BIOLOGICAL REVIEWS
Cambridge Philosophical Society
Biol. Rev. (2023), **98**, pp. 2284–2306.
doi: 10.1111/brv.13005

eIF4E as a molecular wildcard in metazoans RNA metabolism

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ABSTRACT

The evolutionary origin of eukaryotes spurred the transition from prokaryotic-like translation to a more sophisticated, eukaryotic translation. During this process, successive gene duplication of a single, primordial *eIF4E* gene encoding the mRNA cap-binding protein eukaryotic translation initiation factor 4E (eIF4E) gave rise to a plethora of paralog genes across eukaryotes that underwent further functional diversification in RNA metabolism. The ability to take different roles is due to eIF4E promiscuity in binding many partner proteins, rendering eIF4E a highly versatile and multifunctional player that functions as a molecular wildcard. Thus, in metazoans, eIF4E paralogs are involved in various processes, including messenger RNA (mRNA) processing, export, translation, storage, and decay. Moreover, some paralogs display differential expression in tissues and developmental stages and show variable biochemical properties. In this review, we discuss recent advances shedding light on the functional diversification of eIF4E in metazoans. We emphasise humans and two phylogenetically distant species which have become paradigms for studies on development, namely the fruit fly *Drosophila melanogaster* and the roundworm *Caenorhabditis elegans*.

Key words: eIF4E, translation initiation, metazoan, *Drosophila*, *C. elegans*.

CONTENTS

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I. INTRODUCTION: MYRIAD OF eIF4E PARALOGS ACROSS METAZOANS

In 1976, Severo Ochoa's laboratory reported a polypeptide able to bind to the messenger RNA (mRNA) cap structure $m^7G(5')ppp(5')N$, where m^7G is 7-methylguanosine and N is the first nucleotide of mRNA (typically G or A). The polypeptide was identified in high-salt wash fractions of ribosomes from the crustacean *Artemia salina* (Filipowicz *et al.*, 1976). In subsequent studies using a chemical cross-linking approach, a 24 kDa polypeptide was unequivocally isolated from rabbit reticulocytes that specifically binds to the cap, and was later purified to homogeneity using affinity chromatography. This protein was termed eukaryotic initiation factor 4E (eIF4E) (Sonenberg *et al.*, 1978, 1979, 1980). Later work demonstrated that eIF4E forms a complex with eIF4G and eIF4A to promote translation (Tahara, Morgan & Shatkin, 1981). Following these findings, eIF4Es from different metazoan taxa have been characterised (Table 1).

eIF4E is a highly conserved protein across eukaryotes. It is defined by the presence of eight conserved tryptophan residues [Trp-43, Trp-46, Trp-56, Trp-73, Trp-102, Trp-112, Trp-130 and Trp-166 (human protein numbering)] and a cupped-hand three-dimensional shape for cap binding (Aravin & Koonin, 2000; Marcotrigiano *et al.*, 1997; Matsuo *et al.*, 1997). The protein cavity interacts with the mRNA cap sandwiched between Trp-56 and Trp-102. Trp-166 and Asp-103 also contact the cap structure. Arg-112, Arg-157, and Lys-162 directly contact the cap ribose and phosphate moieties. *In silico* studies found that the evolutionary radiation of eukaryotes into many lineages led to the multiplication of a single, primordial eukaryotic *eIF4E* gene into a constellation of orthologs and paralogs in many lineages across eukaryotes (Aravin & Koonin, 2000; Gillespie, Bachvaroff & Jagus, 2016; Hernández & Vazquez-Pianzola, 2005; Joshi *et al.*, 2005). Among metazoans, vertebrates possess 4–6 paralog genes (Evsikov & Marín de Evsikova, 2009; Gillespie *et al.*, 2016; Joshi, Cameron & Jagus, 2004; Joshi *et al.*, 2005), insects possess 2–7 (Hernández *et al.*, 2005; Lasko, 2000; Tettweiler *et al.*, 2012), Nematoda possess 2 (Joshi *et al.*, 2005; Lall *et al.*, 2004), Platyhelminthes possess 1–7 (Joshi *et al.*, 2005; Pereira-Dutra *et al.*, 2019), and echinoderms possess 3 (Gillespie *et al.*, 2016; Morales *et al.*, 2006). Thus, eIF4Es were classified according to variations in the residues equivalent to Trp-43 and Trp-56 of the human protein and were grouped into three classes (Joshi *et al.*, 2005). Class I members contain both Trp residues; Class II members, called eIF4E-homologous protein (4E-HP), eIF4E-like protein (4E-LP), or eIF4E-2, have Tyr, Phe, or Leu at the first position and Tyr or Phe at the second position; and Class III proteins have Trp at the first position and Cys or Tyr at the second position.

The presence of several paralogs of eIF4E in different species led Hernández & Vazquez-Pianzola (2005) to hypothesise that a ubiquitous paralog might perform global translation initiation whereas other isoforms might drive translation in a tissue-, developmental stage- or physiological condition-specific manner. Indeed, recent *in silico* analyses of available human transcriptome and proteome data have provided evidence that the translation machinery possesses a diverse composition across tissues and organs (Anisimova *et al.*, 2023). The scenario has become even more complex as, in addition to translation, eIF4E paralogs are known to be involved in other processes of RNA metabolism, with several functions described for different eIF4Es in yeasts (Ross-Kaschitza & Altmann, 2020) and diverse protist species (Freire *et al.*, 2017; Jagus *et al.*, 2012; Jones *et al.*, 2015; Roy, Jagus & Morse, 2018).

In this review, we describe recent advances in understanding the functional diversification of eIF4E in metazoans, emphasising three paradigm systems: humans, *Drosophila melanogaster*, and *Caenorhabditis elegans*. As all eIF4E activities depend strictly on association of eIF4E with interacting proteins, this overview of eIF4E functions and the proteins with which it interacts across metazoans, leads us to propose that the concept of eIF4E should be re-evaluated. We suggest that it functions not only as a translation factor, but also as a highly versatile, multifunctional, and promiscuous protein involved in different processes of RNA metabolism. In essence, eIF4E functions as a molecular wildcard.

II. CAP BINDING – A VALUABLE SKILL

The earliest and best characterised role of eIF4E is in promoting protein synthesis. Recently, new eIF4E functions have been discovered in RNA metabolism in addition to mRNA translation (Borden, 2016; Hernández, 2022). In many species, a fraction of this protein exists in the nucleus, where it mediates the export of specific mRNAs to the cytoplasm (Hernández, 2022; Osborne & Borden, 2014; Volpon *et al.*, 2017), and eIF4E also is found inside different

Table 1. Biological characterization of eukaryotic initiation factor 4E (eIF4E) from different metazoan species.

Phylum	Species	Name	Class	References
Chordata	*Oryctolagus cuniculus* (rabbit)	eIF4E[a,b]	I	Edery *et al.* (1984); Sonenberg *et al.* (1978, 1979, 1980); Tahara *et al.* (1981)
	Mus spp. (mouse)	eIF4E[a,b]	I	Jaramillo *et al.* (1991); Joshi *et al.* (2004); Marcotrigiano *et al.* (1997); Niedzwiecka *et al.* (2002, 2004)
		eIF4E-1b[c]	I	Evsikov *et al.* (2006); Evsikov & Marín de Evsikova (2009); Kubacka *et al.* (2014)
		4E-HP[d]	II	Joshi *et al.* (2004)
		eIF4E-3	III	Joshi *et al.* (2004); Osborne *et al.* (2013)
	Xenopus laevis (African clawed frog)	eIF4E	I	Kubacka *et al.* (2014); Miyoshi, *et al.* (1999);
		eIF4E(i)[e]		Wakiyama *et al.* (2001*a*,*b*, 1995, 1997)
		eIF4E-1b	I	Kubacka *et al.* (2014); Minshall *et al.* (2007)
	Danio rerio (zebrafish)	eIF4E-1a	I	Fahrenkrug *et al.* (2000); Robalino *et al.* (2004)
		eIF4E-1b		
Arthropoda	*Artemia salina* (brine shrimp)	eIF4E	I	Filipowicz *et al.* (1976)
Nematoda	*Ascaris suum*	eIF4E-3	I	Lall *et al.* (2004); Liu *et al.*, (2011)
Platyhelminthes	*Echinococcus granulosus*	eIF4E	I	Pereira-Dutra *et al.* (2019)
	Schistosoma mansoni	eIF4E	I	Liu *et al.* (2009)
Echinodermata	*Strongylocentrotus purpuratus* (sea urchin)	eIF4alpha	N/D	Huang *et al.* (1987); Jagus *et al.* (1992, 1993)
	Sphaerechinus granularis (sea urchin)	eIF4E	I	Oulhen *et al.* (2007)
	Paracentrotus lividus (sea urchin)	eIF4E-1	I	Chassé *et al.* (2019)
		eIF4E-2	II	
		eIF4E-3	III	
	Pisaster ochraceus (starfish)	eIF4E	I	Lee *et al.* (2000); Xu *et al.* (1993)

N/D, non-determined.
[a]Due to the large amount of work published on this protein, only selected biochemical characterizations are cited.
[b]Also dubbed eIF4E-1 (Joshi *et al.*, 2004); and eIF4E-1a in Tetrapoda (Evsikov & Marín de Evsikova, 2009; Gillespie *et al.*, 2016; Kamenska *et al.*, 2014; Kubacka *et al.*, 2014; Minshall *et al.*, 2007; Robalino *et al.*, 2004; Standart & Minshall, 2008).
[c]Also known as *Eif4eloo* (eukaryotic translation initiation factor 4E-like, oocyte-specific) (Evsikov *et al.*, 2006; Evsikov & Marín de Evsikova, 2009).
[d]eIF4E-homologous protein; also known as eIF4E-2 and 4E-LP (Joshi *et al.*, 2004).
[e]eIF4E isoform.

cytoplasmic foci where it is involved in mRNA storage and decay (Ivanov, Kedersha & Anderson, 2019). Crucially, all biological activities of eIF4E depend on its association with different partner proteins (eIF4E-interacting proteins, 4E-IPs) (Hernández, 2022; Hernández *et al.*, 2016), with the cap-binding ability of eIF4E utilised to perform different cellular processes, making eIF4E a central interface between gene expression and RNA metabolism. Thus, eIF4E is a highly versatile, promiscuous protein that behaves as a molecular wildcard at the crossroads between mRNA translation, transport, processing, storage, and decay, whose biological role is defined by the specific protein with which it interacts (Hernández, 2022).

A key process in evolution is the transformation of a duplicated gene to perform a new function. Jacob (1977) proposed the concept of 'molecular tinkering' to describe this fundamental process: '*…natural selection does not work as an engineer works. It works like a tinkerer —— a tinkerer who does not know exactly what he is going to produce but uses whatever he finds around him, whether it be pieces of string, fragments of wood, or old cardboards…* [Evolution] *would slowly modify his work, unceasingly retouching it, cutting here, lengthening there, seizing the opportunities to adapt it progressively to its new use… It works on what already exists, either transforming a system to give it new functions or combining several systems to produce a more elaborate one*' (Jacob, 1977, pp. 1163, 1164).

During the evolutionary origin of eukaryotes, the emergence of a nuclear membrane represented a disruptive innovation. The resulting spatio-temporal separation between transcription and translation necessitated the development of systems for the protection and nuclear export of transcripts, and may have triggered the emergence of the eukaryote-specific eIF4E and capped and polyadenylated mRNAs (Aravin & Koonin, 2000; Hernández, 2008, 2009). In the absence of any means of interacting directly with the ribosome itself, eIF4E could play a role in translation only after the emergence of a scaffold protein (eIF4G), able to interact with eIF4E (Hernández, 2008, 2009). Thus, eIF4E might first have appeared in early eukaryotes as a promiscuous wild card able to bind to many different proteins. This skill was instrumental to the incorporation of eIF4E into various processes of RNA metabolism. After successive duplications, the new gene copies underwent specialisation for functioning in specific tissues.

The ability of eIF4E to bind the mRNA cap was pivotal during metazoan evolution, with different 4E-IPs co-opting it repeatedly to play a role in the various cellular and developmental processes of RNA metabolism (Fig. 1).

III. eIF4E IN HUMANS

Purification of a cap-binding complex from human cells led to the identification of a polypeptide that was later termed eIF4E (Tahara *et al.*, 1981) (a Class I protein). Subsequently, the human *eIF4E* gene was cloned successfully from lymphocyte and fibroblast λgt11 complementary DNA (cDNA) libraries (Rychlik *et al.*, 1987). More recently, another three eIF4E paralogs in humans have been characterised: Class I eIF4E-1b (Evsikov & Marín de Evsikova, 2009; Kubacka *et al.*, 2014); Class II 4E-HP (Rom *et al.*, 1998); and Class III eIF4E-3 (Joshi *et al.*, 2005; Landon *et al.*, 2014). The three-dimensional (3D) structures of all of these paralogs have also been resolved (Brown *et al.*, 2007; Kubacka *et al.*, 2014; Osborne *et al.*, 2013; Peter *et al.*, 2017). In the following, we describe the diverse roles of human eIF4Es.

(1) Canonical translation

The first and best characterised activity of eIF4E is its ability to promote translation (Sonenberg *et al.*, 1980), perhaps its predominant role in the cell. In eukaryotes, this process is mostly performed by a cap-dependent mechanism that consists of 40S ribosome subunit recruitment to the mRNA cap at the 5′-untranslated region (UTR) through the activity of translation initiation factors (eIFs) (Hershey, Sonenberg & Mathews, 2019; Pelletier & Sonenberg, 2019).

In the current model of canonical translation, the initiation step begins with recognition of the mRNA cap by eIF4E in complex with the scaffolding protein eIF4G and the RNA helicase eIF4A. Together, these three eIFs form the eIF4F complex (Fig. 2A). Simultaneously, a free 40S ribosomal subunit interacts with eIF1, eIF1A, eIF3, eIF5, and the ternary

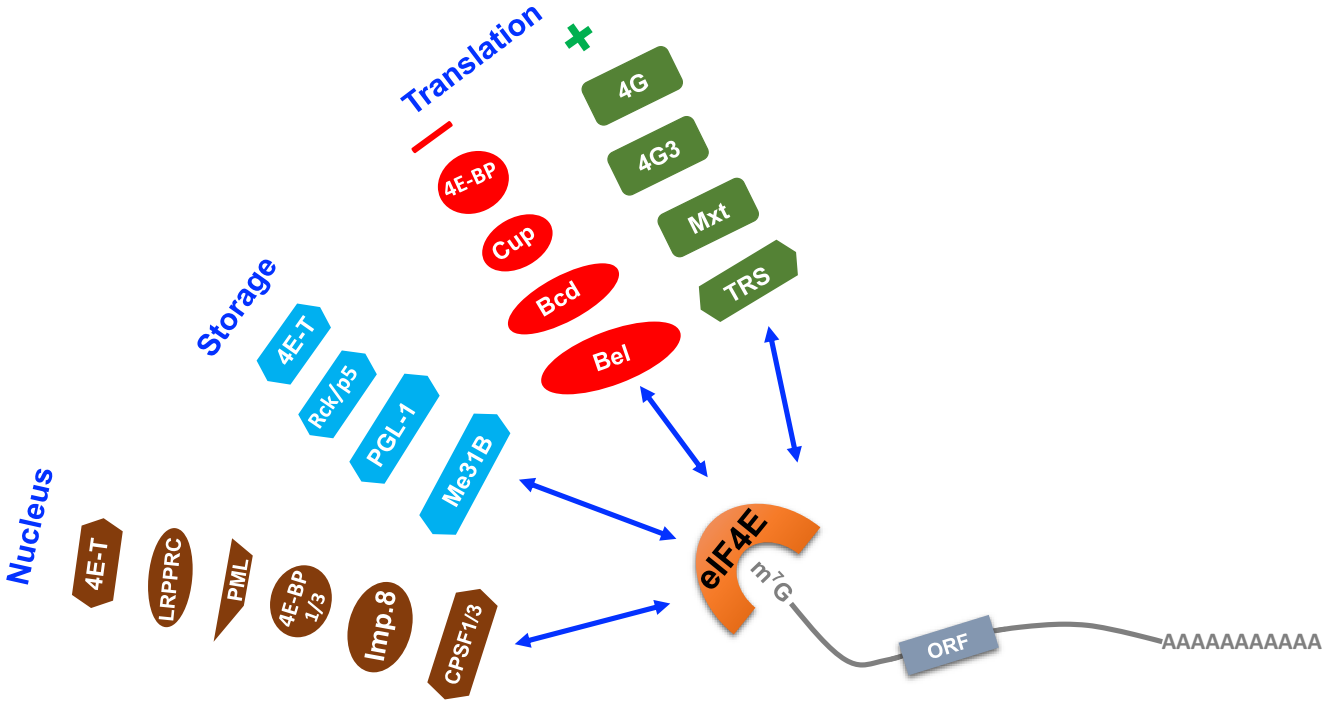

Fig. 1. The cap-binding protein eukaryotic initiation factor 4E (eIF4E) is a promiscuous protein pivotal to RNA metabolism. eIF4E is a wildcard at the interface between nuclear messenger RNA (mRNA) processing, mRNA nuclear–cytoplasm export, storage/decay, and protein synthesis, either promoting (+) or repressing (−) translation, (Hernández, 2022). The biological role of each eIF4E paralog is specified by the proteins with which it interacts. In this figure, eIF4E represents any eIF4E paralog in metazoans. To date, about 50 eIF4E-interacting (4E-IP) proteins are known, and their interaction with eIF4E determines eIF4E activities (Hernández, 2022). Only some of these are depicted. eIF4E-transporter (4E-T), leucine-rich pentatricopeptide repeat protein (LRPPRC), promyelocytic leukemia protein (PML), and eIF4E-binding proteins 1/3 (4E-BP1/3) control mRNA nuclear export or eIF4E nuclear import (depicted in black); cleavage and polyadenylation specific factor (CPSF)1 and CPSF3 are involved in nuclear 3′-end mRNA processing. Importin 8 (Imp8), 4E-T, RNA helicase Rck/p54, P granule-specific protein 1 (PGL-1), and the RNA helicase maternal expression at 31B (Me31B) mediate mRNA storage in cytoplasmic foci. 4E-BP, Bicoid (Bcd), and Belle (Bel) repress translation; eIF4G (4G), eIF4G3 (4G3), Mextli (Mxt), and threonyl-tRNA synthetase (TRS) promote translation. Nuclear 4E-IPs are depicted in brown; 4E-IPs in cytoplasmic foci are in light blue; 4E-IPs repressing translation are in red; and 4E-IPs promoting translation are in green. ORF, open reading frame.

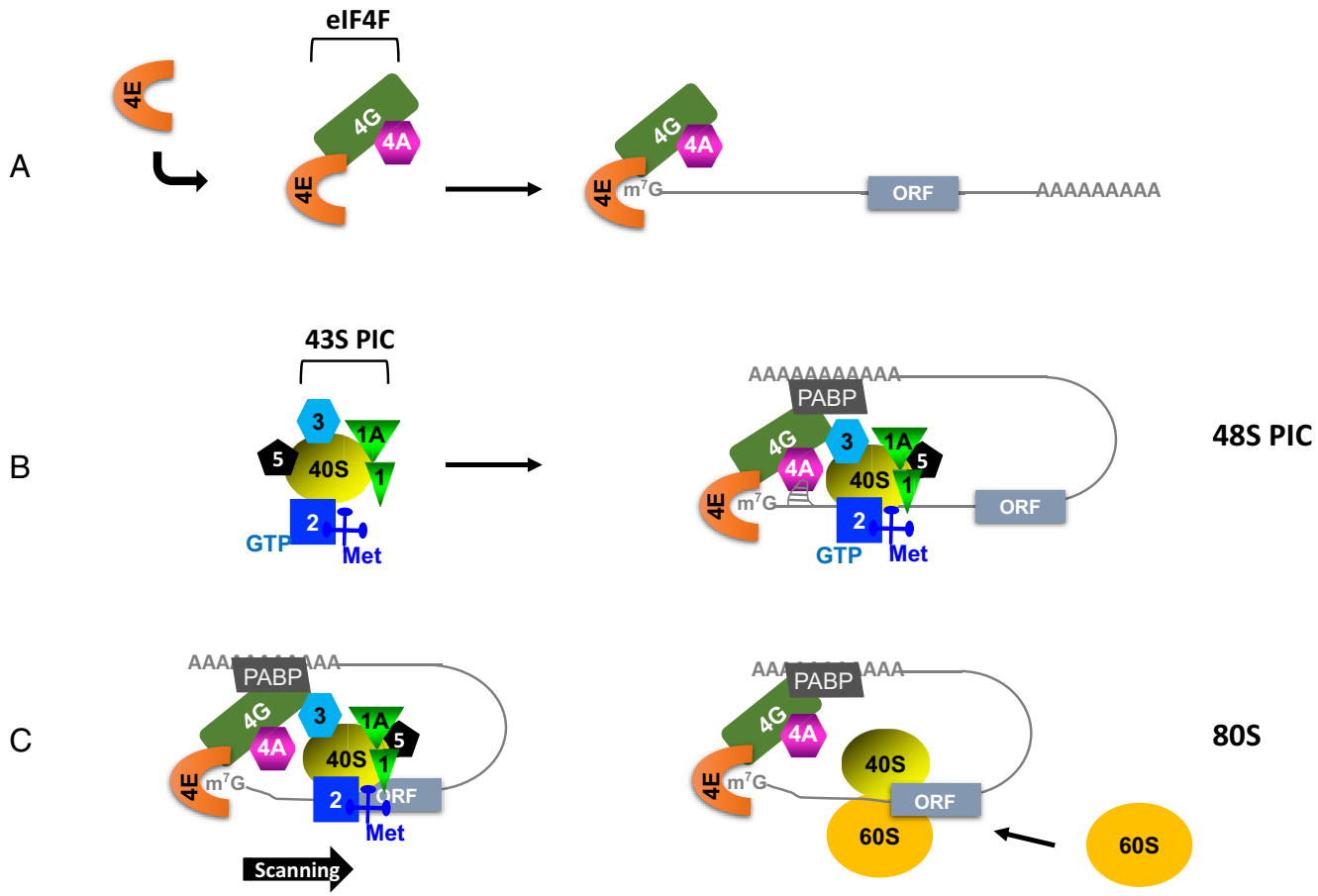

Fig. 2. The canonical process of messenger RNA (mRNA) translation initiation. (A) Eukaryotic initiation factor 4E (eIF4E; 4E) interacts with eIF4G (4G) and the RNA helicase eIF4A (4A) to form the eIF4F complex. eIF4E the recognises the cap (m^7G) at the 5′-end of an mRNA to integrate an mRNA–eIF4F complex. (B) A 40S ribosomal subunit binds a ternary complex [consisting of eIF2 (2) bound to an initiator methionine-$tRNA_i^{met}$ and GTP], eIF3 (3), eIF1 (1), eIF1A (1A), and eIF5 (5) to form a 43S pre-initiation complex (PIC) which is recruited to the mRNA 5′-end in the mRNA-eIF4F complex. These interactions form a 48S PIC. A circularised mRNA competent for translation is established by the interaction of eIF4G, poly(A)-binding protein (PABP), and the mRNA's poly(A) tail (Topisirovic *et al.*, 2011). (C) The 43S PIC scans the mRNA 5′-UTR to reach the translation initiation site (left) that establishes the open reading frame (ORF), ultimately leading to the joining of a 60S ribosomal subunit to the mRNA–ribosome complex (right) to form a translationally active 80S initiation complex.

complex [TC; consisting of eIF2 bound to an initiator methionine-tRNA (Met-$tRNA_i^{Met}$) and GTP] to form a 43S pre-initiation complex (PIC) (Fig. 2B). The 43S PIC is recruited at the 5′-end of the mRNA through the interaction of eIF3 with eIF4G to form a 48S PIC. eIF4G also interacts with the poly(A) binding protein (PABP), promoting mRNA circularization that causes a crosstalk between both the 5′-UTR and 3′-UTR ends of mRNA. eIF4A then unwinds secondary structures present at the 5′-UTR, allowing the 43S PIC to scan the 5′-UTR to reach an AUG start codon. In this step, the initiator Met-$tRNA_i^{Met}$ is positioned in the ribosome's peptidyl site, making base-pairing between the AUG codon and the anticodon of the Met-$tRNA_i^{Met}$. Subsequent reactions drive 60S subunit joining to the 48S PIC to form an active 80S initiation complex (Fig. 2C) (Hinnebusch, 2014; Pelletier & Sonenberg, 2019).

(2) Regulation of eIF4E *via* 4E-BPs and MNKs

Two major pathways signal the initiation phase of translation, namely the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin complex 1 (mTORC1) pathway and the mitogen-activated protein kinases (MAPKs) pathway (Fig. 3A). The mTORC1 pathway senses and drives responses to stress, cellular energy status, nutrient availability, hormones, and mitogens to control cellular proliferation and survival. The mTOR cascade controls translation initiation *via* eIF4E-binding proteins 1, 2, and 3 (4E-BP1/2/3), which bind to eIF4E depending on their phosphorylation status: hypophosphorylated 4E-BPs bind eIF4E and phosphorylated proteins dissociate from eIF4E. Interaction of 4E-BPs to eIF4E block the formation of the eIF4E/eIF4G complex, thereby repressing cap-dependent

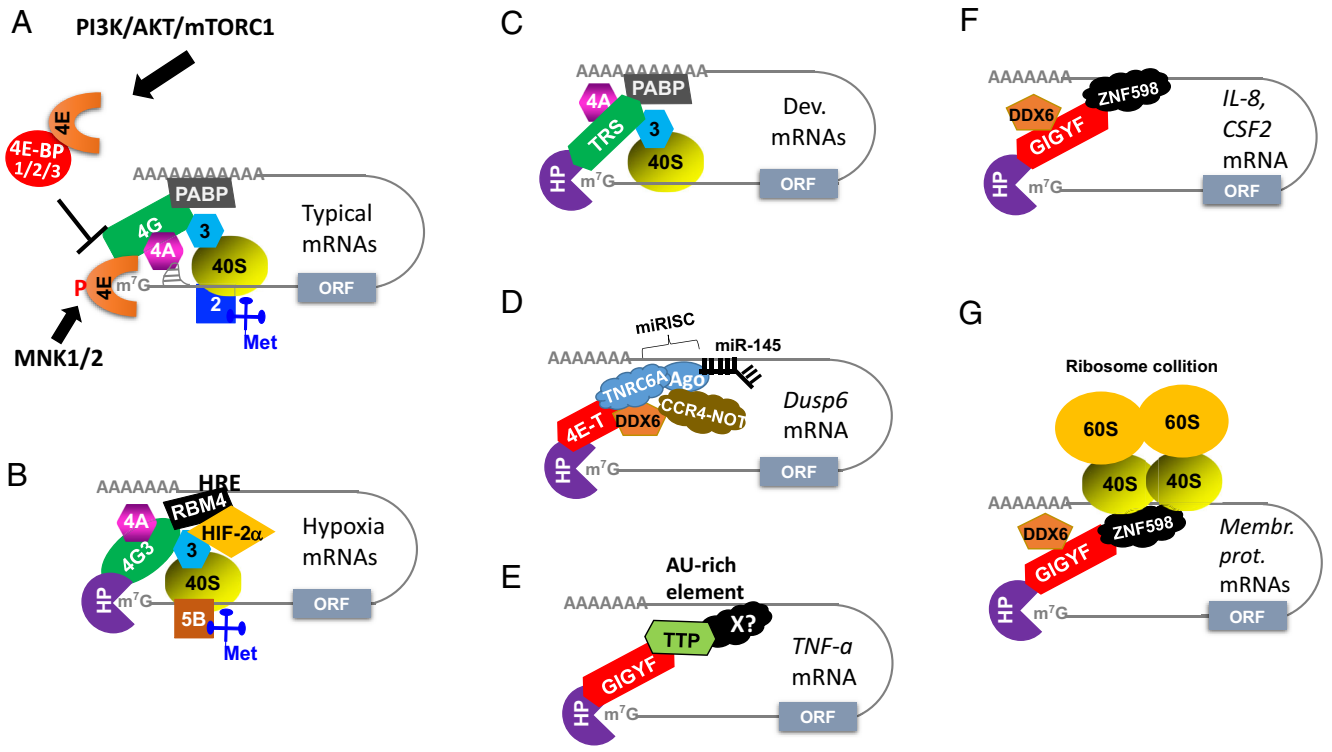

Fig. 3. Cytoplasmic roles of eukaryotic initiation factor 4E (eIF4E) isoforms in the human. (A) Canonical 48S pre-initiation complex (PIC) driving translation of typical messenger RNAs (mRNAs) mediated by eIF4E. This mechanism may account for the translation of most mRNAs. (B, C) Non-canonical 48S PIC driving translation initiation mediated by eIF4E-homologous protein (4E-HP). (B) Translation under hypoxia of hypoxia-responsive element (HRE)-containing mRNAs. (C) Translation of developmental (Dev.) mRNAs involved in skeleton and nervous system formation. (D–G) 4E-HP mediates translational repression of selected mRNAs. 2, eIF2; 3, eIF3; 4A, eIF4A; 4E, eIF4E; 4E-BP1/2/3, eIF4E-binding proteins 1, 2 and 3; 4E-T, eIF4E-transporter; 4G, eIF4G; 4G3, eIF4G-3; 5B, eIF5B; Ago, Argonauta; CCR4-NOT, carbon catabolite repression–negative on TATA-less complex; CSF2, colony stimulating factor; DDX, DEAD-box RNA helicase; Dusp6, Dual specificity phoshatase 6; GIGYF, Grb10-interacting GYF (glycine–tyrosine–phenylalanine domain) protein; HIF-2α, hypoxia-induced factor 2 alpha; HP, 4E-HP; HRE, hypoxia response element; IL-8, interleukin 8; Membr. prot., membrane proteins; miRISC, micro (mi)RNA-induced silencing complex; miR-145, micro (mi)RNA 145; MNK1/2, mitogen-activated protein kinase-interacting kinase 1 and 2; mTORC1, mammalian target of rapamycin complex 1; ORF, open reading frame; P, phosphorylation; PABP, poly(A)-binding protein; PI3K, phosphatidylinositol 3-kinase; RBM4, RNA-binding motif 4; TNF-α, tumour necrosis factor alpha; TNRC6A, trinucleotide repeat containing adaptor 6A; TRS, threonyl-tRNA synthetase; TTP, tristetraprolin protein; X, unidentified protein; ZNF598, E3 ubiquitin ligase.

translation (Fonseca *et al.*, 2016; Roux & Topisirovic, 2018). In the MAPKs pathway, MAPKs respond to mitogens and stress stimuli by activating mitogen-activated protein kinase-interacting kinase 1 and 2 (MNK1/2) that phosphorylate eIF4E at Ser209 (Proud, 2019; Roux & Topisirovic, 2018).

Unlike 4E-BP1/2, rapamycin does not affect the association of eIF4E with 4E-BP3 in the cytoplasm or the nucleus. Upon serum starvation, a decrease in the phosphorylation of 4E-BP3 at Thr23 was seen, concomitant with the dissociation of 4E-BP3 from eIF4E in HEK293 cells (Kleijn *et al.*, 2002). In the cytoplasm, 4E-BP3 dissociated from eIF4E upon serum starvation, and after insulin stimulation, partial recovery of the 4E-BP3/eIF4E association was observed (Kleijn *et al.*, 2002). When mTORC1 is inhibited for an extended period, 4E-BP1 and 4E-BP2 expression decreases, and the transcription factor E3 (TFE3) promotes the expression of 4E-BP3. Thus, upregulation of 4E-BP3 may be a safeguarding mechanism to inhibit the cap-dependent translation (Tsukumo *et al.*, 2016).

(3) Translation under hypoxia

Human 4E-HP shares 28% identity with eIF4E, is ubiquitously expressed and possesses two Trp → Tyr changes in the residues binding to the mRNA 5′ cap structure (Joshi *et al.*, 2004; Rom *et al.*, 1998). 4E-HP binds the cap 100–200-fold more weakly (Rosettani *et al.*, 2007; Zuberek *et al.*, 2007) and is 5–10 times less abundant than eIF4E (Kubacka *et al.*, 2013; Rom *et al.*, 1998). Moreover, 4E-HP does not possess the phosphorylatable Ser209 present in eIF4E, suggesting that it is not regulated by the MAPK

signalling pathway. 4E-HP does not bind eIF4G (Joshi *et al.*, 2004) but it does interact with eIF4G3 (formerly called eIF4GII) (Ho *et al.*, 2016; Timpano & Uniacke, 2016; Uniacke *et al.*, 2012). As is described below, several processes of gene expression are regulated by a variety of 4E-HP complexes (Fig. 2B–G). Interestingly, the different 4E-HP-binding proteins determine whether 4E-HP promotes or inhibits translation.

High hypoxia is a physiological feature occurring within solid tumours. High throughput transcriptomes identified significant overexpression of *4E-HP* mRNA levels in diverse types of cancer, including glioblastoma (GB), oligodendroglioma, and astrocytoma (Digregorio *et al.*, 2019). Under hypoxia, cells depleted of 4E-HP in different types of cancer showed impaired proliferation and increased apoptosis (Uniacke *et al.*, 2014). When GB U87MG cells were grown under highly anaerobic conditions, they repressed eIF4E and switched to an alternative cap-dependent translation mediated by 4E-HP (Ho *et al.*, 2016; Kelly *et al.*, 2017; Timpano & Uniake, 2016; Uniacke *et al.*, 2012). In this process, 4E-HP interacts with eIF4G3 in complex with hypoxia-induced factor 2α (HIF-2α), RNA-binding motif 4 (RBM4), and eIF4A to form an active and alternative hypoxic eIF4F complex termed $eIF4F^{H}$ (eIF4F hypoxia; Fig. 3B). $eIF4F^{H}$ promotes the translation of selected hypoxia-specific mRNAs containing hypoxia response elements within their 3′-UTR which are recognised by the RBM4–HIF-2α complex. Many of these transcripts encode proteins with roles in tumour progression, cell proliferation, invasion, angiogenesis, and survival (Ho *et al.*, 2016; Kelly *et al.*, 2017; Timpano & Uniake, 2016; Uniacke *et al.*, 2012). Indeed, the formation of a hypoxic core in GB tumours requires 4E-HP-directed translation (Uniacke *et al.*, 2014), and the inactivation of $eIF4F^{H}$ significantly reduced tumour growth in a mouse model (Uniacke *et al.*, 2012). Hypoxia also causes the substitution of eIF2 by eIF5B for $Met\text{-}tRNA_i^{Met}$ delivery during translation initiation (Ho *et al.*, 2018). The switch to anaerobic metabolism promoted the formation of new complexes of RNA-binding proteins with mRNAs, including Hu antigen R (HuR), poly(rC)-binding protein 1 (PCBP1), RBM4, and heterogeneous nuclear ribonucleoproteins (hnRNP) A2/B1, for translation activation (Ho *et al.*, 2020).

Formation of the canonical eIF4E/eIF4G complex for translation initiation also has been reported in breast cancer under hypoxic conditions (Timpano & Uniake, 2016; Yi *et al.*, 2013). The hypoxia-inducible factor (HIF)-1α transcribes *eIF4E* to promote eIF4E synthesis and further eIF4E/eIF4G complex formation to promote the translation of selected mRNAs (Yi *et al.*, 2013). The existence of two eIF4F complexes that function under regular and low oxygen tension makes translation possible under a broad range of oxygen concentrations, and both complexes can operate simultaneously within that range (Ho *et al.*, 2016; Timpano & Uniake, 2016; Uniacke *et al.*, 2012). These findings open the possibility that other types of stress could trigger the formation of novel eIF4F complexes to initiate translation.

(4) Translation during development

Because eIF4E does not possess a domain able to recognise the ribosome, eIF4E can only engage in translation when it interacts with eIF4G. Recently, it was discovered that the ability of eIF4E to promote translation also can involve non-eIF4G proteins. In this non-canonical mode of initiation, human threonyl-tRNA synthetase (TRS) directly binds 4E-HP, eIF4A, and PABP, to drive efficient translation initiation by substituting eIF4G as a scaffold protein (Jeong *et al.*, 2019) (Fig. 3C). Most mRNAs regulated by TRS are involved in development of the nervous system, skeleton, and circulatory system. Using vascular endothelial growth factor (VEGF) mRNA, it was observed that TRS binds to the mRNA 5′-UTR *via* an anticodon-like loop structure (Jeong *et al.*, 2019). Similar cases have been documented in other species in which eIF4E paralogs interact with alternative proteins unrelated to eIF4G to form unorthodox 48S PICs able to drive translation. Such is the case for *Drosophila* Mextli (Hernández *et al.*, 2013), *Arabidopsis* CERES (Toribio *et al.*, 2020), *Giardia* eIF2β (Adedoja *et al.*, 2020), and *Leishmania* eIF3a (Meleppattu *et al.*, 2015).

Another eIF4E implicated in human development is eIF4E-1b. The *eIF4E1b* gene resulted from an ancestral duplication of the *eIF4E* locus at the stem of Tetrapoda (Evsikov & Marín de Evsikova, 2009). The association constant of human eIF4E-1b for the m^7GTP cap is threefold lower than that of eIF4E (Kubacka *et al.*, 2014). While the structure and biophysical features of eIF4E-1b have been studied, its biological role in humans has not been addressed. In mice, *Xenopus*, and the zebrafish *Danio rerio* eIF4E-1b is expressed only in ovaries, early oocytes, and early embryos, possibly translating maternal mRNAs (Evsikov *et al.*, 2006; Evsikov & Marín de Evsikova, 2009; Joshi *et al.*, 2004; Minshall *et al.*, 2007; Robalino *et al.*, 2004). In *Xenopus*, eIF4E-1b binds eIF4E-transporter (4E-T) and is part of the cytoplasmic polyadenylation element binding protein (CPEB) mRNA-protein (mRNP) repressor complex along with the RNA-binding proteins perilipin/ADRP/TIP47 (PAT) and like-Sm (Lsm) 14, the *Xenopus* p54 (Xp54) RNA helicase, and specific mRNAs during meiotic maturation of oocytes. In these complexes, the association eIF4E-1b/4E-T represses mRNAs targeted by CPEB (Minshall *et al.*, 2007). Whether human eIF4E-1b performs similar activities is not known.

(5) Translational repression and mRNA stability

In mammals, 4E-HP has emerged as a pivotal molecule regulating mRNA translation and decay. 4E-HP is recruited to the mRNA cap to repress the translation of specific mRNAs in multiple cellular processes (Christie & Igreja, 2023). Most of these transcripts typically contain specific *cis*-acting elements in their UTRs, which provide specificity for eIF4E activity. In humans, the 4E-HP/4E-T complex is a component of the mammalian microRNA (miRNA)-induced silencing complex (miRISC), which mediates gene silencing. It was

demonstrated that miRISC recruits the carbon catabolite repression 4-negative on TATA-less (CCR4-NOT) complex, which associates with the DEAD-box helicase 6 (DDX6), 4E-T, and 4E-HP to inhibit cap-dependent translation of miRNA-targeted transcripts (Chapat *et al.*, 2017; Chen & Gao, 2017; Jafarnejad *et al.*, 2018). Ribosome-profiling experiments led to the discovery of a 4E-HP/miRNA-dependent mechanism that specifically involves miR-145, and represses *Dusp6* mRNA translation (Fig. 3D). *Dusp6* mRNA encodes dual specificity phosphatase 6 (DUSP6), a phosphatase for extracellular signal-regulated kinase (ERK1/2) and a key player in ERK signalling networks. Thus, *Dusp6* mRNA repression increases ERK1/2 phosphorylation, ultimately promoting cell proliferation and reducing apoptosis (Jafarnejad *et al.*, 2018).

Another protein forming a complex with 4E-HP is the zinc finger protein tristetraprolin (TTP), which associates with different proteins to repress translation and is involved in mRNA decay. Tao & Gao (2015) demonstrated that TTP recruits 4E-HP, but not eIF4E, in human HeLa and HEK293 cells to repress the translation of 3′-UTR AU-rich element-containing mRNAs (Fig. 3E). Overexpression of 4E-HP enhanced TTP-mediated mRNA translation repression, and downregulation of endogenous 4E-HP impaired this mechanism, showing the involvement of 4E-HP translational repression of AU-rich element-containing mRNAs. Moreover, TTP promoted 4E-HP binding to target mRNAs containing AU-rich elements, indicating that TTP recruits 4E-HP to compete with eIF4E for cap binding and repress mRNA translation. Overexpression of a 4E-HP mutant unable to bind the mRNA cap impaired TTP-mediated translation repression, indicating that the cap-binding activity of 4E-HP is necessary for its function. Downregulation of either TTP or 4E-HP in macrophage-like THP-1 cells increased synthesis of the cytokine tumour necrosis factor alpha (TNF-α) as well as of pro-TNF (Tao & Gao, 2015). Tollenaere *et al.* (2019) demonstrated that TTP directly binds Grb10-interacting GYF (glycine–tyrosine–phenylalanine domain) proteins 1 and 2 (GIGYF1/2) associated with 4E-HP (Fig. 3E). Previous research in mouse showed that GIGYF1/2 form translational repressor complexes with 4E-HP (Morita *et al.*, 2012). Tollenaere *et al.* (2019) also reported that the E3 ubiquitin ligase ZNF598, a protein involved in triggering mRNA decay, contributes to control of the inflammatory response of cytokine-production cells during the innate immune response. ZNF598 also binds GIGYF1/2 to downregulate the translation of cytokine mRNAs, including interleukin 8 (IL-8) and colony-stimulating factor 2 (CSF2), among others (Fig. 3F).

The 4EHP/GIGYFs complexes also couple mRNA translation with decay. During the elongation step, damaged mRNAs, misfolded or aberrant nascent peptides, and defective ribosomes perturb ribosome movement on the mRNA and may result in ribosome stalling and collision. These defects are recognised by helicase DDX6 associated with the CCR4-NOT complex, which triggers mRNA degradation to avoid the accumulation of toxic polypeptides (Joazeiro, 2019). Thus, failures in elongation often result in recruitment of the decay machinery to the translating mRNA. In this process, the 4EHP/GIGYF2 complex, along with DDX6 and ZNF598, are recruited to the mRNA cap structure to mediate translation-coupled mRNA decay of transcripts encoding membrane proteins or tubulin subunits (Weber *et al.*, 2020) (Fig. 3G).

(6) mRNA storage in cytoplasmic foci

During the mRNA life cycle, mRNAs can be translationally repressed and stored, or even degraded in large, membraneless ribonucleoprotein aggregates termed mRNP granules or cytoplasmic foci, which include processing-bodies (PBs), stress granules (SGs), and germ granules (GGs) among others (An, Merietens & Shelkovnikova, 2021; Layana, Corujo & Rivera-Pomar, 2016). Within the foci, eIF4E-based mechanisms of translation repression are pivotal for mRNA storage and decay across metazoans (Fig. 4). The different properties of human eIF4E paralogs also extend to specific localization within different RNA foci under different stresses (Frydryskova *et al.*, 2016).

PBs from *Xenopus* (Minshall & Standart, 2004), the planarian *Dugesia japonica* (Rouhana *et al.*, 2010), *D. melanogaster* (Ferrero *et al.*, 2012; Layana *et al.*, 2023), *C. elegans* (Rieckher *et al.*, 2018), and human cells (Akao *et al.*, 2006; Andrei *et al.*, 2005; Ferraiuolo *et al.*, 2005; Frydryskova *et al.*, 2016; Kubacka *et al.*, 2013) contain different isoforms of eIF4E in complexes with silent mRNAs and different proteins. Human 4E-T binds eIF4E, which is required for PB

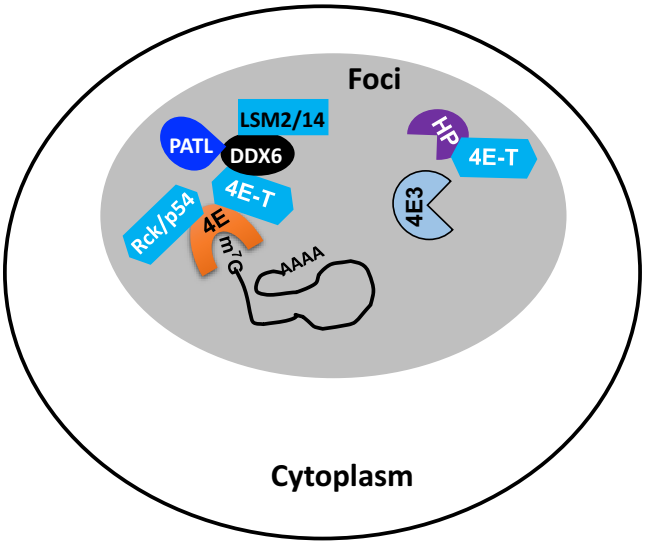

Fig. 4. Human eukaryotic initiation factor 4E (eIF4E) isoforms in cytoplasmic foci. Messenger RNA (mRNA) storage in cytoplasmic processing-bodies and stress granules is represented. For simplicity, the nucleus is not depicted. 4E, eIF4E; 4E3, eIF4E-3; 4E-T, eIF4E-transporter; DDX6, DEAD-box RNA helicase 6; HP, 4E-HP; LSM2/14, LSm RNA binding protein 2/14; ORF, open reading frame; PATL, patellin; Rck/p54, DEAD-box RNA helicase p54.

formation (Andrei *et al.*, 2005; Ferraiuolo *et al.*, 2005; Kubacka *et al.*, 2013; Nishimura *et al.*, 2015; Räsch *et al.*, 2020), and with the DEAD-box RNA helicase Rck/p54 (Akao *et al.*, 2006; Andrei *et al.*, 2005). In addition to binding eIF4E, 4E-T interacts with the mRNA decapping and decay factors DDX6, PAT1 homolog, processing body mRNA decay factor (PATL1), LSM14, and LSM2. Indeed, 4E-T must interact with eIF4E to trigger mRNA decay (Nishimura *et al.*, 2015) (Fig. 4).

eIF4E-3 was initially identified only in chordates. It is expressed in a few tissues, including heart, skeletal muscle, lung, and spleen. In contrast to eIF4E, eIF4E-3 interacts with eIF4G but not with 4E-PBs (Joshi *et al.*, 2004). To date, no other proteins have been found to interact with eIF4E-3. Structural and biophysical studies of the mouse protein showed that eIF4E-3 recognises the mRNA cap with a binding affinity 10- to 40-fold lower than eIF4E (Osborne *et al.*, 2013). Unlike eIF4E, human eIF4E-3 localises to SGs but not PBs upon both heat shock and arsenite stress. 4E-HP is also recruited to PBs during both stresses by 4E-T, whereas it is redirected to SGs only upon heat shock (Frydryskova *et al.*, 2016; Kubacka *et al.*, 2013). Altogether, these observations suggest different roles of eIF4Es in cellular stress responses (Fig. 4).

(7) The hectic life of nuclear eIF4E

Nuclear eIF4E was first identified in monkey cells. Cellular fractionation and immunofluorescence analyses showed that 12–33% of total eIF4E is localised to the nucleus of monkey COS-1 and CV-1 cells (Lejbkowicz *et al.*, 1992). Cells from some other metazoans also possess one or more eIF4E paralogs partially located in the nucleus, including mouse NIH 3T3 fibroblasts (Lai & Borden, 2000; Rosenwald *et al.*, 1995; Rousseau *et al.*, 1996), mouse embryo fibroblasts (MEFs) (Rong *et al.*, 2008), *Xenopus* cells (Strudwick & Borden, 2002), and *Drosophila* embryo cells (Cohen *et al.*, 2001). As we describe below, eIF4E plays different roles in the nucleus of human cells. The nuclear localization and function of the different eIF4E paralogs are ruled by their interaction with different proteins (Fig. 5). Indeed, eIF4E has a busy agenda inside the nucleus.

Nuclear eIF4Es have been studied primarily in model metazoan species and carcinogenic cells. To date, no nuclear

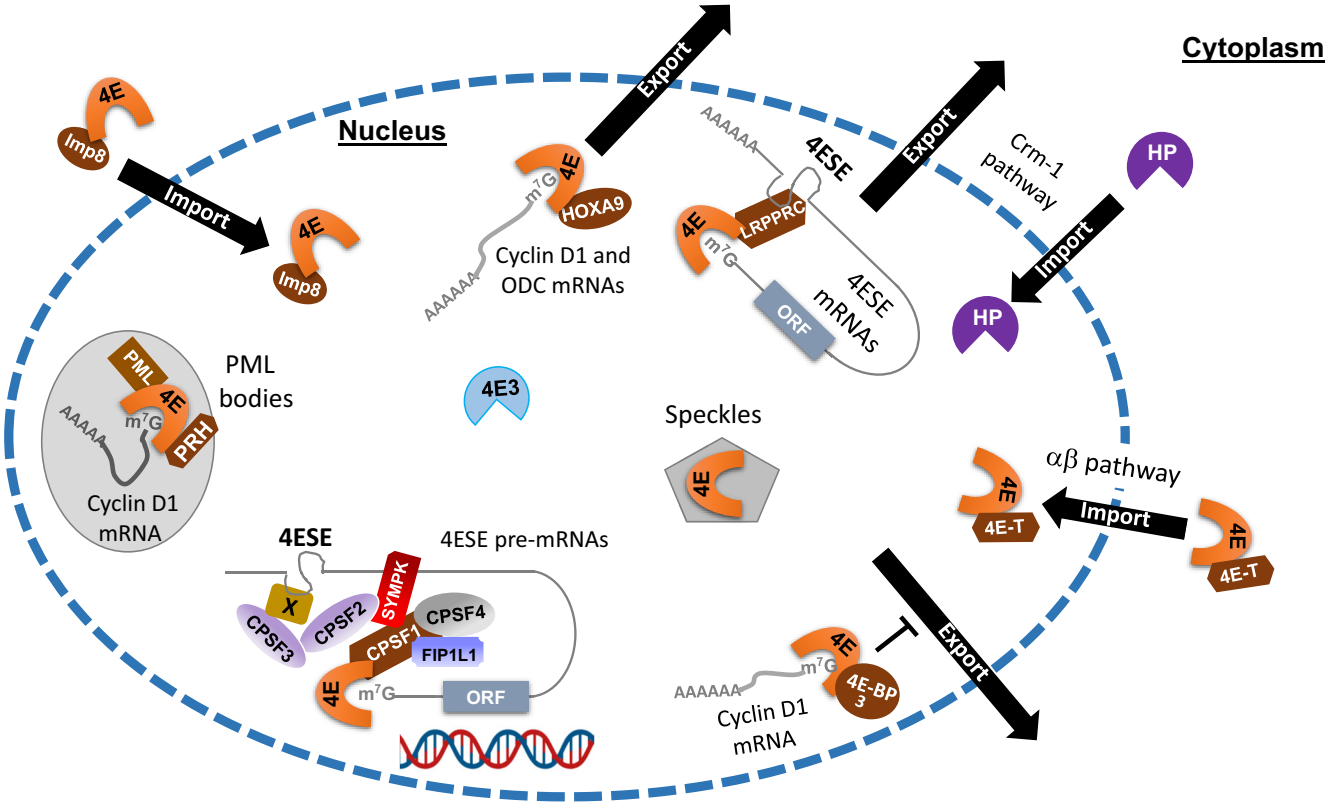

Fig. 5. Human eukaryotic initiation factor 4E (eIF4E) within the cell nucleus. eIF4E nuclear import and the different eIF4E paralogs involved in messenger RNA (mRNA) export, storage in promyelocytic leukaemia protein (PML) bodies, or pre-mRNA 3′end processing are shown. 4E, eIF4E; 4E-BP3, eIF4E-binding protein 3; 4ESE, eIF4E sensitivity element; 4E-T, eIF4E-transporter; CPSF, cleavage and polyadenylation specific factor; Crm-1, chromosome region maintenance 1/exportin1/Exp1/Xpo1; FIPIL1, factor interacting with PAPOLA and CPSF1;HOXA9, human homeodomain protein 9; HP, 4E-HP; Imp8, importin 8; LRPPRC, Leucine-rich pentatricopeptide repeat protein; ODC, ornithine decarboxylase; ORF, open reading frame; PML, promyelocytic leukaemia protein; PRH, prolin-rich homeodomain protein; SYMPK, symplekin.

eIF4E paralog has been reported in multicellular fungi or plants, most probably due to the lack of studies in these kingdoms. Further investigation in different species and healthy cells will allow a better understanding of the biology of eIF4E in the nucleus.

(*a*) *Nuclear import of eIF4E and 4E-HP*

4E-T was identified and isolated from human placental and brain cDNA libraries and further characterized as a nucleocytoplasmic shuttling protein mediating nuclear import of eIF4E in HeLa cells *via* the importin αβ pathway (Dostie *et al.*, 2000*a*) (Fig. 5). A second mechanism of shuttling proteins from the nucleus to the cytoplasm is *via* the nuclear export chromosome region maintenance (Crm)-1-mediated pathway. This mechanism can be blocked with leptomycin B (LMB), an inhibitor of the Crm1. Experiments in HeLa cells treated with LMB showed that, unlike eIF4E, 4E-HP shuttles into the nucleus in a Crm1-dependent manner in the absence of 4E-T (Kubacka *et al.*, 2013). Indeed, in LMB-treated cells but in the presence of 4E-T, eIF4E became highly nuclear. By contrast, 4E-HP accumulated in the nucleus in LMB-treated cells in the absence of 4E-T, and eIF4E remained cytoplasmic. Thus, although 4E-T binds both eIF4E and 4E-HP, 4E-T is a transporter for eIF4E only (Kubacka *et al.*, 2013) (Fig. 5). In addition, 4E-BPs also mediate nuclear localization of eIF4E. In mouse embryo fibroblasts, 4E-BP1 and 2 mediate eIF4E localization within the nucleus (Rong *et al.*, 2008). However, whether this is also true for human cells has not been addressed.

In patients with acute myeloid leukaemia (AML), eIF4E levels are highly elevated and accumulate within the nucleus. Importin 8 (Imp8) is a protein that directly binds eIF4E and imports it into the nucleus (Fig. 5). In AML patients, Imp8 is highly expressed, leading to nuclear eIF4E accumulation (Volpon *et al.*, 2016). In AML, the dysregulation of Imp8 affects eIF4E-dependent mRNA nuclear export. It was observed that dysregulated nuclear trafficking of oncoproteins, including eIF4E, contributes to cancer. In particular, Imp8 knockdown led to cytoplasmic retention of eIF4E and nuclear accumulation of eIF4E nuclear mRNA targets, such as avian myelocytomatosis viral oncogene homolog (c-Myc) and myeloid cell leukemia-1 (Mcl-1), with a concomitant reduction of levels of those proteins. Thus, the reduction of Imp8 impaired eIF4E-dependent mRNA export. Conversely, Imp8 overexpression stimulated eIF4E-dependent mRNA export of c-Myc and Mcl-1 mRNAs, leading to a final increase in levels of the proteins (Volpon *et al.*, 2016).

Human eIF4E-3 is located in the nucleus and the cytoplasm (Frydryskova *et al.*, 2016; Osborne *et al.*, 2013). In contrast to eIF4E, eIF4E-3 was found to repress expression of a pool of target mRNAs in common with eIF4E, reducing their oncogenic potential (Osborne *et al.*, 2013). Because eIF4E-3 associates with eIF4G in mice (Joshi *et al.*, 2004) and humans (Frydryskova *et al.*, 2016), how eIF4E-3 inhibits the expression of specific mRNAs is unknown. The biological role of eIF4E-3 in the nucleus is also not known.

(*b*) *mRNA export*

Nuclear eIF4E modulates the nuclear–cytoplasmic export of selected mRNAs. Early studies in humans reported that eIF4E is present throughout the nucleoplasm but is concentrated in speckles where it colocalizes with the splicing factors Sm and U1 small nuclear ribonucleoprotein particle (U1snRNP) in HeLa cells, a phenomenon conserved in monkey CV-1 cells (Dostie, Lejbkowicz & Sonenberg, 2000*b*). It was later found that eIF4E also concentrates in nuclear bodies where it interacts with the promyelocytic leukaemia protein (PML) in human leukaemia U937, K562, and NB4 cells (Cohen *et al.*, 2001). PML reduces the affinity of eIF4E for the mRNA cap. As eIF4E requires cap binding for the transport of cyclin D1 mRNA, the eIF4E/PML complex in PML nuclear bodies downregulates cyclin D1 mRNA export and protein levels (Cohen *et al.*, 2001) (Fig. 5). Moreover, proline-rich homeodomain protein (PRH), which functions in haematopoiesis, directly binds eIF4E in both the nuclear PML bodies and the cytoplasm of myeloid U937 cells. Through this interaction, PRH inhibits eIF4E-dependent nuclear export of cyclin D1 mRNA and concomitant cell proliferation. The finding that the eIF4E/PRH interaction is tissue specific opens the possibility that other proteins associate with eIF4E to modulate its activity in specific tissues (Topisirovic *et al.*, 2003). Human homeodomain protein 9 (HOXA9) also interacts directly with eIF4E in the nucleus of primary AML blood cells and normal bone marrow cells, where HOXA9 stimulates eIF4E-dependent nucleocytoplasmic export of cyclin D1 and ornithine decarboxylase (ODC) mRNAs. Competition between HOXA9 and PRH for eIF4E binding appears important in maintaining normal cell growth control (Topisirovic *et al.*, 2005).

In human HEK293, HeLa, and primary T cells, 4E-BP3 associates directly with eIF4E in both the nucleus and cytoplasm. 4E-BP3 localization did not change upon LMB treatment, indicating that 4E-BP3 is not transported out of the nucleus *via* a chromosome region maintenance 1/exportin1/Exp1/Xpo1 (CRM)-1-dependent pathway (Kleijn *et al.*, 2002). In complexes with eIF4E, 4E-BP3 acts as a negative regulator of eIF4E-dependent mRNA export, mainly under cellular stress or DNA damage (Chen, Lee & Chang, 2012; Kleijn *et al.*, 2002). In human U2OS and MCF7 cells, altered expression of 4E-BP3 significantly affected cyclin D1 protein levels. 4E-BP3 also affected a subset of growth-promoting mRNAs exported in an eIF4E-dependent manner (Chen *et al.*, 2012). Interestingly, the naked mole rat *Heterocephalus glaber*, a rodent that lives in tunnels under hypoxic conditions, appears not to possess a gene encoding 4E-BP3 (Maldonado & Hernández, 2021). Thus, the role of the eIF4E/4E-BP3 complex in nucleocytoplasmic mRNA transport of cyclin D1 mRNA (and perhaps many other mRNAs) is not conserved across all mammals. Indeed, the *4E-BP3* gene could have been secondarily lost in *H. glaber* due to its adaptation to permanent hypoxia.

Nuclear eIF4E also mediates mRNA export of other mRNAs (Fig. 5). The eIF4E-dependent pathway of mRNA

nuclear export is based on the action of Crm-1, leucine-rich pentatricopeptide repeat cassette protein (LRPPRC), the RNA helicases 56-kDa U2AF-associated protein (UAP56), DDX3, and hnRNPA1, and is LMB-sensitive – features that differ from bulk mRNA export which is mostly based on the protein complexes nuclear cap-binding complex (CBC), tip-associated protein (TAP)/NXF1 or REF/Aly (Topisirovic *et al.*, 2009). In some carcinogenic and normal human cells, including HEK 293, U937, K562, and U2O2, eIF4E interacts with about 3500 mRNAs in the nucleus. In addition, a subset of mRNAs targeted for export, including cyclin D1, cyclin B1, Pim-1, c-Myc, and VEGF mRNAs, contain a ∼50–100-nt element in the 3′-UTR, called the eIF4E sensitivity element (4ESE) (Culjkovic *et al.*, 2005, 2006; Culjkovic-Kraljacic *et al.*, 2016; Topisirovic *et al.*, 2009) and are exported in a Crm-1- and eIF4E-dependent manner. LRPPRC directly binds the 4ESE RNA element, eIF4E, and the nuclear pore receptor Crm1 to perform eIF4E-dependent nuclear–cytoplasmic mRNA export (Topisirovic *et al.*, 2009; Volpon *et al.*, 2017).

(*c*) *Pre-mRNA processing*

Within the nucleus, maturation of pre-mRNAs involves cleavage of the 3′ end by the endonuclease cleavage and polyadenylation specific factor 3 (CSPF3). It was recently discovered that, in human osteosarcoma cells, nuclear eIF4E stimulates 3′-end processing of a subset of mRNAs containing 4ESE in the 3′-UTR (Davis, Delaleau & Borden, 2019). Before nuclear mRNA is exported, eIF4E directly interacts with cleavage and polyadenylation specific factor (CPSF)1 and CPSF3 to make a functional complex with the cleavage and polyadenylation (CPA) machinery. This complex targets 4ESE-containing mRNAs, including myeloid leukemia 1 (MCL1) and cyclin D1 (CCND1) transcripts, and promotes cleavage (Fig. 5). In addition to 3′-end cleavage, for a subset of transcripts, poly(A) tail length and polyadenylation signal (PAS) selection may also be modulated by eIF4E (Davis *et al.*, 2019). eIF4E involvement in pre-mRNA maturation was discovered in oncogenic cells, but its relevance for carcinogenesis remains to be investigated. In addition, whether eIF4E performs these activities in non-cancerous cells remains unknown.

IV. eIF4E IN *DROSOPHILA*

Drosophila has become an important organism in studies of the role of eIF4Es during development. Upon fertilisation, the oocyte forms the embryo, which further gives rise to three consecutive instar larvae, a pupal stage, and finally to the adult. During embryogenesis, the global axes and body patterning are established by the action of several proteins named morphogens.

D. melanogaster possesses seven *eIF4E* genes encoding eight proteins. eIF4E-1 was first purified (Maroto & Sierra, 1989) and cloned (Hernández & Sierra, 1995) from embryos. By alternative splicing, the *eIF4E-1/2* gene encodes eIF4E-1 and eIF4E-2 (Hernández *et al.*, 1997; Lavoie *et al.*, 1996). Subsequently, the genes *eIF4E-3*, *-4*, *-5*, *-6*, *-7*, and *4E-HP* were identified *in silico* (Lasko, 2000), further cloned, and characterised (Hernández *et al.*, 2005). Analysis of genome sequences revealed that *eIF4E* is a single-copy gene across insects (Tettweiler *et al.*, 2012). Strikingly, during the explosive evolutionary radiation of the genus *Drosophila* in the Tertiary, the *eIF4E* locus underwent multiple duplications giving rise to species with three, four, five, six, or seven cognate genes. Moreover, all insects contain a unique *4E-HP* ortholog gene. Apart from *4E-HP*, all insect cognates belong to Class I. No Class III *eIF4E* has been found in this taxon.

All *eIF4E* genes show differential expression patterns throughout development. *eIF4E-1/2* mRNAs strongly accumulate in pole cells at the posterior end of the early embryo, a region that gives rise to the adult germline (Hernández *et al.*, 1997). Later on, mRNAs are highly expressed throughout the life cycle. By contrast, *eIF4E-3*, *eIF4E-4*, *eIF4E-5*, and *eIF4E-7* transcripts are detected only from the third larval instar onwards, and *eIF4E-6* mRNA is barely expressed over the complete life cycle (Hernández *et al.*, 2005). The biochemical properties of all *D. melanogaster* eIF4E proteins are well characterised. Homology modelling and fluorescence binding assays determined that all proteins possess cap-binding ability (Zuberek *et al.*, 2016), albeit with a 14-fold affinity difference among them. eIF4E-3 and eIF4E-4 exhibited the highest and the lowest cap-binding affinities, respectively, and eIF4E-3 and eIF4E-5 bind the second nucleoside of the cap in an unusual manner *via* stacking interactions with histidine or phenylalanine, respectively.

Further studies on gene expression in specific tissues (Graveley *et al.*, 2011) and the characterised roles of each eIF4E cognate in different biological processes are described below. A global picture of the functional diversification of eIF4Es is depicted in Fig. 6. For eIF4Es with no biological role yet known (eIF4E-2, eIF4E-6, and eIF4E-7), Table 2 shows their reported features.

(1) Global translation

The eIF4E-1 protein is the best-characterised isoform in *Drosophila*. It promotes translation *in vitro* (Maroto & Sierra, 1989; Zapata, Martínez & Sierra, 1994), binds eIF4G for eIF4F formation (Hernández *et al.*, 1998, 2004*a*, 2005; Zapata *et al.*, 1994), is detected ubiquitously throughout the complete life cycle (Hernández *et al.*, 2012), and is suggested to support global translation throughout the *Drosophila* life cycle (Hernández *et al.*, 2005).

Functional (Maroto & Sierra, 1989; Zapata *et al.*, 1994) and proteomics (Hernández *et al.*, 2005) analyses of the cap-binding complex in embryos identified eIF4E-1 as the sole protein binding to the cap and promoting translation during this developmental stage. Accordingly, a null mutant of the *eIF4E-1/2* gene (Hernández *et al.*, 2004*b*, 2005) is embryonic lethal and results in the induction of widespread apoptosis in

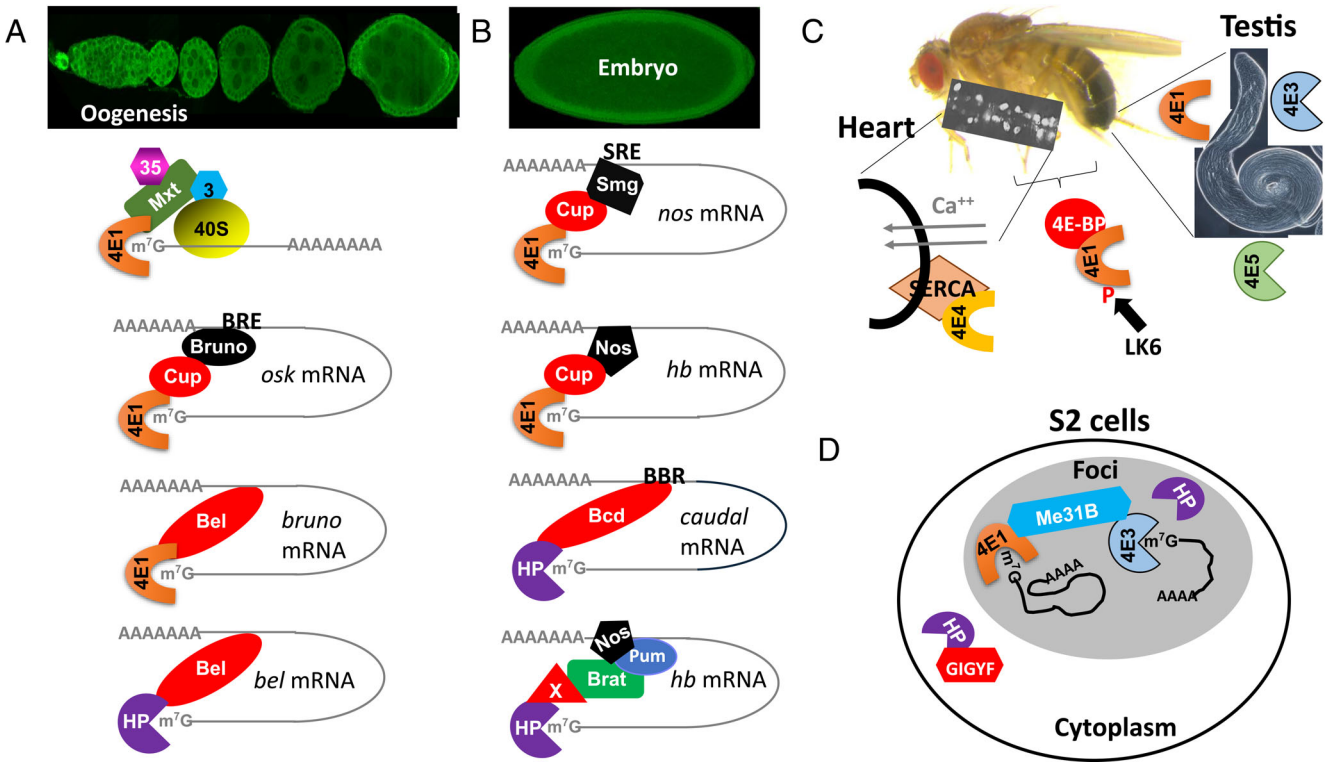

Fig. 6. The roles of eukaryotic initiation factor 4E (eIF4E) paralogs in *Drosophila melanogaster*. eIF4E-1 (4E1) and 4E-HP (HP) bind to specific proteins either to promote or to repress translation during oogenesis (A), embryogenesis (B), in the adult (C), and in cultured S2 cells (D). (A) eIF4E-1 binds the scaffold Mxt to promote cap-dependent, eIF4G-independent translation. eIF4E-1 and 4E-HP bind different proteins to repress the translation of selected mRNAs involved in development. (B) eIF4E-1 and eIF4G drive global translation during embryogenesis (not depicted). eIF4E-1 and 4E-HP bind different proteins to repress the translation of selected mRNAs encoding morphogens to establish body patterning. (C) In the adult, eIF4E-4 (4E4) plays a role in calcium handling in heart cardiomyocytes *via* binding to the ATPase SERCA of the calcium pump. In this process, eIF4E-4 may be regulated by 4E-BP. In testis, eIF4E-1, eIF4E-3 (4E3), and eIF4E-5 (4E5) play a role during spermatogenesis. (D) mRNA storage in cytoplasmic foci of S2 cells. For simplicity, the nucleus is not depicted. 3, eIF3; 35, RNA helicase 35; 4E-BP, eIF4E-binding protein; BBR, Bicoid binding region; Bcd, Bicoid; Bel Belle; Brat, brain tumour; BRE, Bruno response element; GIGYF, Grb10-interacting GYF (glycine–tyrosine–phenylalanine domain) protein; hb, hunchback; LK6, serine/threonine protein kinase 6; Me31B, Maternal expression at 31B; Mxt, Mextli; nos, Nanos; ORF, open reading frame; osk, Oskar; P, phosphorylation; Pum, Pumilio; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; Smg, Smaug; SRE, Smaug response element; X, unidentified protein.

late embryos from stages 10 to 12. Lachance *et al.* (2002) observed that different *eIF4E1/2* mutant alleles arrest growth during early larval development, and some showed embryonic lethality. Delayed lethality and absence of apoptosis in early embryonic stages in some allelic combinations might result from persistence of the eIF4E-1 maternal contribution (Hernández *et al.*, 2004*b*). Altogether, these observations demonstrated that the bulk of translation throughout embryogenesis relies solely on eIF4E-1.

The role of eIF4E-1 phosphorylation in *Drosophila* development has also been addressed (Lachance *et al.*, 2002). *Drosophila* transgenic individuals expressing a non-phosphorylatable Ser251Ala eIF4E-1 mutant in an *eIF4E1/2* mutant background showed reduced viability. These data indicate that eIF4E phosphorylation in Ser251 is biologically relevant and essential for normal growth and development. It was subsequently demonstrated that the kinase LK6 is a functional homolog of mammalian MNK kinases and phosphorylates eIF4E-1 (Arquier *et al.*, 2005; Parra-Palau *et al.*, 2005; Reiling *et al.*, 2005). Confirming the biological importance of eIF4E-1 phosphorylation (Lachance *et al.*, 2002), *lk6* mutants exhibited reduced viability, slow development, and smaller adult size, demonstrating that Lk6 activity is required for organismal growth and development (Arquier *et al.*, 2005; Parra-Palau *et al.*, 2005; Reiling *et al.*, 2005).

The biological relevance of eIF4E-1 in cell growth has also been studied in the context of the developing adult eye, which is composed of a precise number of cells forming a lattice of ommatidia and sensitive chaetae. eIF4E-1 loss-of-function mutants inhibited cell growth but did not affect differentiation (McNeill, Craig & Bateman, 2008). On the contrary, ectopic overexpression of eIF4E-1 led to cell proliferation of the ommatidia, abnormal cell growth, and the presence of extra chaetae (Hernández *et al.*, 2005).

Table 2. Characterised features of *Drosophila melanogaster* eukaryotic initiation factor 4E (eIF4E) proteins with as yet unknown biological roles.

Protein	Proposed biological function	Complements yeast eIF4E?	Known interactors	References
eIF4E-2	Cap-binding activity	Yes	eIF4G 4E-BP Mextli SERCA	Hernández *et al.* (2005, 2013); Lavoie *et al.* (1996); Santalla *et al.* (2022); Zuberek *et al.*, (2016)
eIF4E-6	Cap-binding activity; mRNA enriched in male reproductive organs and third-instar larval imaginal disc; little or no mRNA expression in other tissues	No	Not determined	Brown *et al.* (2014); Graveley *et al.* (2011); Hernández *et al.*, (2005)
eIF4E-7	Cap-binding activity; mRNA highly enriched in male reproductive organs; mRNA also expressed in fat body of pupae and third-instar larval imaginal disc; eIF4E-7 protein is present in the testes forming a complex with Doublefoult, a protein that controls mRNA translation in premeiotic spermatocytes	Yes	eIF4G eIF4G-2 4E-BP Mextli	Brown *et al.* (2014); Graveley *et al.* (2011); Hernández *et al.*, 2005, 2012, 2013; Sechi *et al.* (2019); Zuberek *et al.* (2016)

4E-BP, eIF4E-binding proteins; mRNA, messenger RNA; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase.

Thor, the *Drosophila* homolog of 4E-BPs, negatively regulates eIF4E-1 downstream of the PI3K/Akt/TOR signalling pathway to control cellular and organismal growth in response to environmental, endocrinological, and nutritional inputs (Bernal & Kimbrell, 2000; Flatt *et al.*, 2008; Miron, Lasko & Sonenberg, 2003; Miron *et al.*, 2001). Whether the different eIF4E isoforms are also regulated by the PI3K/Akt/TOR cascade remains to be investigated. The exception is eIF4E-3, a testis-specific *Drosophila* isoform that is essential for spermatogenesis and does not bind 4E-BP (Hernández *et al.*, 2012).

(2) Translational control during development

During the early stages of development, transcription is silent due to chromosome condensation, yet gene expression must still be tightly regulated. Thus, gene expression relies significantly on the translational control of pre-existing mRNAs transcribed maternally and deposited into the egg (Kong & Lasko, 2012; Vazquez-Pianzola & Suter, 2012). During *Drosophila* oogenesis and embryogenesis, most maternal mRNAs are asymmetrically distributed and translationally repressed, becoming activated by the action of various morphogens according to developmental programs (Kong & Lasko, 2012; Lécuyer, Yoshida & Krause, 2009). Coupling localization of mRNAs to translational control provides a mechanism to achieve the spatial and temporal distribution of proteins essential to establishing the embryo axes and body patterning (Kong & Lasko, 2012; Vazquez-Pianzola & Suter, 2012). Local concentrations of the different morphogens are pivotal to establishing the embryo antero-posterior and dorsoventral axes (Kong & Lasko, 2012; Lécuyer *et al.*, 2009).

eIF4E-1 plays critical roles in development. During oogenesis and early embryogenesis, eIF4E-1 interacts with Mextli (Mxt) to form a cap-binding protein complex (Hernández *et al.*, 2005, 2013). Mxt is a scaffold, non-phylogenetically related to eIF4G, that also binds eIF3 and the DEAD-box RNA helicase (DHX) 35 but not PABP. Thus, the eIF4E-1/Mxt interaction drives the formation of unorthodox 48S PIC to perform eIF4G-independent translation. *Mextli* plays a role in oogenesis and early embryogenesis, and functions in germline stem cell (GSC) maintenance during oogenesis (Hernández *et al.*, 2013) (Fig. 6A).

Different metazoan species utilise eIF4E-based mechanisms of translational repression to govern the correct spatial–temporal expression of specific mRNAs. In *Drosophila* development, eIF4E-1 and 4E-HP are crucially involved in these mechanisms, in which they interact with diverse morphogens tethered to the 3′-UTR of selected mRNAs for translational repression. These processes are required for generating protein gradients that are essential for specifying embryo patterning (Kong & Lasko, 2012).

4E-HP cannot interact with eIF4G (Hernández *et al.*, 2005; Zuberek *et al.*, 2016) and thus inhibits translation initiation by out-competing eIF4Es on the cap for a subset of mRNAs. During oogenesis (Fig. 6A), 4E-HP binds the DEAD-box RNA helicase Belle (Bel). The complex 4E-HP/Bel is recruited to the *belle* (*bel*) mRNA cap preventing Bel translation at the anterior oocyte border. Subsequently, *bel* mRNA is transported to the posterior region of the oocyte, where the loss of 4E-HP repression alleviates translation of *bel* mRNA (Yarunin *et al.*, 2011). eIF4E-1 also directly binds Bel to inhibit Bruno mRNA translation at the posterior of the oocyte (Yarunin *et al.*, 2011). When *bruno* translation is alleviated, Bruno interacts with Cup to recruit eIF4E-1 to *oskar* (*osk*) mRNA. Simultaneously, Bruno recognises *osk* mRNA to prevent Oskar (Osk) expression (Nakamura, Sato & Hanyu-Nakamura, 2004; Wilhelm *et al.*, 2003). Altogether, these interactions control the localization and translation of

specific mRNAs determining the oocyte axis required in the female germline for fertility.

Upon fertilization, the oocyte gives rise to the embryo, a stage in which both eIF4E-1 and 4E-HP bind different proteins to repress mRNA translation of selected morphogens governing embryogenesis (Fig. 6B). eIF4E-1 binds Cup, which simultaneously interacts with Smaug tethered on *nanos* (*nos*) mRNA for translation repression at the anterior of the embryo (Nelson, Leidal & Smibert, 2004). These interactions drive abdominal segmentation in the embryo. eIF4E-1 also binds Cup in complex with Nanos (Nos) on sequences in the 3′-UTR of *hunchback* mRNA to promote the formation of the abdomen and posterior structures (Zappavigna *et al.*, 2004). In a different interaction, Bicoid (Bcd) binds the mRNA 3′-UTR of *caudal* (*cad*) mRNA. The simultaneous binding of 4E-HP to the cap of *caudal* mRNA and Bcd blocks *cad* translation at the anterior, thereby generating an antero-posterior protein gradient (Cho *et al.*, 2005). At the posterior of the embryo, 4E-HP also makes a complex with brain tumour (Brat), Pumilio (Pum), and Nos to repress translation of *hunchback* (*hb*) mRNA to form an antero-posterior protein Hunchback (Hb) gradient along the longitudinal axis of the embryo. The opposing gradients of Caudal and Hunchback establish the head and thoracic segments of the fly (Cho *et al.*, 2006; Sonoda & Wharton, 1999, 2001). Phenotype analysis of *4E-HP* null mutants showed that this gene is essential for viability and completion of development (Valzania *et al.*, 2016).

After embryogenesis, *4E-HP* also regulates ecdysone biosynthesis, the major hormone regulating moulting, metamorphosis and reproduction (Valzania *et al.*, 2016). RNA interference (RNAi) experiments targeting *4E-HP* in the prothoracic gland disrupted ecdysone synthesis, blocking the transition from the larval to pupal stages and affecting the expression of steroidogenic enzymes. Thus, *4E-HP*-null larvae showed delayed development, failed to grow, and died (Valzania *et al.*, 2016). The molecular mechanism of 4E-HP in the ecdysone synthesis pathway remains unknown.

4E-HP is widespread across metazoans, plants, and some fungi (Joshi *et al.*, 2005). In the plant *Arabidopsis thaliana*, the worm *C. elegans*, and humans, 4E-HP orthologs bind eIF4G-like proteins and promote the translation of specific subsets of mRNAs (Dinkova *et al.*, 2005; Ho *et al.*, 2016; Ruud *et al.*, 1998; Uniacke *et al.*, 2012). Thus, the evolution of an ancestral and widespread eIF4E into 4E-HP as a translational repressor only took place in *Drosophila* (Hernández, Altmann & Lasko, 2010).

(3) Spermatogenesis

In *Drosophila*, growing evidence shows that, compared to somatic cells, the translation process in undifferentiated, pluripotent cells such as GSCs and primordial germ cells (PGCs) is performed by non-canonical components of the translational machinery (Kai, Williams & Spradling, 2005). Alternative factors include Mextli (Hernández *et al.*, 2013) and the variant ribosomal proteins RPS5b, RPS10a, RPS19b, and RPL22-like (Kong *et al.*, 2019). As discussed below, this is also the case for spermatogenesis.

Spermatogenesis progresses when the stem cells at the testis tip develop towards the basal end, where mature sperm will exit. In brief, male GSCs undergo several rounds of mitosis to generate a cyst of 16 primary spermatocytes. After they grow, they undergo meiosis to form a cyst of 64 haploid spermatids, which progress through cell elongation and individualization to give rise to mature sperm through a process called spermiogenesis (Fabian & Brill, 2012). In the male, translational control is crucial for spermatogenesis (White-Cooper, 2010). Accordingly, mRNAs from the non-canonical isoforms *eIF4E-3*, *eIF4E-4*, *eIF4E-5*, and *eIF4E-7* are highly enriched in the testis (Graveley *et al.*, 2011; Krause *et al.*, 2022). eIF4E-3 and eIF4E-5 proteins are specifically expressed in the testis and, together with eIF4E-1, play crucial roles in different processes to produce mature sperm. Indeed, eIF4E-1, eIF4E-3, and eIF4E-5 proteins are essential for spermatogenesis, and loss-of-function or mutation analyses showed that males lacking eIF4E-1, eIF4E-3, or eIF4E-5 are sterile (Ghosh & Lasko, 2015; Hernández *et al.*, 2012; Shao *et al.*, 2023) (Fig. 6C).

eIF4E-1 is expressed throughout many stages of spermatogenesis. Early expression in the germ cells and the surrounding somatic cyst cells is essential for gonad morphogenesis and germline development. Later, in spermatocytes, eIF4E-1, together with eIF4E-3, regulates chromosome condensation during meiosis and spermatid maturation (Ghosh & Lasko, 2015). eIF4E-3 is expressed in germline cells from the primary spermatocyte stage to the early differentiating spermatids immediately before the onset of spermiogenesis, and is required for cytokinesis and meiotic chromosome segregation and sperm individualization (Hernández *et al.*, 2012). eIF4E-5 localises to the distal ends of elongated spermatid cysts and is required for post-meiotic stages, including spermatid cyst polarisation and sperm individualization (Shao *et al.*, 2023).

eIF4E-1 and eIF4E-3 interact with eIF4G and the non-canonical eIF4G-2 in the testis to drive translation during spermatogenesis (Baker & Fuller, 2007; Franklin-Dumont *et al.*, 2007; Hernández *et al.*, 2012). However, eIF4E-3 appears not to be regulated by 4E-BP in this tissue (Hernández *et al.*, 2012). Recently, the RNA helicase maternal expression at 31B (Me31B) was shown to play an essential role in spermatogenesis (Jensen *et al.*, 2021). Interestingly, Me31B and eIF4E-3 colocalize in S2 cells, the testis stem cells niche, and spermatocytes. Furthermore, both proteins coimmunoprecipitate from testis extracts, providing evidence that both proteins physically interact in this tissue, rendering Me31B a possible negative regulator of translation in spermatogenesis (Layana *et al.*, 2023).

Yeast two-hybrid assay (Hernández *et al.*, 2005; Shao *et al.*, 2023) and fluorescent binding assays with a short eIF4G peptide containing the eIF4E-binding motif (Zuberek *et al.*, 2016) reported positive interactions between eIF4E-5 and 4E-BP, eIF4G, eIF4G-2, 4E-T, and Cup. Thus, eIF4E-1, eIF4E-3, and eIF4E-5 may form various complexes to regulate translation initiation during sperm development.

(4) Heart physiology

In mammals and invertebrates, heart performance relies on a tightly regulated handling of Ca^{2+}, a process in which the ATPase sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) plays a pivotal role (Gambardella *et al.*, 2018). Recent evidence showed that SERCA is regulated in the *Drosophila* heart by 4E-BP during cardiac function (Santalla *et al.*, 2022) (Fig. 6C). Flies over-expressing 4E-BP showed improved cardiac performance in young individuals associated with incremented SERCA activity. Moreover, SERCA interacted with eIF4E-4 in the two-hybrid system. Indeed, eIF4E-4 mRNA and protein are present in the cardiac tissue of adult flies. The specific identification of eIF4E-4 in the heart supports the notion that the interaction of SERCA with elF4E-4 might underlie the cardiac effects observed in 4E-BP-over-expressing flies associated with incremented SERCA activity (Santalla *et al.*, 2022). Thus, eIF4E-4 might be a negative regulator of SERCA. However, whether eIF4E-4 promotes translation in the heart tissue remains unclear.

(5) mRNA storage and decay

eIF4E-based mechanisms of translation repression are also important for mRNA storage and degradation in *Drosophila*. As in other eukaryotes, non-translated mRNAs stored in PBs have been described in *Drosophila*. In this process, Me31B assembles with mRNAs and various proteins, including Exuperantia, CUP, and eIF4E-1, to form translationally repressed mRNPs in GCs and PB (DeHaan *et al.*, 2017; McCambridge *et al.*, 2020; Thomson *et al.*, 2008; Wang *et al.*, 2017). Indeed, Me31B is a key protein for PB assembly (Eulalio *et al.*, 2006), where it interacts with eIF4E-1 and eIF4E-3 in S2 cells (Ferrero *et al.*, 2012; Layana *et al.*, 2023). Remarkably, further biochemical analyses showed that Me31B is the first dual eIF4E-interacting protein, i.e. it binds both eIF4E-1 and eIF4E-3 *via* two eIF4E-binding sites located on different, distantly located protein domains (Layana *et al.*, 2023) (Fig. 6D).

Layana *et al.* (2023) also reported that 4E-HP colocalizes with Me31B in PBs in S2 cells. Accordingly, Ruscica *et al.* (2019) discovered that 4E-HP makes a complex with GIGYF protein to couple repression of mRNA translation with stability in cultured S2 cells. GIGYF acts as a scaffold that recruits Me31B, the decapping activator HPat and the CCR4-NOT deadenylase complex to repress translation, and promote mRNA decay (Ruscica *et al.*, 2019) (Fig. 6D).

V. eIF4E IN *CAENORHABDITIS ELEGANS*

In *C. elegans*, ∼60% of protein-coding mRNAs have an unusual $m_3^{2,2,7}$GpppN (2,2,7-trimethylguanosine) cap, resulting from trans-splicing of the pre-mRNA 5′-UTR. Trans-splicing enhances translational efficiency by shortening the 5′-UTR and weakening secondary structures (Yang *et al.*, 2017). *C. elegans* possesses five eIF4E paralogs, termed IFE-1, IFE-2, IFE-3, IFE-5 (Class I), and IFE-4 (Class II) (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000), which have undergone functional divergence.

IFE-3 is the most closely related isoform to mammalian eIF4E and *Drosophila* eIF4E-1. IFE-3 and IFE-4 bind only the canonical m^7GpppN cap but not $m_3^{2,2,7}$GpppN. By contrast, IFE-1, IFE-2, and IFE-5 proteins bind both m^7GpppN and $m_3^{2,2,7}$GpppN caps (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000). Molecular modelling of 3D structures with mono- and trimethylated caps showed that changing just two amino acid residues can alter the cap discrimination of IFE-5 to resemble that of IFE-3. This change results in a reduction of the cap-binding cavity size (Miyoshi *et al.*, 2002). Their different abilities in recognising two types of caps contribute to the recruitment of selected subsets of mRNAs for translation in different cellular processes (Dinkova *et al.*, 2005; Friday *et al.*, 2015; Henderson *et al.*, 2009; Song *et al.*, 2010). RNAi experiments in *ife* genes showed that *ife-3* is the only one essential for viability. Separate inactivation of any of *ife-1*, *ife-2*, *ife-4*, or *ife-5* showed no embryonic lethality. However, simultaneous inactivation of *ife-1*, *ife-2*, and *ife-5* resulted in 99% embryonic lethality. IFE-4 is most closely related to 4E-HP and is not essential for viability. Indeed, none of the combinations in which *ife-4* was inactivated was harmful to the worms, including the simultaneous inactivation of *ife-4*, *ife-1*, and *ife-5*. Thus, *ife-1*, *ife*-2, and *ife*-5 are partially redundant for viability, but at least one isoform is required for cell survival (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000). As we describe in the following, three IFEs function in the germline (IFE-1, 3, and 5) and one (IFE-4) exclusively in somatic cells. IFE-2 is active in oogenesis and somatic cells.

(1) Global translation

C. elegans is endowed with a single eIF4G gene, *ifg-1*, which encodes two IFG-1 isoforms, namely $4G_L$ (long, or p170) and $4G_S$ (short, p130). *ifg-1* null mutants arrest germ cell proliferation and larval development (Contreras *et al.*, 2008). $4G_L$ possesses an extra N-terminal domain containing PABP- and eIF4E-binding sites absent in $4G_S$. Both isoforms are present in total lysates in equal amounts and are equally distributed in embryos, larvae, adult germline, and adult somatic cells. Interestingly, only $4G_L$ forms part of canonical cap-binding complexes, and thus only $4G_L$ binds all IFE proteins. It is proposed that $4G_L$ sustains global cap-dependent translation and is required for oocyte differentiation. $4G_S$ might sustain cap-independent translation for basic somatic functions (Contreras *et al.*, 2008). Some IFEs promote spatial and temporal translation of selected pools of transcripts. The most studied IFEs are described below.

Analyses of the mRNAs engaged in translation of the strain *ife-1(bn127)* lacking IFE-1 identified a subset of maternal mRNAs encoding proteins required for germ cell differentiation and maturation during oogenesis, fertilisation, and embryogenesis, including *gld-1*, *vab-1*, *vpr-1*, *pos-1*, *pal-1*,

mex-1, and *oma-1* among others. It is hypothesised that IFE-1 is a binding partner of IFG-1 in the cap-binding complex for mRNA translation (Friday *et al.*, 2015; Henderson *et al.*, 2009) (Fig. 7A).

IFE-2 is diffusely expressed in the oocyte. Loss of this protein caused meiotic disorders because of defects in chromosome repair. Accordingly, IFE-2 recruits transcripts involved in meiotic chromosome segregation, such as *msh-4* and *msh-5* (Song *et al.*, 2010). IFE-2 is highly expressed in all somatic tissues of post-embryonic developmental stages and throughout adulthood. RNAi knockdown experiments of this paralog reduced global protein synthesis and protected the animals from oxidative stress. Interestingly, the loss of *ife-2* resulted in a substantial extension of lifespan and further extended the longevity of mutants for other genes controlling lifespan, such as *daf-2*, *clk-1*, and *age-1*, indicating that IFE-2 functions in the same genetic pathway controlling age. Caloric restriction further enhanced the *ife-2* age phenotype. Thus, IFE-2 might perform routine translation from the embryo stage onwards, and a reduction in global translation decreases energy requirements, leading to an extended lifespan (Syntichaki, Troulinaki & Tavernarakis, 2007) (Fig. 7A).

IFE-4 is expressed in somatic tissues and was present in 48S initiation complexes, indicating that it promotes protein synthesis initiation. Furthermore, microarray analysis of polysome-engaged mRNAs isolated from *ife-4*-knockout animals showed that IFE-4 selectively recruits a subset of mRNAs encoding proteins related to egg laying, including *daf-12*, *egl-15*, and *kin-29* transcripts (Dinkova *et al.*, 2005) (Fig. 7A).

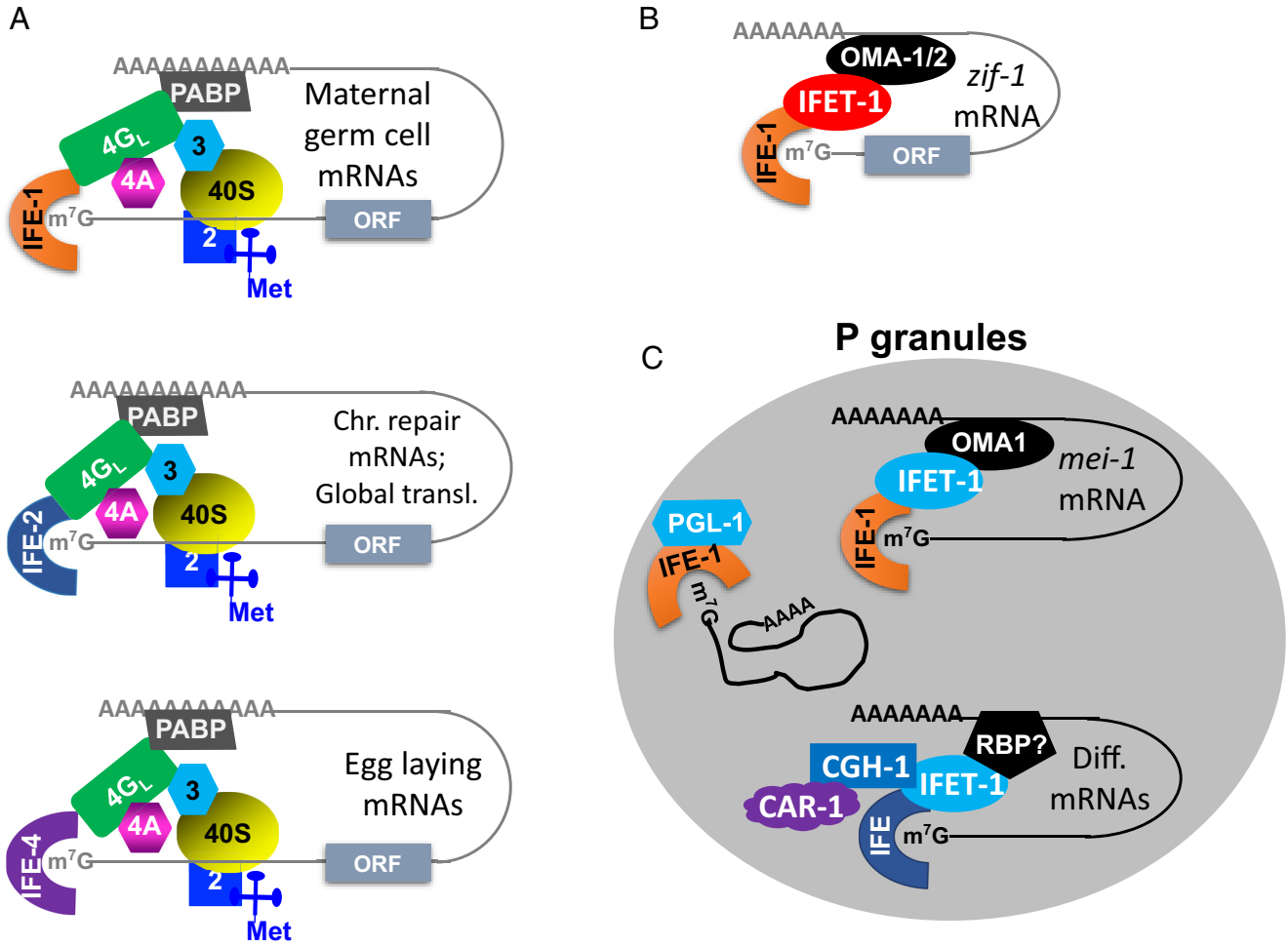

Fig. 7. The roles of eukaryotic initiation factor 4E (eIF4E) paralogs in *Caenorhabditis elegans*. (A, B) Cytoplasmic activities of IFEs. (A) Protein complexes promoting translation. (B) Protein complexes repressing translation. IFE-2 drives the translation of chromosome repair (Chr. repair) mRNAs in the oocyte and performs global and ubiquitous translation (Global transl.) in embryos and adults. (C) mRNA storage within P granules. 2, eIF2; 3, eIF3; 4A, eIF4A; $4G_L$, IFG-1 full length (p170); CAR-1, cytokinesis, apoptosis and RNA protein; CGH-1, conserved germline RNA helicase; Diff, mRNAs encoding proteins involved in cell differentiation; IFE-T1, IFE-transporter-1; mRNA, messenger RNA; OMA-1/2, oocyte maturation defective 1 and 2 proteins; ORF, open reading frame; PABP, poly(A)-binding protein; PGL-1, P-granule-specific protein 1; RBP?, hypothetical RNA-binding protein.

(2) Translational control during development

IFE-1 is key for germline development. It is expressed primarily in the gonad of maturing worms, plays an essential role in late spermatogenesis in both males and hermaphrodites, and also promotes oocyte maturation by translating maternal genes (Amiri *et al.*, 2001; Henderson *et al.*, 2009). IFE-1 has robust expression in spermatocytes, is present in GGs (also termed P granules in this species) in spermatocytes and oocytes, and promotes late spermatogenesis. In addition, IFE-1 is required to complete cytokinesis in secondary spermatocytes and for spermatid maturation. It also recruits germline-specific mRNAs that encode proteins for oocyte or embryo differentiation, including *mex-1*, *oma-1*, *glp-1*, *gld-1*, *pos-1*, *pal-1*, and *rab-7*, among others (Friday *et al.*, 2015; Henderson *et al.*, 2009).

In early oogenesis, transcription is silent in all germline blastomeres. In this stage, the maternally supplied proteins oocyte maturation defective-1 (OMA-1) and -2, together with PIE-1, globally repress transcription by inactivating the RNA polymerase II complex. Degradation of PIE-1 in somatic cells is carried out by a cullin (CUL)-2-containing E3 ligase, a process that activates transcription. The substrate-binding subunit of E3 ligase, zinc finger-interacting factor 1 (ZIF-1), binds to PIE-1. For oocyte maturation, OMA-1/2 also repress translation of *zif-1* mRNA by binding to both the 3′-UTR and IFE-transporter 1 (IFET-1) (formerly called SPN-2 or PQN-45), the *C. elegans* ortholog of 4E-T (Guven-Ozkan *et al.*, 2010). In oocytes, *zif-1* mRNA encodes a subunit of E3 ligase that degrades PIE-1. OMA proteins maintain high levels of PIE-1 protein by binding to and repressing translation of *zif-1* mRNA, thereby preventing PIE-1 degradation, critical for oocyte maturation. Phosphorylation of OMA proteins displaces IFET-1 from the *zif-1* 3′-UTR, releasing translational repression (Guven-Ozkan *et al.*, 2010) (Fig. 7B).

Upon fertilisation, ubiquitin-mediated proteolysis often degrades oocyte proteins that are harmful in the embryo, targeting them for degradation by the 26S proteasome. During oogenesis, the meiotic spindle formation protein-1 (MEI-1)–katanin microtubule-severing complex is involved in the control of meiosis. In *C. elegans*, the CUL-3-based ubiquitin ligase regulates the meiosis-to-mitosis transition of fertilised embryos by triggering the degradation of MEI-1 (Bowerman & Kurz, 2006). In *C. elegans*, P granules are required for oocyte and sperm differentiation and proliferation (Philips & Updike, 2022). Upon oocyte fertilisation, the transition to embryogenesis triggers downregulation of the MEI-1–katanin microtubule-severing complex by a Cullin-dependent degradation pathway. One mechanism to repress MEI involves translational repression of *mei-1* mRNA by IFET-1 localised to the cytoplasm and P granules. IFET-1 binds to both IFE-1 and OMA-1 tethered to the *mei-1* 3′-UTR (Li *et al.*, 2009) (Fig. 7C).

Sengupta *et al.* (2013) showed that interaction with IFET-1 is required in gonadal perinuclear P granules to control the translation and storage of various maternal mRNAs for normal oocyte development (Fig. 7C). The different mRNAs include *puf-5*, *rme-2*, *lip-1*, *pal-1*, *snp-4*, *mex-3*, *daz-1*, and *pin-1*. After transitory retention in P granules, mRNAs are released into the cytoplasm for translation. In P granules, the complex IFET-1 functions alongside the translational regulators CGH-1 (the homolog of Me31b/Rck/p54 RNA helicase), CAR-1, and PATR-1 (Sengupta *et al.*, 2013). The authors hypothesised that this IFET-1 action requires interaction with an IFE paralog. In this model, specificity for any given mRNAs would be caused by the simultaneous association of IFET-1 with any IFE and a sequence-specific RNA binding protein (RBP) tethered to the target mRNAs (Sengupta *et al.*, 2013). However, other mechanisms of translational repression are also possible.

In P granules, IFE-1 associates with the P granule-specific protein 1 (PGL-1) found in spermatocytes and oocytes that store maternal/paternal mRNAs determinant for germline function. It is speculated that sequestering IFE-1 within P granules might regulate the level and availability of IFE-1 during spermatogenesis (Amiri *et al.*, 2001).

IFE-3 plays an important role in germline sex determination (GSD), where it promotes oocyte cell fate. IFE-3 is expressed throughout the germline, localises to GGs, and facilitates oocyte growth and viability (Friday *et al.*, 2015; Henderson *et al.*, 2009; Huggins *et al.*, 2020). Maternal *ife-3* is essential for body growth and embryogenesis, and promotes the transition from spermatogenesis to oogenesis in the germline (Mangio, Votra & Pruyne, 2015).

VI. TOWARDS A NEW CLASSIFICATION OF eIF4ES ACROSS EUKARYOTES

The first classification of eIF4Es was based on variations in the residues equivalent to Trp-43 and Trp-56 of human eF4E (Joshi *et al.*, 2005). This classification grouped these proteins into three classes: Class I contain both Trp residues; Class II contain Tyr, Phe, or Leu at the first position and Tyr or Phe at the second position; and Class III contain Trp at the first position and Cys or Tyr at the second position. However, due to the presence of various insertions, deletions, and residue changes at key functional positions, eIF4E sequences from the most recently studied taxonomic groups (all protists), including dinoflagellates, ciliates, heterokonts (Roy *et al.*, 2018; Jones *et al.*, 2015; Jagus *et al.*, 2012), and the kinetoplastids *Trypanosoma* and *Leishmania* (Freire *et al.*, 2011, 2014, 2017; Jagus *et al.*, 2012) do not fit into this classification.

Dinoflagellate eIF4Es are now classified into three alternative clades (eIF4E-1, eIF4E-2, and eIF4E-3) divided into nine sub-clades (Jagus *et al.*, 2012; Jones *et al.*, 2015; Roy *et al.*, 2018), whereas the ciliate and heterokont proteins are included only in clade eIF4E-1 (Jones *et al.*, 2015; Roy *et al.*, 2018). Clade eIF4E-1 proteins contain extended sequences between Trp-72 and Trp-73, and Trp-130 to Trp-166 compared to eIF4E-2 and -3. Clade eIF4E-1 and

Clade eIF4E-2 proteins have a Tyr substitution at the position equivalent to Trp-56 in human Class I eIF4E. The Arg position at 112 has been substituted with a His in Clade eIF4E-1 and with a non-conserved Cys in Clade eIF4E-3 proteins. Clade eIF4E-1 and eIF4E-3 proteins possess conserved Arg-157 and Lys-162 of human eIF4E Class I that are involved in binding the cap pyrophosphate. Clade eIF4E-2 have conserved Arg-157 and Lys-162 of human eIF4E Class I (Jones *et al.*, 2015; Roy *et al.*, 2018). The ability of dinoflagellate, ciliates and heterokonts eIF4Es to bind cap and different 4E-IPs (including eIF4G orthologs) has not been experimentally investigated.

Kinetoplastids *Trypanosoma* and *Leishmania* eIF4Es are classified into three groups (Freire *et al.*, 2011, 2014, 2017). Group 1 (eIF4E-1 and -2) are found at low levels and have a similar size and high homology to human eIF4E, but cannot form eIF4F-like complexes nor perform general translation. Group 2 (eIF4E-3 and eIF4E-4) consists of more abundant proteins containing long N-terminal extensions that show low homology with the human eIF4E sequence. They form eIF4F complexes involved in general translation and are essential for cell survival. Group 3 (eIF4E-5 and eIF4E-6) are very small proteins with low homology to human eIF4E, are able to bind to eIF4G homologs to form novel eIF4F-like complexes, but are possibly not involved in general translation (Freire *et al.*, 2011, 2014, 2017).

Taken together, these observations make evident the need for a new classification of eIF4Es that includes the highly divergent protist and recently discovered giant virus proteins. Moreover, eIF4Es from the different phyla of the Fungi and from many protist lineages, remain totally unknown. The new classification might be based on functional aspects of eIF4Es, such as protein–protein interactions and the eIF4E interaction surface, rather than possessing specific amino acids.

VII. CONCLUSIONS

(1) eIF4E has emerged as a versatile wildcard in the regulation of different processes of RNA metabolism in which the role it plays is determined by a partner protein (4E-IPs). To date, about 50 different eIF4E partner proteins have been described in disparate taxa, most of them in metazoans (Hernández, 2022).
(2) The promiscuity of eIF4E for recurrent association with different proteins has fuelled its functional diversification in RNA metabolism throughout metazoan evolution. The lack of phylogenetic relationships among most 4E-IPs and the existence of some of them only in specific lineages supports this notion (Hernández *et al.*, 2010, 2016).
(3) An ancestral eIF4E might have emerged early in eukaryotic evolution as a cap-binding protein with no specificity for mRNA translation, transport, or storage, but rather as a multifunctional, highly versatile, and promiscuous protein able to bind many partner proteins to accommodate different processes of RNA metabolism.
(4) eIF4E, eIF4E-1, and 4E-HP are multi-tasking proteins in humans and *Drosophila*, where their involvement in multiple different processes results from association with many 4E-IPs. By contrast, other eIF4E paralogs are specialised to function in specific tissues or developmental stages to recruit pools of selected mRNAs.
(5) Most nuclear activities of the eIF4E paralogs also have been found in humans. Thus, research in other species could lead to a better understanding of eIF4E roles in the cell nucleus across eukaryotes, with the potential for novel functions to be discovered. Indeed, eIF4E-1 or -2 might be involved in intron splicing reactions in *Drosophila* (Graham *et al.*, 2011).
(6) Novel eIF4E activities and 4E-IPs may be found in the future in scarcely explored taxa, such as multicellular fungi and protists.
(7) A new classification of eIF4E paralogs across eukaryotes is needed, to be able to accommodate all sequences from the most recently studied taxonomic groups (mostly unicellular species). This new classification should be based on functional aspects of eIF4Es, such as protein interactions and the cellular processes in which eIF4E paralogs are involved, rather than the possession of specific amino acids.

VIII. ACKNOWLEDGEMENTS

G. H. was supported by the intramural funding program of the National Institute of Cancer (Instituto Nacional de Cancerología, INCan), Mexico. The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be considered a potential conflict of interest. We thank Paola Ferrero for images of the *Drosophila* heart and Alison Cooper for proofreading the manuscript. We also thank the anonymous reviewers for their valuable criticism and insightful comments that significantly improved this review.

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(*Received 9 February 2023; revised 1 July 2023; accepted 25 July 2023; published online 8 August 2023*)