for translation[65]. Nevertheless, when haematopoietic stem cells engineered to stably express *Myc* plus either an MNK1 mutant or an eIF4E derivative were injected into irradiated mice, the incidence of lymphomas in the recipient mice was much higher with a constitutively active MNK1 mutant than with a dominant-negative MNK1 mutant, and also higher with wild-type eIF4E than a non-phosphorylatable eIF4E, which had a Ser209 to Ala mutation[66]. Thus, it appears that excessive eIF4E phosphorylation can promote malignancy.

Phosphorylation of several other eIFs (eIF1, eIF2β, eIF2Bε, several eIF3 subunits, eIF4G, eIF4B, eIF4H, eIF5 and eIF5B) and of rpS6 has also been recorded[64], and in many cases increases under conditions in which translation is activated; for example, following serum addition to quiescent cells. However, there is no solid evidence that any of these phosphorylation events are the cause of such activation. On the contrary, in the case of rpS6 phosphorylation, although the correlation with increased translation seems particularly striking, cells derived from the embryos of rpS6 kinase 1 and rpS6 kinase 2 double knockout mice, or knock-in of an rpS6 gene with all five phosphorylation sites mutated to Ala residues, show normal regulation of translation[67,68]. These cases of eIF4E and rpS6 phosphorylation should serve as warnings against attaching too much significance to what are merely suggestive correlations.

Regulation by RNA-binding proteins

Regulation by a given sequence-specific RNA-binding protein is selective for those mRNAs that contain the relevant RNA sequence motif in an appropriate position, and is (almost) invariably inhibitory, except for the interaction of PABP with the poly(A) tail. Activation of translation of such mRNAs, therefore, requires sequestration or degradation of the inhibitory protein, inactivation of its RNA-binding potential, or disruption of its interactions with essential corepressor proteins.

Regulation by specific 5′ UTR–protein interactions. Regulation by protein–RNA interactions in the 5′ UTR is surprisingly rare and there is just one well-studied example — ferritin mRNAs[69]. The general principle to emerge from this paradigm is that strong inhibition of initiation requires the protein–RNA interaction to occur at a cap-proximal location, which prevents loading of the 43S complex onto the mRNA[70] but not eIF4F binding to the capped 5′ end. Inhibition is much weaker, or even non-existent, if the critical protein-binding RNA motif is moved to a more cap-distal position, suggesting that if the 43S complex can be loaded, its subsequent scanning

will displace the bound protein[71]. However, this position effect may depend on the affinity of the protein–RNA interaction, since *PABP* mRNA translation is autoregulated by excess free PABP binding to clustered oligo(A) motifs that are ~ 70–130 nucleotides downstream of the cap[72]. In this case, therefore, bound PABP can apparently block scanning 43S complexes without being displaced by them.

5′ UTR sequences are undoubtedly crucial for the regulation of ribosomal protein and translation elongation factor mRNAs, a large group of abundant and exceedingly important mRNAs[73]. Their translation is poor in quiescent cells, but is strongly and rapidly activated on serum re-feeding, by insulin and by amino acid availability. This property can be conferred on a reporter by transplanting any ribosomal protein 5′ UTR, which are unusual in that they all start with m^7GpppC followed by a run of pyrimidines; hence their name, 5′ terminal oligopyrimidine tract (5′ TOP) mRNAs. This 5′ TOP motif is necessary and must be in its native, 5′ terminal position for proper regulation, but it may not always be sufficient without the rest of the 5′ UTR. The mechanism of regulation remains a mystery, largely because some key parameters differ according to cell type and conditions used. For example, sensitivity of TOP mRNA translation to rapamycin varies from almost no effect to a strong (but never complete) inhibition[74]. Current opinion is that regulation is unlikely to be by a straightforward repressor mechanism similar to that in the ferritin mRNA paradigm, that rpS6 kinases and rpS6 phosphorylation are unlikely to be directly involved and that the phosphoinositide 3-kinase (PI3K) pathway plays an essential part[73].

Stimulation by PABP binding to the 3′ poly(A) tail. It is often said that a 3′ poly(A) tail with bound PABP is essential for initiation, and that PABP can therefore be considered a canonical eIF[75]. As supporting evidence, such statements usually cite the fact that deletion of the *PAB1* gene in yeast is normally lethal, but this ignores the important caveat that there are numerous bypass suppressor mutations that allow yeast to grow (albeit slowly) in the complete absence of Pabp[76]. Experiments in systems as diverse as yeast poly(A) polymerase mutants and rabbit reticulocyte lysates have shown that the translational advantage of polyadenylated over non-polyadenylated mRNAs is greatest under conditions of strong competition for limiting eIFs and/or ribosomes[77,78], suggesting that the PABP–poly(A) effect is stimulatory rather than essential.

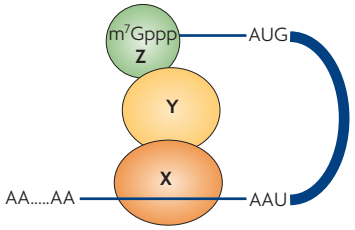
This stimulatory effect appears to be mainly due to the potential of PABP's second RRM domain to interact with the eIF4G component of eIF4F, which would normally be bound to the 5′ end of the mRNA[79,80]. The resulting circularization of the mRNA, in the 'closed loop' configuration, is commonly believed to aid recycling of ribosomes on the mRNA (see Supplementary information S5 (box)), but there is a simpler explanation. Anchoring of eIF4F to the 3′ poly(A) tail by the PABP bridging interaction ensures that eIF4F will remain tethered to the mRNA even if its contacts with the 5′ end of the mRNA are disrupted, whereas it would be lost in the absence of PABP or a poly(A) tail and would need to be recruited *de novo* from the free eIF4F pool. This consideration alone is sufficient to explain why poly(A) tails confer a particular advantage under competitive conditions. In effect, the naturally occurring poly(A)–PABP–eIF4F interactions are equivalent to the tethering experiments discussed in the following two sections. Indeed, the translation of an mRNA that lacks a poly(A) tail is greatly enhanced by artificially tethering PABP to its 3′ UTR[81].

The importance of the tethering (and the closed loop) is shown by the fact that there are analogous interactions with somatic cell histone mRNAs, which lack a 3′ poly(A) tail yet are efficiently translated despite the competition from bulk (polyadenylated) mRNA. All replication-dependent histone mRNAs have a conserved stem loop structure near the 3′ end, which binds stem loop-binding protein (SLBP). SLBP interacts with SLBP-interacting protein 1 (SLIP1; sometimes designated MIF4GD), which in turn interacts with the N terminus of eIF4G, close to the PABP-binding site[82] (Fig. 3a). These interactions, which result in the tethering of eIF4F to the 3′ end of histone mRNAs, stimulate histone synthesis.

Regulation by specific 3′ UTR–protein interactions. In contrast to the paucity of examples of regulation of initiation by specific protein–RNA interactions in the 5′ UTR, there are numerous cases, most of them important in development, of control by 3′ UTR–protein interactions. It was once widely believed that such regulation was entirely dependent on changes in poly(A) tail length (which could provide a rationale for why regulatory proteins bind to the 3′ UTR), because the regulated mRNAs usually had a short tail when they were translationally repressed and activation coincided with lengthening of the tail. However, there are clear exceptions (for example, mouse protamine 1 mRNA, which maintains a long tail throughout the seven days when its translation is repressed during spermatogenesis[83,84]), and cases where translation can be activated without any lengthening of short poly(A) tails[85], which together led to the hypothesis that there must be mechanisms whereby 3′ UTR–protein interactions regulate initiation more directly than by changes in the polyadenylation status.

Many of the better understood examples conform to the generic model shown in BOX 2 (for specific individual examples, see REF. 86), in which sequence-specific binding of protein X to the 3′ UTR results in formation of an inhibitory closed loop involving protein Y and protein Z. In many cases, protein Y is CUP (in *Drosophila melanogaster* embryos)[86] or its vertebrate homologue, eIF4E transporter (4E-T; sometimes designated EIF4ENIF1)[86,87], an eIF4E-interacting protein that was first identified as a transporter of eIF4E across the nuclear membrane but also has a large cytoplasmic presence. In some cases protein Z is the canonical eIF4E, eIF4E1a, but in the *Xenopus laevis* oocyte cytoplasmic polyadenylation element-binding protein (CPEB)–4E-T system, protein Z is a paralogue, eIF4E1b[87], which is restricted to oocytes, eggs and early embryos and, surprisingly, has weak

Box 2 | **Generic model for the regulation of initiation by 3' UTR–protein interactions**



Protein X binds in a sequence-specific manner to a specific 3′ UTR motif of mRNA and interacts with an intermediate bridging protein (protein Y), which in turn interacts with a cap-binding protein (protein Z), leading to the formation of an inhibitory closed loop that precludes access of eukaryotic initiation factor 4F (eIF4F) to the 5′ end (see the figure). As protein X is the only sequence-specific RNA-binding protein amongst the three, the identity of protein X in the complex differs more widely between different mRNAs or groups of mRNAs than the identities of protein Y and protein Z (see the table). The functions of protein X and protein Y can be embodied in a single protein (for example, Bicoid) or in a group of proteins (Nanos, Pumilio and Brat)[86]. It should be noted that although maskin has been claimed to be protein Y in *Xenopus laevis* oocytes[121], its interactions with cytoplasmic polyadenylation element-binding protein (CPEB) have not been seen in some laboratories[87], the motif by which it is supposed to interact with eIF4E is not conserved in maskins from other species, and it is only expressed in the late stages of oogenesis[87].

Organism	mRNA	Protein X	Protein Y	Protein Z
Xenopus laevis oocytes	Cyclin B1 (and others)	CPEB*	4E-T‡	eIF4E1b
Drosophila melanogaster embryos	Nanos	Smaug	CUP	eIF4E1a§
	Oskar	Bruno	CUP	eIF4E1a§
	Caudal	Bicoid‖	Bicoid‖	4EHP
	Hunchback	Nanos, Pumilio and Brat‖	Nanos, Pumilio and Brat‖	4EHP

CPEB, cytoplasmic polyadenylation element-binding protein; 4EHP, eIF4E homologous protein; eIF, eukaryotic initiation factor; eIF4E-T, eIF4E transporter. *CPEB homologues with the same function are present in *D. melanogaster* (ORB) and *Caenorhabitditis elegans* (FOG-1), but not in budding yeast. ‡4E-T homologues with the same function are present in *D. melanogaster* (CUP) and *C. elegans* (SNP-2), but not in budding yeast. §*D. melanogaster* has eight eIF4E-like proteins, including one 4E-HP, but no equivalent of mammalian eIF4E1b. eIF4E1a is by far the most abundant species in embryos, and thus is likely to be protein Z. ‖Fulfils the functions of both protein X and protein Y.

intrinsic affinity for 5′ caps. In *D. melanogaster* embryos (which lack eIF4E1b) and mouse oocytes, there are examples of repression in which protein Z is another eIF4E paralogue, eIF4E homologous protein (4EHP), which also has low intrinsic affinity for caps and cannot bind eIF4G[86–88]. In addition to the inhibitory closed loop, oligomerization of repressed mRNAs into ill-defined aggregates may provide a further layer of repression[89].

Several potential corepressors are often found associated with this protein X–protein Y–protein Z complex: in the case of *X. laevis* oocytes, a DEAD box helicase (RCK; sometimes designated DDX6 and p54), PAT1, and two nonspecific RNA-binding proteins, RAP55 and FRGY2 (sometimes designated YBX2A)[87]. Homologues of these occur in *D. melanogaster* embryos (Me31B, PAT1, Trailer hitch and YPS, respectively) and in *C. elegans* (CGH-1, PATR-1, CAR-1 and CEY, respectively), and genetic analyses in both organisms have strongly implicated the first three in the mechanism of repression (reviewed in REF. 87). In the case of *X. laevis* oocytes, which are not amenable to such genetic analyses, tethering experiments (FIG.5) have shown that anchoring p54, RAP55 or 4E-T to an mRNA causes the mRNA to be specifically repressed[87,90,91]. Interestingly, in *S. cerevisiae*, which has homologues of RCK, PAT1 and RAP55 (known as Dhh1, Pat1 and Scd6, respectively), the Dhh1 helicase and Pat1 seem to act as partially overlapping regulators of global mRNA translation[92], as deletion of both encoding genes prevents the rapid inhibition of initiation that normally occurs on glucose withdrawal.

On progesterone-induced maturation of *X. laevis* oocytes, many of these players become phosphorylated (CPEB, 4E-T, PAT1 and maskin) and then extensively degraded[87] (at least for CPEB and PAT1), and these changes are likely to be the key to activation of translation, which must involve disruption of the inhibitory closed loop.

It is striking that the same proteins are implicated in all organisms from worms to vertebrates, which suggests that there is a universal mechanism, subject to relatively minor variations. Moreover, although these models are based on regulation in development, they are unlikely to be confined to such situations. For example, CPEB paralogues have been found in somatic cells, particularly neuronal tissue, where they are thought to play an important part in synaptic plasticity[93].

There are two cases of regulation through the 3′ UTR in somatic cells that do not conform to the above model, but point to a defect in a late stage in initiation, at or soon after the commitment step. First, binding of the KH domain-containing proteins hnRNP-K and hnRNP-E1 (also known as PCBP1) to tandem 19 nucleotide CU-rich repeats in the 3′ UTR of erythroid 15-lipoxygenase mRNA represses its translation until the late reticulocyte stage[94]. Second, binding of ZBP (another KH domain-containing protein) to the ~ 54 nucleotide 'zip-code' motif, located downstream of the stop codon of β-actin mRNA, represses translation until the mRNA is properly localized at the lamellipodia of fibroblasts[95]. In both cases, repression can be recapitulated in an *in vitro*

KH domain
(K-homology domain). A protein domain, originally identified in the human hnRNP-K protein, that is important for RNA binding and probably binds RNA directly.

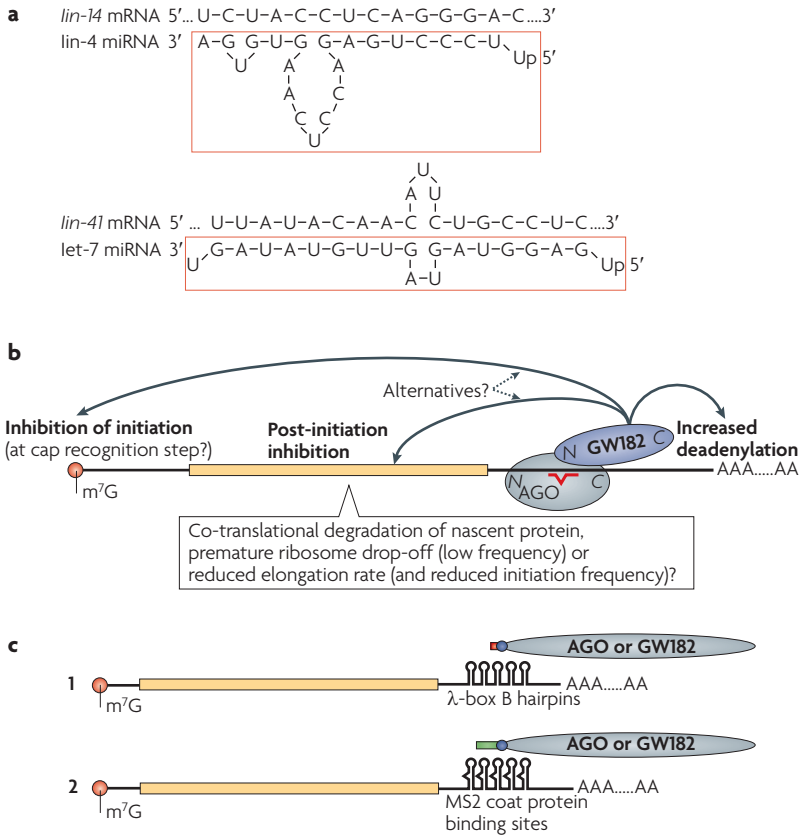


Figure 5 | **Models of miRNA-mediated repression of translation of target mRNAs.** **a** | Examples of the imperfect complementarity between microRNAs (miRNAs; boxed) and their mRNA target sites (upper line) for two validated *Caenorhabditis elegans* miRNA–mRNA interactions. The interaction typically involves perfect contiguous base pairing of miRNA residues 2–8 (the seed match), in some cases extending to residues 1–9, followed by mismatch bulges in either the miRNA or mRNA (or both), and then irregular base pairing of the miRNA 3′ end to the mRNA. **b** | Schematic depiction of the different mechanisms by which miRNAs might regulate their target mRNAs. For clarity, only a single miRNA target site is shown and the other proteins in the complex with agonaute (AGO) and GW182 (the most downstream effector of repression identified so far) have been omitted. There are four AGO paralogues and three GW182 paralogues (commonly designated TRNC6A, TRNC6B and TRNC6C) in mammals. **c** | Tethering experiments showing repression by tethered AGO or GW182. The 3′ untranslated region (UTR) of the reporter mRNA has multiple bacteriophage λ-box B motifs, or bacteriophage MS2 high affinity sites for coat protein, and the test protein (AGO or GW182) is expressed as a fusion with an epitope tag (blue) to allow monitoring of expression levels, and either λ-N-peptide (red) or MS2 coat protein (green). Controls have the epitope tag but lack N-peptide or MS2 coat protein sequences. Tethering a translational activator to the 3′ UTR by the same method results in stimulation of translation, for example, tethering poly(A)-binding protein (PABP) to an mRNA that lacks a 3′ poly(A) tail[81].

system, in which formation of 80S initiation complexes in the presence of GTP is strongly inhibited, whereas 48S complex formation (with the 40S subunit at the initiation codon) in the presence of the non-hydrolysable GTP analogue GMPPNP is not. This suggests that the eIF5B-catalysed reaction and/or subunit joining is abortive, leading to unproductive release of 40S subunits from the mRNA. However, before this model is taken as gospel, we need to be sure that 48S complex formation is also unaffected when such complexes are formed in the presence of GTP (but with eIF5B and 60S subunits absent), to eliminate the possible artefact of GMPPNP stabilizing an intermediate that doesn't actually exist when GTP is used. Nevertheless, the possibility that the eIF5B reaction might be regulated is raised by the provocative finding that the *D.melanogaster* embryo DEAD box helicase, Vasa, binds eIF5B, and that this interaction is required for activation of *gurken* mRNA translation[96].

Argonaute
A family of proteins that are characterized by the presence of two homology domains: PAZ and PIWI. These proteins are essential for diverse RNA silencing pathways.

Translation regulation by miRNAs

miRNAs are another means of repression through the 3′ UTR and can even act in conjunction with sequence-specific RNA-binding proteins, as has been found for cationic amino acid transporter 1 (*CAT1*; sometimes designated *SLC7A1*) mRNA regulation in liver cells[97]. The interaction of the ~ 21 nucleotide miRNA with its target sites takes the form shown in FIG.5a. The degree of repression increases with the increasing number of miRNAs associated with the 3′ UTR, irrespective of whether or not they are identical[98]. Repression efficiency might also be influenced by the distance and sequence between miRNA target sites and by their position in the 3′ UTR.

An argonaute protein (AGO), of which there are four mammalian isoforms, is intimately associated with the paired miRNA–mRNA interaction, and many other proteins are present more peripherally, including the RCK helicase discussed in the previous section and GW182 proteins, of which there are three mammalian paralogues, commonly designated TNRC6A, TNRC6B and TNRC6C[98]. miRNAs, therefore, act as adaptors that confer sequence-specific mRNA binding on AGO. In fact, repression can be recapitulated, even in the absence of any miRNA target site, by tethering AGO to the 3′ UTR[99] (FIG.5c). Moreover, tethering any of the three human GW182 paralogues can by-pass the requirement for both AGO and miRNA[100,101]. These assays show that repression is mediated by the C-terminal ~ 33% of GW182 (the silencing domain)[100], whereas the GW repeat-containing N-terminal domain binds AGO[101]. Thus, miRNAs recruit AGO, which in turn recruits GW182 — the most downstream effector identified so far.

The mechanism of repression seems to have two components[98]: a true repression of mRNA translation, and an accelerated rate of mRNA degradation through the normal deadenylation-dependent pathways[102]. The relative importance of these two components seems to vary between different miRNA–mRNA pairs for unknown reasons, but in tethering assays the same GW182 silencing-domain was necessary and sufficient for both outcomes[101]. Two recent reports have shown that this GW182 domain binds PABP[103,104] (although they disagree over which domain of PABP is involved), and this in turn can recruit the complex of deadenylating enzymes.

The actual mechanism of true repression of translation remains controversial. Some authors find the repressed mRNA displaced from large polysomes into small polysomes or sub-polysomal particles, which is indicative of inhibited initiation. Others find the repressed mRNA in polysomes that are a similar size to those present when the reporter mRNA is not repressed, implying inhibition at a post-initiation stage. A recent provocative report, yet to be independently confirmed, suggests that the initiation or post-initiation outcome is

determined by the identity (but not the efficiency) of the promoter used to drive reporter mRNA synthesis[105], for reasons that remain unknown.

The mechanism underlying the post-initiation lesion remains a mystery. One suggestion is specific co-translational degradation of the nascent protein, because polysome-associated nascent protein N-terminal sequences could not be detected by immunoprecipitation[106]. If confirmed, this would eliminate the other two suggestions of premature ribosome drop-off (which would have to be infrequent to maintain polysome size) and a reduced rate of elongation (which would cause polysome size to increase unless it was coupled with a quantitatively similar reduction in initiation frequency).

As for inhibition of initiation, previous suggestions that AGO itself may interact with the 5′ cap, or that the repression mechanism might impact on eIF6, seem to have both been soundly refuted[107]. Those who observe inhibition of initiation are generally agreed that strong repression is only seen if the mRNA has the normal m^7Gppp cap, and not if this is replaced by Appp, nor if the mRNA has a viral IRES, suggesting that it may be the cap–eIF4F interaction that is the proximal target of the repression mechanism[108–110], superficially similar to the model depicted in BOX 2 for regulation by other 3′ UTR–protein interactions. Because the GW182–PABP interaction mentioned above seems to compete against the eIF4G–PABP interaction that maintains the closed loop[103,104], the consequent disruption of the closed loop may contribute to translational repression. This cannot be the complete answer, however, as translation of mRNAs that lack poly(A) tails can also be repressed by miRNAs[104,111].

Conclusions and perspectives

The picture which emerges from this Review is one of steady progress on most fronts. On mechanisms of initiation, further advances can be expected from the approaches that have previously proved most informative: yeast genetics and kinetic analysis in yeast cell-free systems, and mammalian *in vitro* systems that recapitulate all the steps of translation with purified factors. Further insights into initiation complex structure can be expected from cryoelectron microscopy and biochemical mapping of initiation factor binding sites on 40S subunits, relying on modelling that is based on the eubacterial ribosome crystal structure (because crystal structures of eukaryotic ribosomes are unlikely to be available for some time).

A rather urgent problem is to resolve the controversies over the mechanisms of miRNA-mediated repression so that a solid molecular interpretation can then be placed on the current flood of bioinformatic, microarray and proteomic data, which are aimed at elucidating the regulatory networks dependent on the several hundred miRNAs present in vertebrates. Apart from *D. melanogaster* and *C. elegans* genetic analysis, further pursuit of which proteins interact with AGO and, particularly, with GW182, would seem to be the most promising way forwards, coupled with tethered function assays (FIG. 5c). The same approaches also seem to be the best for gaining further insights into the mechanisms of regulation by protein–3′ UTR interactions. In addition, RNA interference and antisense approaches to knock down specific proteins will undoubtedly have an increasingly important role in research on these topics.

The biggest gap in knowledge remains the mechanism of regulation of vertebrate TOP mRNAs, for which lower eukaryote genetics cannot offer any insights. This topic is possibly proving so difficult because we don't yet fully understand how the PI3K signalling pathway impacts on translation. Another reason may be that regulation of this group of mRNAs is so important that there are multiple overlapping, partially redundant pathways as a type of fail-safe mechanism.

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Competing interests statement
The authors declare competing financial interests: see web version for details.

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