

**THE DISTINCT ROLES OF TWO eIF4E ISOFORMS AND THEIR COGNATE 4EIPS IN
THE GERMLINE OF *C. elegans***

BY

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Biological information becomes functional at the step of mRNA translation when the protein product is synthesized. Translational regulation of mRNAs is critically important for proper modulation of gene expression in germ cells, gametes, and embryos. The ability of the nucleus to control gene expression in these systems may be limited due to spatial or temporal constraints, as well as the breadth of gene products they express to prepare for the rapid development that follows. During development, germ granules are hubs of post-transcriptional regulation of mRNAs. They assemble and remodel messenger ribonucleoprotein (mRNP) complexes that control translational repression and activation. Recently, mRNPs have been appreciated as discrete regulatory units, whose function is dictated by the many positive and negative acting factors within the complex. Repressed mRNPs must be remodeled and activated for translation on ribosomes to introduce novel proteins into germ cells. The eIF4E:eIF4E-interacting protein (4EIP) node controls many aspects of mRNP fate including localization, stability, poly(A)-elongation, deadenylation, and translational

activation/repression. Furthermore, plant and animal species have evolved to express multiple functionally distinct eIF4E and 4EIP variants within germ cells, giving rise to different modes of translational regulation. Here we investigate the physiological and translational functions of two distinct eIF4E isoforms, IFE-1 and IFE-3, and their cognate 4EIPs (PGL-1 and IFET-1 respectively) in the germline of the nematode *C. elegans*. We find that deficiencies in IFE-1 and IFE-3 result in unique terminal phenotypes, suggesting non-redundant functions of these two closely related proteins. Each isoform contributes to development by regulating the translation of a unique pool of mRNAs. We provide biochemical evidence that IFE-1 and IFE-3 reside in discrete mRNPs with their cognate 4EIPs, that have P granule and P body related functions respectively. Additionally, we find that these 4EIPs control the localization of their respective eIF4E and likely their differential translational functions.

**The Distinct roles of two eIF4E isoforms and their cognate 4EIPs in the germline
of *C. elegans***

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East Carolina University

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Doctor of Philosophy in Biochemistry and Molecular Biology

by

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August, 2020

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CHAPTER I

BACKGROUND

The contents of this chapter are adapted from Huggins and Keiper, Frontiers in Cell and Developmental Biology, doi: 10.3389/fcell.2020.00562, Published 7 July 2020.

Introduction

Post-transcriptional regulation of mRNAs is frequently used to modulate gene expression throughout development in all plants and animals [1]. Particularly during cellular growth and differentiation, chromosome dynamics and/or spatial and temporal constraints may limit the ability of transcriptional regulation alone to adequately govern gene expression [2, 3]. This is especially true of germ cells, embryos, and plastic neurons. As a result, steady-state mRNAs levels often correlate poorly with their protein products in developing tissues [4]. The question that arises is: How do germ cells selectively determine which mRNAs are repressed, degraded, or activated for translation in a spatial and temporal manner? Activation will ultimately produce the functional gene product and thus could be considered the more critical step in gene expression. Current models suggest that RNA-regulatory networks composed of RNA-binding proteins (RBPs), small non-coding RNAs, and translation initiation factors (eIFs) regulate nearly all aspects of mRNA metabolism [1, 5]. Yet it remains unclear how these regulatory networks coordinate translational repression/activation switches of individual mRNAs in response to developmental stimuli. Failure to coordinate the translation of mRNAs encoding the many, sometimes opposing, signals results in aberrant

development [6]. The beginning of an answer appears to be that mRNAs are assembled into specialized messenger ribonucleoprotein (mRNP) complexes as they transit from the nucleus. The assembled mRNP controls most aspects of the transcript's life, including transport, localization, storage, translation, and stability. These protein-RNA complexes are hypothesized to be the discrete regulatory units of germline transcripts. Appropriate regulation is accomplished by remodeling mRNPs in germ granules or in the cytoplasm (see below). This broader model challenges earlier notions that focused on individual components of the complex, and their solitary roles in repression or decay. This chapter will provide a brief overview of some regulated mRNPs in various developmental models and draws parallels for their regulation between each organism. Although the individual mRNAs or their regulators may be different between systems, some common functional themes begin to emerge.

Translational repression of mRNAs by RBPs has been studied extensively; most bind to sequence specific elements within 5' or 3'-UTRs to interfere with ribosome recruitment by blocking the translation initiation machinery or to recruit the Ccr4-Not1 deadenylase complex causing shortening of the poly(A) tail [7-9]. The latter is generally accepted as the first step in targeted mRNA decay. Other RBPs, like PUF (Pumilio and FBF) family members, can also interact with components of the small-RNA pathway to interfere with the elongation step of protein synthesis by mechanisms that are not yet well understood [10]. By contrast, a few RBPs act positively for expression. The Dazl group of proteins have been shown to stimulate the translation of their mRNA targets either directly or indirectly, by their interaction with poly(A)-binding protein [11]. RBPs can therefore be thought of as modular regulators that recruit other factors to assemble

more complex functional mRNPs. The composite mRNPs may have very different functions depending on cellular context, specific sequences within the mRNA, or association of other trans-acting factors.

The functions of small-RNA pathways on post-transcriptional control have also been well characterized, where they have been shown to mediate mRNA turnover, as well as inhibit translation through multiple mechanisms [12 , 13, 14]. Broad regulation of post-transcriptional gene expression by small RNAs is beyond the scope of this chapter, but a comprehensive description of these mechanisms during germline development is found in [15]. Briefly, most miRNAs imperfectly match their targets and regulate gene expression post-transcriptionally by inhibiting translation or by initiating mRNA turnover via decapping and deadenylation [16]. The mechanisms by which siRNAs induce gene silencing differ from those of miRNAs; siRNAs match their targets perfectly and use distinct Argonats to induce endoribonucleolytic cleavage of their targets *i.e.* “slicing” [17]. piRNAs interact with the PIWI class of Argonaut proteins and aid in distinguishing self from non-self (transposable element repression), transcriptional regulation, mRNA regulation, and transgenerational inheritance by similar “slicing” mechanism as those described for siRNAs [18]. Interestingly, during *Drosophila* embryogenesis, transposon-derived piRNAs bind imperfectly to *nos* mRNA in an mRNP involving Aubergine, Smaug, and eIF4E complexes to initiate Ccr4-Not1 mediated deadenylation [19] (see below).

Regardless of the repression mode, translational activation of the target mRNA must follow in order to introduce novel proteins and their functions, particularly in differentiating germ cells. For any developing cell type this is arguably the more critical

step of post-transcriptional gene expression. Eukaryotic translation initiation factors (eIFs) play a fundamental role in the activation step via their ability to recruit the ribosome to repressed or stored mRNPs. Recent studies have begun to shed light on their unique roles in RNA-regulatory networks across diverse phyla. A model has emerged in which eIF4 factors reside in such mRNPs in both their repressed and active states (Fig. 1.1). Data from metazoan and plant developmental systems are consistent with this model showing multiple eIF4 group members participate in mRNP remodeling and coordinate activation/repression switches that drive selective mRNA translation [20-29]. This chapter focuses on known roles for isoforms of the mRNA cap-binding protein eIF4E, and cognate binding partners, the eIF4E-interacting proteins (4EIPs) in regulating mRNP localization, storage, turnover, and translational activation in germ cells and embryos (Fig. 1.1). Recent studies show that germ cells often express multiple isoforms of eIF4E and 4EIPs [23, 29]. Over the course of evolution, each has adopted specialized function in regulating translation during development. This review seeks to outline the contribution of eIF4E:4EIP complexes in germ cells and embryos that control translational repression/activation switches. Such switches are vital for the progression of germ cells throughout meiosis, for example [25, 28]. For clarity, the nomenclature 4E-interacting protein (4EIP) is used for all eIF4E-sequestering proteins other than the canonical 4EBPs 1 and 2 that are released upon phosphorylation by mTOR kinase. However, the 4EIPs may also regulate cap-dependent initiation, but in response to developmental rather than nutritional cues. A comprehensive understanding of how mRNP remodeling mechanisms are integrated within the cell to drive selective protein

synthesis and cell fate decisions is critical to understanding the regulation of gene expression in germ cells.

eIF4 and regulation of cap-dependent translation

Canonical members of the eIF4 complex are the first to bind mRNAs at the 5' end and include: the m⁷Gppp cap-binding protein eIF4E; the scaffold protein eIF4G, which supports mRNA circularization via interactions with eIF4E and poly(A)-binding protein (PABP), while also bridging eIF4 to the ribosome and recruiting eIF4A, the DEAD-box RNA helicase used to unwind secondary structure [30]. Additionally, eIF4B stimulates eIF4A activity although it is not part of eIF4 *per se* [31]. These factors assemble on mRNAs (that presumably are part of preformed mRNPs) and stimulate translational activation by recruiting the 43S activated small ribosomal subunit to form the 48S preinitiation complex (PIC). Subsequent 5' to 3' scanning of the untranslated region and recognition of the AUG start codon, followed by 60S subunit joining leads to productive protein synthesis. Initiation of most cellular mRNAs relies on recognition of the 5' m⁷Gppp cap and therefore the eIF4E:eIF4G interaction. mRNAs that show strong cap-dependence (CD) are those with long and/or complex 5'-UTRs, and consequently rely on the complete, intact eIF4 complex for robust translation [32]. Because 48S PIC assembly is rate-limiting for translation, initiation rates of these structurally complex mRNAs benefit from eIF4A helicase activity as well as the circularization induced by eIF4E:eIF4G:PABP interactions between 5' and 3' ends of the message (Fig. 1.1). It is now accepted that circularization leads to more efficient re-initiation and ribosome recycling following termination [33, 34]. Furthermore, eIF4E stimulates eIF4A helicase activity by binding an inhibitory region in eIF4G, indicating that eIF4E provides multiple

activities within the eIF4 complex to stimulate translation of mRNAs with complex 5' UTRs [35]. Other less complex mRNAs and those containing an internal ribosome entry site (IRES) translate cap-independently (CI) by binding eIF4G/eIF3 and the 43S PIC directly via conserved sequences or structural motifs [36]. Internal binding mechanisms effectively circumvent the need for eIF4E-mediated recruitment; in many cases the eIF4E-binding region of eIF4G is cleaved off or missing in the CI mechanism. Importantly, the CI initiation mechanism is also utilized in animal germ cell development. However, it is beyond the scope of this review, but has been reviewed elsewhere [37].

The balance between CD and CI synthesis at the eIF4E:eIF4G node is regulated by nutrients in somatic tissues by target of rapamycin (TOR) kinase via phosphorylation and inhibition of canonical eIF4E-binding proteins (4EBPs), which were among the first 4EIPs discovered but have a much narrower definition, and will not be the primary focus of this review [38]. Regulation of this node is consistent with the downstream effects of TOR activity, which simulates proliferation and differentiation via eIF4E-sensitive mRNAs like cyclins, VEGF, and Myc [39, 40]. TOR also contributes to global protein synthesis by phosphorylating p70 S6 kinase (S6K), which stimulates translation by phosphorylation and activation of RPS6 and eIF4B [41]. Hypo-phosphorylated 4EBPs compete with eIF4G for binding the dorsal surface of eIF4E, through a structurally conserved alpha-helix motif (Tyr-X₄-Leu-Φ: where X is variable and Φ is hydrophobic) [38]. Additional non-canonical eIF4E-binding motifs like those found in *Drosophila* 4EIPs (Thor, Cup, and 4ET) recognize the lateral surface of eIF4E, displacing eIF4G in preformed eIF4E:eIF4G complexes [42]. Thus, eIF4E-sequestering proteins are considered “broad-scale” translational repressors that disrupt eIF4E:eIF4G

interactions/eIF4 formation and subsequent CD protein synthesis. However, translational control by TOR may also performs an mRNA-selective role in mammalian germ cells. Studies in mouse spermatogonia have shown that mTOR is required for differentiation and proliferation, presumably due to its activity in the retinoic acid (RA)-induced translational activation of mRNAs encoding KIT, SOHLH1, and SOHLH2. This provides an example of the translational machinery responding to a developmental stimulus (e.g. RA) by activating translation of distinct mRNAs [43]. Whether or not translation of these mRNAs relies on mTORs ability to inhibit 4EBPs *per se* and promote CD synthesis remains unclear but seems likely. These regulatory paradigms involving eIF4E and 4EBP provide the cell with the ability to rapidly alter the ratio of CD to CI translation, and therefore selectively determine which pools of mRNA have a competitive advantage for being translated on ribosomes. The ability of a particular 4EIP to inhibit translation initiation via disruption of the eIF4E:eIF4G interaction should be addressed individually, but a number of 4EIPs, like *Drosophila* Cup, are able to compete with eIF4G for eIF4E binding and inhibit protein synthesis (see below). Whether or not certain 4EIPs are TOR regulated is largely unknown, yet remains an interesting question.

To add to the complexity to the broad CD vs CI translational control outlined above, gene duplication events over evolutionary time have given rise to multiple isoforms of eIF4E and 4EIP in both plant and animal species [44-47]. The evolution of reproductive schemes and embryonic development leading to diverse tissues in complex animals and plants appears to have taken advantage of these diversified isoforms for developmentally relevant translational control. eIF4E and 4EIP isoforms are often

expressed in a tissue specific manner, and null mutation analysis in several species have shown that they have distinct non-redundant roles during development, particularly in germ cells [29, 46-51]. Considering that these factors were classically thought to catalyze generalized mRNA initiation that allowed cells to maintain homeostatic levels of general protein synthesis, it is interesting that recent investigations show their unique developmental functions. Several interesting observations come out of the phenotypes derived from deficiencies in eIF4E and 4EIP genes in worms. One might expect that for proteins whose functions antagonize one another, opposite phenotypes should arise. Quite the contrary, the phenotypes caused by loss of each partner in the eIF4E:4EIP pair are remarkably similar, and each pair appears to facilitate a different developmental process [29, 52-54]. In *C. elegans*, multiple eIF4E isoforms (IFE-1 – IFE-5) are expressed in a tissue specific manner and each has unique developmental functions [44, 49, 50, 52]. The two major germ cell isoforms, IFE-1 and IFE-3, have distinct roles in translational regulation of unique subsets of mRNAs [25, 29, 48]. By measuring translation of mRNAs in worms lacking either IFE-1 or IFE-3, it was found that IFE-1 is critical for translating specific mRNAs related to oocyte maturation (*pos-1*, *vab-1*, *mex-1*) and mRNAs involved in spermatocyte cytokinesis [25, 48]. In fact, *ife-1* mutant animals display defects in oocyte maturation and in sperm division, linking its biochemical function to phenotype [25, 48]. IFE-3 on the other hand, is the canonical eIF4E-1 isoform from worms, yet is responsible for the translational repression of some germline sex-determination (GSD) mRNAs (*fog-1*, *fem-3*, *daz-1*), as observed by polysome profiling [29]. Importantly, this repression similarly relied on the IFE-3 cognate 4EIP, IFET-1 (4ET/Cup homologue) suggesting that together they participate in a

repressive mRNP that regulates this subset of mRNAs [29]. Importantly, both *ife-3* and *ifet-1* mutant worms show major defects in germ cell sex-determination, again linking their biochemical functions to their mutant phenotypes [29, 53]. Interestingly, IFE-3 was found associated with the *C. elegans* Tudor domain protein TOFU-6 in 21U piRNA-generating mRNPs [55]. Whether or not IFE-3 represses GSD mRNA translation via piRNA biogenesis remains to be determined. Similarly, studies from *Drosophila* show that eight distinct eIF4E isoforms are expressed in diverse tissues [46]. Each of these binds m⁷Gppp caps, however, eIF4E-6 and -8 bind with significantly lower affinity, due to conserved and non-conserved substitutions in the cap-binding region [46]. Fly eIF4E-8 was found to be most similar to human and mouse eIF4E-2/4EHP type that was predicted to be unable to bind eIF4G and 4EIP [46]. However, eIF4E-8 was shown to participate in a repression complex on Caudal mRNA by binding to the 5' cap structure and the regulatory 3'-RBP Bicoid [56]. This demonstrates an alternative eIF4E:4EIP mRNP repression mechanism that contributes to Caudal asymmetry in the egg. Expression of multiple eIF4E and 4EIP isoforms being co-opted during evolution to regulate translation of distinct mRNAs during germ cell development is not limited to nematodes and flies. Studies from vertebrate models, most notably mice and *Xenopus*, show that multiple eIF4E isoforms are expressed and exhibit similar use of eIF4Es and 4EIPs to regulate the spatial and temporal translation or degradation of specific mRNAs via functional mRNP complexes (discussed below).

Figure 1.1

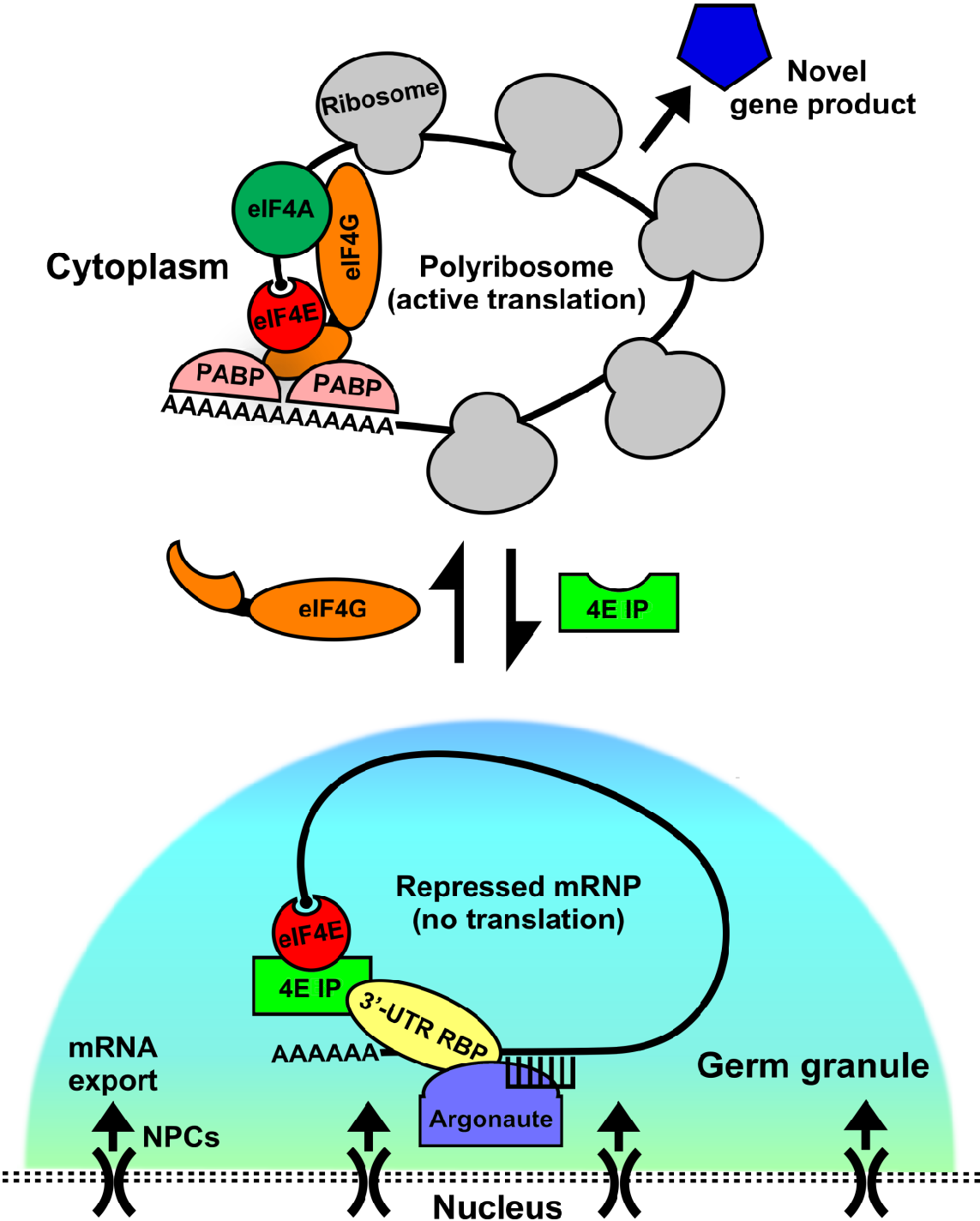


Figure 1.1. Germ granules as assembly sites for eIF4E:4EIP mRNP complexes to direct repression and activation of translation. In germ cells, mRNAs are exported out of the nucleus through the nuclear pore complex (NPC). As they exit they come under control by the germ granule environment. These conserved perinuclear structures act as hubs of post-transcriptional gene regulation, where they act as sites for the assembly and remodeling of diverse messenger ribonucleoprotein complexes (mRNPs). Germ granules are primarily sites of translational repression, as they do not contain ribosomes. However, recent evidence suggests that both repression and activation events may be set up here as a manner of mRNA sorting or licensing. Translation factors like eIF4E reside in complexes both within (repressed) and outside (activated) of the granule, potentially giving it a dual role. When eIF4E is in germ granules it binds to its cognate eIF4E-interacting protein (4EIP) in a complex with sequence specific 3'-UTR binding proteins. This complex prevents cap-dependent translation initiation by preventing eIF4G binding to eIF4E. Little is known about how the “decision” to activate translation is made, but our models suggests that a handoff from the granule to the cytoplasm occurs by mRNP remodeling. 4EIP is displaced by eIF4G and leads to initiation and recruitment to polyribosomes. Other factors participate in the repression to activation switch, like helicases (eIF4A, VASA, *etc.*) that unwind mRNA secondary structure, poly(A)-polymerases (PAP) that extend 3'-poly(A) tails, and poly(A)-binding protein (PABP), which both protects transcripts and enhances translation via its interaction with eIF4G. Activating a repressed mRNP is the first step in producing novel gene products that can dictate the fate and function of germ cells.

Importance of germ granules in translational control

Germ granules are conserved large mRNPs that act as hubs of post-transcriptional regulation in the germline of most metazoans studied [57]. They represent an environment in which mRNAs exiting the nucleus come under stringent post-transcriptional regulation before entering the bulk cytoplasm. Additionally, mRNPs that form in the cytoplasm may transit to germ granules for remodeling and to adopt appropriate post-transcriptional control. Germ granules contain mRNAs that are important for development, small-RNAs that regulate message silencing, and RBPs that control storage, stability, and ultimately translation of resident transcripts [58]. Importantly, germ granules in *C. elegans* appear distinct from P bodies, whereas in mouse spermatogonia evidence points to the structures being more closely related [59-61]. Thus, the overlap in functions and associated factors one might expect when comparing P bodies to germ granules is species-dependent. It is generally recognized that P bodies and stress granules have major functions in mRNA turnover and translational stalling, respectively, while germ granules have a much broader role in regulating the exit of transcripts from the nucleus, interaction with small-RNA pathways, mRNP remodeling, and maintenance of germline integrity. Recently, an additional germ granule called a Z granule has been identified in *C. elegans* that is distinct from P granules and has unique functions in transgenerational epigenetic inheritance [62]. It is likely that many or all of these granules have substantial crosstalk in the regulation and maintenance of mRNP metabolism in germ cells. The first insights into germ granule structure showed that where they are electron dense, non-membrane bound organelles that associate with the nuclear periphery and nuclear pore complexes (NPC) [63, 64].

Later advances in fluorescent microscopy led to a better understanding of the highly dynamic nature of these germ granules [65]. We now know that they are liquid-liquid phase separating condensates, which can exchange components rapidly with the cytoplasm [66]. It is thought that germ granules act broadly to control formation, remodeling, and shuttling of smaller nuclear and cytoplasmic mRNPs so that they may come under the appropriate translational regulation (Fig. 1.1). Conserved components of germ granules that regulate post-transcriptional regulation of mRNAs include: RNA-helicases (Vasa and related DEAD box proteins), RISC (RNA-induced silencing complex) components like AGO1/CSR-1 (Argonaute), 3'-UTR RBPs that generally repress translation but may also activate translation (Nanos, Pumilio, and CPEB), eukaryotic initiation factors (eIF4E, eIF5B), and 4EIPs (4E-T, Cup, Maskin, PGL-1, Bicoid) [67]. This collection of factors uniquely equips germ granules with the ability to regulate the diverse fates of developmentally important mRNAs as they are shuttled into the cytoplasm. While most of the RBPs take part in repression or turnover, the eIFs are known to activate translation. Importantly, the absence of detectable rRNAs or ribosomal proteins suggests that they lack ribosomes and no active protein synthesis occurs within the granule [68, 69]. Therefore, a simple model for germ cell translational control is that the granule to cytoplasm exchange sets up translational repression/activation switches, where repression happens in the granule and mRNAs that are activated for translation leave the granule as activated mRNPs and join with ribosomes (Fig. 1.1). Likewise, it is conceivable that the inverse transit occurs to sequester populations of mRNPs whose expression must be quenched for subsequent development. Such a model then begs the question of whether factors are used for both

repression and activation, and how either function is selected. Furthermore, what mechanisms exist to ensure these functions occur in the correct spatial and temporal manner in the intricate steps of germ cell and embryo development. It is clear from recent studies, however, that eIF4Es and 4EIPs are integral to this process of activation and repression [29, 70-72].

Germ granules and RBPs have been extensively studied in the nematode, *C. elegans*. One constitutive component of the so-called “P granule” is a self-associating RGG-motif RBP named PGL-1 [54]. PGL-1 is thought to be involved in the repression of germ cell mRNAs. It enhances the translational silencing of mRNAs bound to the PUF protein FBF-2, indicating that repressive 3'-UTR-RBPs can use PGL-1 as a cofactor to repress translation [73]. PGL-1 is also the non-canonical cognate 4EIP of IFE-1 [52]. The sequestration of an eIF4E isoform indicates yet another way that RBPs in germ granules control the translation of distinct mRNAs, by inhibiting the CD translational machinery itself. PGL-1 maintains a close functional relationship with IFE-1; it dictates the localization of this eIF4E isoform to perinuclear granules in early germ cells, causes its migration during oocyte meiotic maturation, and releases IFE-1 from granules in late spermatocytes [29, 52]. We have suggested that PGL-1 helps IFE-1 to locate and bind its target mRNA, and simultaneously prevents IFE-1 from binding to eIF4G to initiate translation [26], just as canonical 4EBPs are known to do in other systems [21, 74, 75]. It is clear that PGL-1 sequesters a substantial portion of IFE-1 from the bulk cytoplasm in early germ cells, and releases it later in gametogenesis. Although direct competition of IFG-1 (eIF4G) and PGL-1 for IFE-1 has not been demonstrated, PGL-1 is not found in polyribosomes, unlike IFE-1 and IFG-1 [26]. There is some suggestion that IFE-1 may

also be involved regulation of spermatogenic mRNAs with Argonautes like CSR-1, ALG-3, and ALG-4 (Fig. 1.1), though it is unclear if these are linked or sequential steps in mRNA licensing [76-78].

eIF4E:4EIP complexes distinctly influence differentiation

One of the clearest examples of the link between granule dynamics, translational control, and germ cell differentiation occurs in the latter stages of *C. elegans* germ cell development where multiple eIF4E:4EIP complexes control translation of distinct mRNAs and control cell fate. During the latter stages of spermatogenesis the 4EIP PGL-1 is rapidly degraded in secondary spermatocytes, releasing IFE-1 from the P granule into the bulk cytoplasm [29, 79]. There IFE-1 (and its cargo mRNAs) have the opportunity to associate with ribosomes and activate translation [25, 48]. Importantly, IFE-1 is the only eIF4E isoform in *C. elegans* that binds PGL-1, indicating that other eIF4Es may be subject to different modes of regulation or have alternative cognate 4EIPs [52]. Recent studies provide direct evidence of the latter. RBPs isolated from worm extracts in their native mRNP complexes show that another 4EIP, IFET-1, associates exclusively with IFE-3 *in vivo* (Peter Boag, personal communication and our unpublished observations) [80]. Furthermore, IFET-1 is responsible for proper IFE-3 localization to germ granules that are distinct from IFE-1:PGL-1 granules [29]. Importantly, these IFE-3:IFET-1 complexes control translation of germline sex-determination mRNAs which are not subject to control by IFE-1 [26, 29]. Similar examples of unique eIF4E:4EIP regulation are found in *Drosophila* germ cells, where the IFET-1 homologue, Cup, facilitates eIF4E nuclear-cytoplasmic shuttling, localization and function in fly oocytes [81]. Cup is also required for posterior localization of several

mRNAs (*osk*, *grk*, *nos*, *etc.*), and maintains their translational repression, which is critical for body axis patterning during embryogenesis (discussed below). It seems clear that all animal germ cells contain multiple unique eIF4E:4EIP complexes. Regulating the transit and activation of these complexes contributes to differential mRNA translational control used to drive physiological germ cell processes in divergent animal systems.

Both PGL-1 and IFE-1 are required for fertility at elevated temperatures, most critically affecting late sperm development. The interpretation of both the biological and biochemical data indicate that IFE-1 recruits a subset of mRNAs whose translation is necessary for completion of sperm (and oocyte) differentiation [26, 82]. Likewise, IFET-1 and IFE-3 are required for normal germline sex-determination, where they prevent ectopic translation of sperm fate mRNAs and enhance translation of at least one oocyte mRNA [29]. These represent just a few instances in which eIF4E:4EIP mRNPs participate in translational repression/activation switches and that controls germ cell and embryo development in quite divergent animal species.

Linking localization of mRNAs to translational regulation

Localization of mRNAs is highly orchestrated in germ cells and embryos. A regimented set of sequence-specific RBPs bound to mRNAs to be localized often coincides with spatial and temporal translational regulation of the transcript [83]. These events also rely on eIF4E:4EIP complexes for proper localization and correct message utilization [84, 85]. The clearest examples are found during *Drosophila* oocyte development and early embryogenesis. *oskar* mRNA (*osk*) becomes enriched in the posterior pole (germ plasm) during mid-oogenesis and *nanos* mRNA (*nos*) becomes enriched in the same region during late oogenesis (Fig. 1.2). Other fly mRNAs such as

gurken (*grk*) and *bicoid* (*bcd*) also exhibit unique and dynamic localization during the maternal-zygotic transition [86, 87]. Each mRNA is simultaneously subject to complex translational control that restricts their regional expression. The protein products form a morphogen gradient in the oocyte or embryo that is critical for anterior-posterior differentiation. However, the process by which each is localized and translationally regulated appear to differ in each case, suggesting that multiple mechanisms are used during development to control spatial and temporal abundance of mRNAs and their translation. Such processes are not restricted to invertebrates. Similar examples can be found in vertebrate models, such as in *Xenopus* oocytes, where Vg1, VegT, Nanos-1 mRNAs all localize to the vegetal pole of the frog oocyte [88].

The combination of mRNA localization and translational control by these mRNP complexes is of critical developmental importance. For example, mis-localization of regional *osk* mRNA translation during oogenesis results in embryos that lack an abdomen and have no germline [89]. *osk* is also subject to complex translational control mediated by the oocyte eIF4E:Cup complex to prevent protein accumulation in the anterior portion of the oocyte. Suppression of initiation events is maintained by multiple mechanisms as the mRNP is being properly localized to the germ plasm [90, 91]. Cup is recruited to *osk* mRNA by Bruno, which both represses and protects the transcript from degradation (Fig. 1.2). Interestingly, the complex recruits the Ccr4-Not1 deadenylase complex to shorten the poly(A) tail but does not destabilize the mRNA [91]. Interestingly, translational repression by Cup does not appear to require the canonical eIF4E-binding motif, but instead translational repression and transcript protection rely on secondary non-canonical eIF4E binding motifs [91]. Evidence shows that the eIF4E:Cup interaction

prevents degradation of *osk* by directly blocking de-capping of the mRNA [91]. Similar to Cup, recent evidence in human cells shows that eIF4E-T (4E-T) is able to repress translation and recruit Ccr4-Not1 deadenylase complex while preventing de-capping and targeted decay, preserving the mRNA in a repressed form [92]. Therefore, the eIF4E:4EIP cap-binding complex is necessary to protect the mRNA while it is coming under appropriate repression by the Ccr4-Not1 machinery in multiple systems. Once *osk* mRNP is properly localized to the posterior region of the oocyte, pre-bound eIF4E in the mRNP joins with eIF4G to initiate translation. Regional synthesis of functional Oskar protein directs patterning in the early embryo. Regulation of *osk* mRNA localization and translation are therefore a stepwise process involving repression/protection, transport, and ultimately translational activation in its complete program. All steps use the eIF4E:4EIP complex to ensure proper timing of the repression/activation switch. Complexes that likely first assemble in the nucleus co-transcriptionally or during splicing also play a role in setting up the localization/fate of mRNPs as they enter the cytoplasm and germ granules. Tsunagi and Magonashi are core components of the exon-junction complex (EJC) that marks mature mRNAs at those ligation points as they exit the nucleus [93]. Both proteins are required for *osk* mRNA microtubule-dependent localization, indicating that 3'-UTR sequences do not act alone to localize mRNAs during development [94].

Figure 1.2

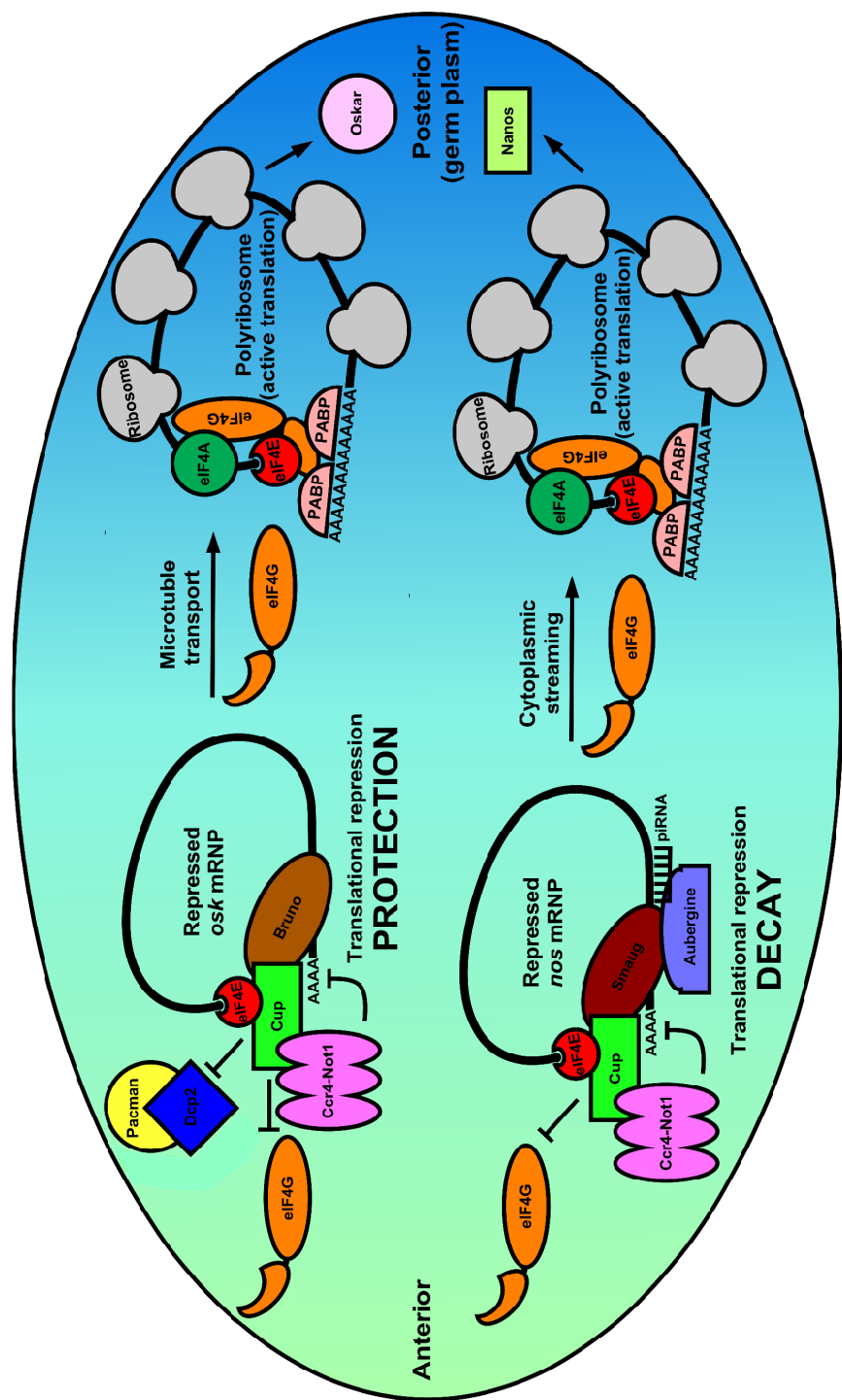


Figure 1.2. Germ cell mRNPs use the same eIF4E:4EIP core but different RBPs for altered modes of local translation in the *Drosophila* oocyte. During the latter stages of *Drosophila* oogenesis many germline mRNPs like *osk* and *nos* are localized to and specifically translated in the germ plasm (posterior pole). These regulated mRNPs use the eIF4E:4EIP core, but each employs a unique mechanism to control mRNA stability, localization, and eventual translation. In the anterior region of the oocyte *osk* mRNPs are circularized by a core complex containing the oocyte eIF4E, Cup (4EIP), and Bruno (3'-UTR-binding protein). Cup prevents eIF4E:eIF4G binding while simultaneously recruiting the Ccr4-Not1 deadenylase complex. Because eIF4E:Cup also prevents de-capping and 5'-3' decay by the Dcp2/Pacman complex, *osk* mRNA is protected and translationally silent while being shuttled to the posterior pole via microtubules. Similarly, *nos* mRNPs in the anterior region of the oocyte are circularized by a eIF4E:Cup:Smaug complex, which again prevents translation initiation and recruits Ccr4-Not1. Unlike *osk*, however, *nos* mRNA localizes by cytoplasmic streaming that is inefficient. Therefore, Smaug recruits Aubergine and associated piRNA machinery to degrade *nos* mRNA outside the germ plasm. Little is known about the events that promote translational activation of these two distinct mRNPs. However, it is likely that a remodeling occurs in which Cup is displaced by eIF4G leading to polyribosome recruitment. Additionally, Oskar protein translationally activates the *nos* mRNP, indicating a complex interplay of translational regulation regimens that ensure that germ cell determinants are synthesized in correct time and place.

In contrast to the efficient localization seen with *osk* mRNA, *nos* mRNA localization appears to be inefficient. Only a 4% enrichment of the mRNA in the posterior end of the early embryo is observed [95]. Consequently, restriction of Nanos to the germ plasm is accomplished by different means, involving both translational repression and targeted mRNA decay [86, 96]. Specifically, Smaug binds a 90-nt element within the 3'-UTR of *nos* mRNA deemed the translational control element (TCE) [97]. In the circularized mRNP structure, Smaug interacts with the Cup:elF4E (Fig. 1.2). This interaction is required for Smaug-dependent translational repression of *nos* mRNA indicating repression at the level of initiation, as well as regulation by Ccr4-Not1 [98]. Thus, *nos* mRNA appears to be repressed and localized by inhibition of CD initiation and targeted mRNA decay outside of the germ plasm, quite different from the repression, protection, and active transport regulation seen with *osk* mRNA, yet still utilizing elF4E:4EIP complexes. Once synthesized in the germ plasm, Oskar protein takes part in the translational activation of repressed *nos* mRNPs by inhibiting deadenylation, indicating there is a complex interplay of germline determinants within the mRNP translational control network in fly oocytes [99].

Additional insights into these translational repression/activation switches that rely on localization can be found in the regulation of Gurken mRNA (*grk*). During egg development *grk* mRNA also relies on Cup for proper localization, but in contrast to *osk* and *nos* mRNAs, it localizes to the dorsal anterior portion of the egg [100]. Developmental defects and biochemical analysis show that a closed-loop mRNP complex involving elF4E, Cup, Bruno, and Squid mediates translational repression of *grk* mRNA that has not localized anteriorly. This repressive elF4E:4EIP complex

becomes activated for translation initiation by PABP55B recruitment of eIF4G once properly localized to the dorsal anterior region [100]. Mechanisms of *osk* and *grk* mRNA translational repression and localization have mechanistic parallels, even using many of the same trans-acting factors. So how is it possible that these shared factors are distinguished in order to localize these two transcripts to different regions of the egg? A protein named Encore, which binds to PABP55B may be key in answering this question [100]. Importantly, Encore is involved in the proper localization and translational activation of *grk* mRNA, but has no role in *osk* mRNA localization and activation. This suggests that each mRNP has trans-acting factors that provide context for activation. What is clear is that once mRNPs are properly localized, initiation factors like eIF4E and eIF4G are responsible for ribosome recruitment. However, the steps necessary and sufficient for initiation factors to overcome repressive trans-acting factors are largely unknown.

Similar examples of mRNP network crosstalk that leads to cell fate decisions can be found in *C. elegans* germline sex-determination [6]. Here too, mRNA localization and translational control pathways work in conjunction with one another to ensure proper *de novo* synthesis of specific gene products regionally. Importantly, even after each mRNA is localized to the correct place at the correct time, the necessary synthesis of the protein products has still not been accomplished. We know very little about the ensuing translational activation of repressed, localized mRNPs (Fig. 1.2). What is clear is that members of the translation initiation apparatus already reside in the mRNP complex during repression and transport. Germline eIF4E isoforms appear to be ubiquitous in

these repressed mRNPs and warrant further investigation as they are likely to be a driving force behind activation.

Polyadenylation/deadenylation also modulate germ cell translational control

In germ cells, deadenylation occurs mostly via the Ccr4-Not1 complex [101-103]. The activity of this multi-subunit complex represents a major mechanism by which germ cells regulate the stability, localization, and translatability of mRNAs. Poly(A) removal may also be mediated by a family of proteins called PARNs, which require removal of the 5'-cap structure (discussed below). The examples cited above from *Drosophila*, involving *osk* and *nos* mRNAs, shows that mechanisms of poly(A)-regulation are used in oocytes and embryos to control the local translation of messages that are required to establish the germ plasm. Similar examples can be found in nematodes, frogs and mice, indicating the prevalence of poly(A)-mediated mRNA regulation throughout germ cell biology [101-104]. Transcripts may also have existing poly(A) tails elongated in the cytoplasm in order to activate translation initiation and/or enhance transcript stability [105, 106]. The choice to remove poly(A) tails or elongate them on an mRNA in a sequence-specific manner imposes another translational switch that may behave similar to a rheostat, controlling the overall “current” of translation (enhancing initiation frequency leading to greater total protein output on those mRNAs with elongated poly(A) tails. It is now clear that eIF4E:4EIP complexes actively participate in poly(A) regulation as well [53, 103, 107, 108]. Polyadenylation of nascent mRNA transcripts first occurs co-transcriptionally in the nucleus. Cleavage of the nascent-mRNA downstream of the polyadenylation signal (PAS) by cleavage and polyadenylation specificity factor (CPSF) precedes addition of 200-250 adenine residues by nuclear poly(A)-polymerases

(PAPs) [109-111]. Poly(A) tail formation is coordinated with transcription termination and nuclear export [112]. Polyadenylation also takes place in the cytoplasm of germ cells and embryos and is highly regulated in order to control the balance of translational activation vs. mRNA decay [113-115]. Poly(A)-extension in the cytoplasm of germ cells is catalyzed predominantly by a highly conserved cytoplasmic poly(A)-polymerase, GLD-2 [115, 116]. Enhancing poly(A) tail length promotes binding and multimerization of poly(A)-binding protein (PABP), which in turn facilitates mRNA closed-loop formation and increased translation through a direct interaction with eIF4G in the eIF4E:eIF4G cap-binding complex [117, 118]. Multimerization of PABP along the poly(A) tail also serves to protect transcripts from targeted decay [119, 120]. This dynamic may be more complicated, however, as recent evidence shows an unexpected dual role for PABPs in mediating both protection and poly(A)-decay, depending on which accessory factor it recruits, deadenylases and TOB proteins [121]. Dynamic changes in poly(A) tail length and subsequent PABP multimerization can remodel mRNPs quite dramatically and lead to nearly a hundred-fold increase in translation initiation rate in response to developmental stimuli [117, 118]. This process is most well understood for mRNAs containing U-rich cytoplasmic polyadenylation elements (CPEs) in *Xenopus* oocytes (Fig. 1.3). There are at least four subclasses of CPEs active during the oocyte to embryo transition in the frog; these are classified according to the developmental step at which they are used [122, 123]. Not all poly(A)-regulated maternal mRNAs are polyadenylated simultaneously in the maturing oocyte. Therefore, the selective activation of groups of CPE-containing mRNAs at various times during development ensures the proper progression of meiosis and sets up the egg to embryo transition

[124]. The most recognized CPE-containing mRNAs encode cell cycle regulators (Cyclins A, B, E, and CDK2) and the proto-oncogene c-Mos [113, 125-128].

Figure 1.3

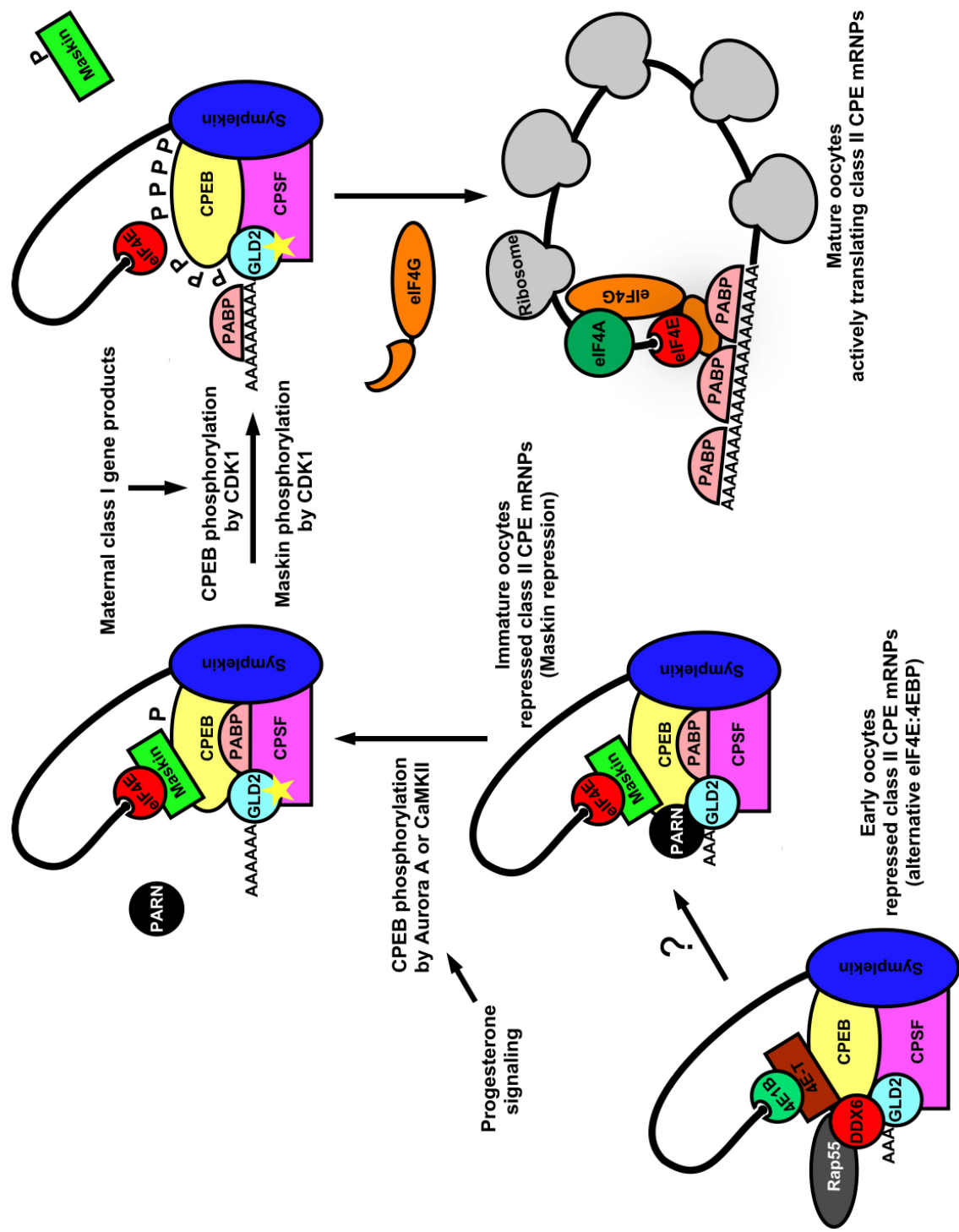


Figure 1.3. Dynamic mRNP remodeling promotes alternate mRNA repression, poly(A)-elongation, and ribosome recruitment during *Xenopus* oocyte maturation.

In immature *Xenopus* oocytes, class II CPE mRNAs are translationally repressed by eIF4E:Maskin cap-binding complexes directed by the CPEB, CPSF, Symplekin 3'-UTR-binding module. These complexes also contain PABP, PARN, and GLD2 indicating the potential for both poly(A)-elongation and deadenylation. At the 5'-end, Maskin (4EIP) inhibits cap-dependent initiation by competing with eIF4G for eIF4E binding. At the 3'-end PARN shortens the poly(A) tail. Although GLD2 is resident within the complex, PARN activity appears to be dominant. It also may be that CPE-containing mRNAs cycle between deadenylation and polyadenylation. Upon progesterone stimulation, CPEB is phosphorylated by Aurora A kinase or CaMKII which leads to remodeling of the complex and ejection of PARN. GLD2 then catalyzes extension of the mRNA poly(A) tail. Concurrently, class I CPE containing mRNAs are translated and lead to activation of CDK1. CPEB and Maskin are phosphorylated by CDK1, and PABP is recruited to the poly(A) tail for further stabilization. Maskin is displaced and PABP associates with eIF4G, allowing more opportunity for a productive eIF4E:eIF4G interaction. This stepwise remodeling leads to translational activation of mRNAs critical for germinal vesicle breakdown and maturation. In early *Xenopus* oocytes CPE containing mRNAs are repressed by an alternative eIF4EB1:4E-T cap-binding complex along with P body components (DDX6 and Rap55), CPEB, CPSF, and scaffold protein Symplekin. PARN is not found in this complex, suggesting that translational repression is caused by inhibition of cap-dependent initiation. P body components resident may also act to degrade CPE these early class II transcripts.

The sequence of events worked out by several labs is as follows: Cytoplasmic polyadenylation element binding protein (CPEB) binds the CPE of target mRNAs, a step essential for progesterone-induced oocyte meiotic maturation [129]. During *Xenopus* oocyte maturation, CPEB acts as a central factor in large closed-loop mRNPs containing CPSF, Symplekin, eIF4E, and a 4EIP called Maskin to mediate translational repression of CPE containing mRNAs (Fig. 1.3) [21, 130]. Maskin binds eIF4E to compete with eIF4G and inhibits translation at the step of ribosome recruitment [21, 107]. Somewhat surprisingly, these large mRNPs also contain a PARN isoform and GLD-2, indicating the potential to carry out both deadenylation and poly(A)-elongation respectively [131]. It may be that CPE-containing mRNAs cycle between deadenylation and polyadenylation states due to the opposing action of PARN and GLD-2, or that either may be exclusive on a single substrate mRNA, or even that PARN inhibits GLD-2 activity enzymatically or sterically. Thus, translational repression in quiescent *Xenopus* oocytes operates at both ends of the mRNA; inhibiting access to eIF4E on the cap and preventing enzymatic activity on the 3'-poly(A) tail. With the induction of meiotic maturation, CPEB becomes phosphorylated on multiple residues, in response to progesterone signaling [132]. Prevailing models suggest that a cascade of mRNP remodeling events culminates in ejection of PARN from the complex, freeing the GLD-2 polymerase to execute poly(A)-elongation (Fig. 1.3). The extended tail recruits more PABP, which in turn binds and recruits eIF4G. Tethered eIF4G provides more opportunity for a productive eIF4E:eIF4G interaction by enhancing proximity, and allowing it to displace Maskin. In younger oocytes PARN and Maskin are not detectable, so other mechanisms are in place to set up very early CPE-dependent repression of

maternal mRNAs [72, 133]. Interestingly, a biochemically separable repression complex was identified in immature oocytes that includes the eIF4E1b isoform (oocyte/embryo specific), eIF4E-T (4EIP, 4E-T), DDX6, and Rap55 (Fig. 1.3). eIF4E1b binds only weakly to m7G-caps and does not appear to interact with eIF4G or Maskin [134]. Thus, in the absence of PARN, repression appears to be mediated by a eIF4E1b:eIF4E-T complex that prevents recruitment to ribosomes, similar to *nos* mRNA repression in *Drosophila* oocytes and in humans as outlined above (Fig. 1.2). Indeed, several studies in other model organisms show that eIF4E isoforms can act as translational repressors. The eIF4E homologous protein (4EHP) takes part in Bicoid-dependent repression of *cad* mRNA during fly embryogenesis [56], and IFE-3 (the eIF4E1 ortholog) mediates IFET-1 (eIF4E-T)-dependent translational repression of germ line sex-determination mRNAs (*fog-1* and *fem-3*) in worms [29]. Additionally, eIF4E:4EIP complexes have been linked to translational repression and mRNA decay during the maternal-zygotic transition in mice, where an oocyte-specific eIF4E was shown to interact with the 4EIP, BTG4, and recruits a catalytic subunit of the Ccr4-Not1 deadenylase complex [135].

Clearly, the mechanisms that control translational repression/activation switches in maturing *Xenopus* oocytes are complex, but like other animal germ cells they utilize eIF4E:4EIP complexes to mediate translation repression/activation switches. Simple thematic paradigms can be constructed from the modes of action that are conserved across most species. Early germ cell translational repression modes rely on preventing eIF4E:eIF4G interactions and thereby inhibiting cap-dependent translation initiation. Late germ cell repression modes not only inhibit the cap-dependent mechanism, but also remove poly(A) tails, which reduces translational efficiency or enhances

degradation. Thus, germ cell systems institute translational repression early, then maintain that repression until intrinsic or extrinsic signals drastically change cell fate (e.g. maturation). The signaling cascades activate translation by stimulating the eIF4 factors already resident in the mRNP and thereby disrupt the repressive effects of 4EIPs and RBPs, and activate GLD-2 to elongate the poly(A) tails of their bound mRNAs.

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CHAPTER II

IFE-3 IS REQUIRED FOR GERMLINE SEX-DETERMINATION IN *C. elegans*

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Summary

Germ cells use both positive and negative mRNA translational control to regulate gene expression that drives their differentiation into gametes. mRNA translational control is mediated by RNA-binding proteins, miRNAs, and translation initiation factors. We have uncovered the discrete roles of two translation initiation factor eIF4E isoforms (IFE-1 and IFE-3) that bind m⁷GTP mRNA caps during *C. elegans* germline development. IFE-3 plays important roles in germline sex determination (GSD), where it promotes oocyte cell fate and is dispensable for spermatogenesis. IFE-3 and its 4EIP, IFET-1 regulate translation of several GSD mRNAs (*fog-1*, *fem-3*, and *daz-1*), but not those under control of IFE-1 (*mex-1*) or housekeeping genes (*tbb-2* and *gpd-3*). IFE-3 also appears to have roles beyond GSD, where it contributes to normal meiotic progression of the oocyte, and is required for embryogenesis. Distinct mutant phenotypes and differential mRNA translation suggest independent dormant and active periods for each eIF4E isoform in the germline.

Introduction

In germ cells, translation control of mRNAs that leads to new protein synthesis has been shown to be critical for stem cell maintenance, meiotic entry, completion of meiosis, and gamete differentiation [26, 136, 137]. Current models suggest that RNA regulatory networks composed of RNA-binding proteins (RBP), miRNAs, and translation initiation factors (eIFs) coordinate splicing, transport, storage, translation, and degradation of mRNAs by the assembly of messenger ribonucleoprotein (mRNP) complexes. Repression of mRNAs by RBPs and miRNAs has been extensively studied in various developmental systems [138-140]. However, repression is only part of overall translational control. Recent evidence suggests that selective activation of repressed mRNPs by eIF4 factors that recruit them to ribosomes represents a vital step in translational control [48, 141]. However, the mechanisms for the transition remains poorly understood. The prevalence of positive mRNA translational control by eIF4 factors in development has only recently begun to be appreciated [26, 37].

Translation initiation factors in the eIF4 group are the first to bind mRNAs and recruit them to ribosomes [142]. Two regulated components are eIF4E and eIF4G, which bind the mRNA 7-methylguanosine 5'-cap (m7G) and act as a scaffold for ribosome association, respectively. The eIF4E:eIF4G interaction is a critical step for mRNA recruitment [143]. This node is regulated by eIF4E interacting proteins (4EIPs) that compete with eIF4G for eIF4E interaction and usually inhibit cap-dependent translation [144]. We postulate that the eIF4E:eIF4G interaction may actively dissociate 4EIPs from repressed mRNPs and activate translation. To add greater complexity, multiple eIF4E isoforms and 4EIPs are expressed in all invertebrate, vertebrate, and

plant species studied [46, 75, 145, 146]. Five eIF4E isoforms (IFE-1-IFE-5) are expressed from the *C. elegans* genome. Individual null strains are mostly viable, and strikingly, loss of each IFE produces a unique phenotype [44]. We previously showed that IFE-1 is required for completion of spermatogenesis and to a lesser extent oocyte maturation [25, 48].

In contrast to IFE-1's role in the germline (primarily driving spermatogenesis) our lab initially showed that IFE-3 was the only eIF4E essential for embryogenesis in *C. elegans* [44]. Mutations in *ife-3* also disrupt germline sex determination (GSD; [147]. In *C. elegans*, larval stages (L1-L4) are defined by four successive cuticle molts. During the fourth larval stage (L4) the first 200-250 germ cells that undergo differentiation in the gonad become spermatocytes, which eventually bud off as spermatids; 10-18 hours later, as the animal reaches adulthood, the germ cell differentiation program switches to produce oocytes for the remainder of the lifecycle (Fig 2.1). This programmed germ cell differentiation switch results in a self-fertile worm, who's fecundity is limited by the number of sperm produced during the L4 stage. The genetic pathway that controls the germ cell switch relies mostly on translational control of fate determining mRNAs, implicating the prescribed role of IFE-3 (see below).

Figure 2.1

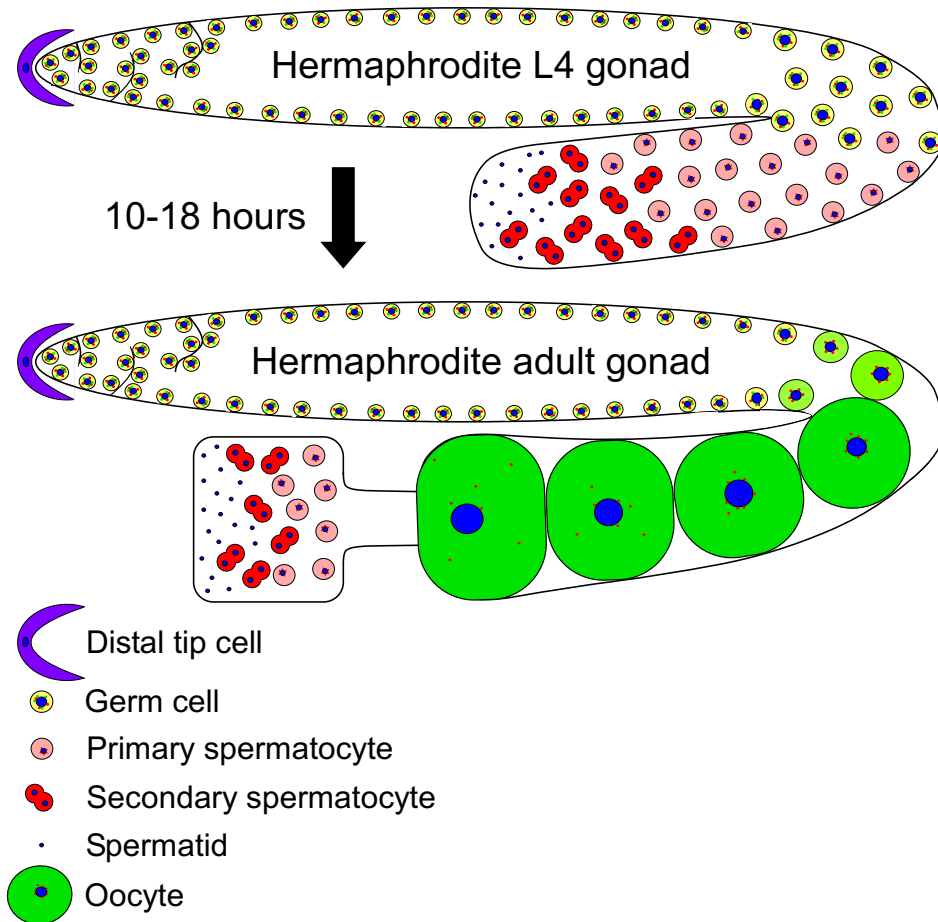


Figure 2.1. Larval and adult germline development in *C. elegans*. A cartoon depicting both larval and adult gonad development in the hermaphroditic nematode *C. elegans*. Larval stages (L1-L4) in the worm are defined by four successive cuticle molts. During the fourth larval stage (L4), germ cells (yellow), begin to differentiate into spermatocytes (pink and red) and then bud off to become spermatids (small blue); 10-18 hours later as adults, the germ cell differentiation program switches to produce oocytes (green). This germ cell switch results in a self-fertile animal. The distal tip cell (DTC; purple) is a somatic cell that controls proliferation of the germline stem cell pool via Notch signaling.

This study describes the unique germline roles of IFE-3 in the sperm to oocyte switch and oocyte growth and contrasts them with IFE-1's very different roles in sperm cytokinesis and oocyte maturation. The GSD pathway relies heavily on mRNA translational control as a means of integrating fate determining gene expression (Fig. 2.2). This pathway allows larval animals to transiently produce sperm, then switch to oogenesis. The GLD-1/FOG-2 complex is an upstream factor in GSD that binds the 3'-untranslated region (3'-UTR) of oocyte promoting *tra-2* mRNA and represses its translation, which initially promotes larval sperm production by allowing FEM proteins to carry out their function in the germline [148-150]. The FEM proteins (FEM-1,-2,-3) promote sperm fate by degrading TRA-1, the transcriptional repressor of terminal sperm promoting genes *fog-1* and *fog-3* [151]. In contrast, oocyte cell fate is promoted by TRA-2 inhibition of FEM-3, which allows TRA-1 to repress *fog-1* and *fog-3* [152]. *fem-3*-binding factors (FBFs) also promote oocyte cell fate by binding the 3'-UTRs and repressing translation of *fem-3*, *fog-1/-3*, and other mRNAs involved in GSD [153, 154] (Fig. 2.2). Translational control obviously dominates GSD gene expression at many levels, however the means by which opposing fate signal mRNAs, like *tra-2* and *fem-3*, are coordinated with such precise timing during development are unclear. How are these signals integrated via negative and positive translational control to allow sperm production in larvae, followed by oogenesis in the adults? Are eIF4E isoforms and their cognate 4EIPs playing a role in this translational control? Based on the primary phenotypes associated with *ife-3* and *ifet-1* mutations, the answer appears to be that they are intimately involved.

Figure 2.2

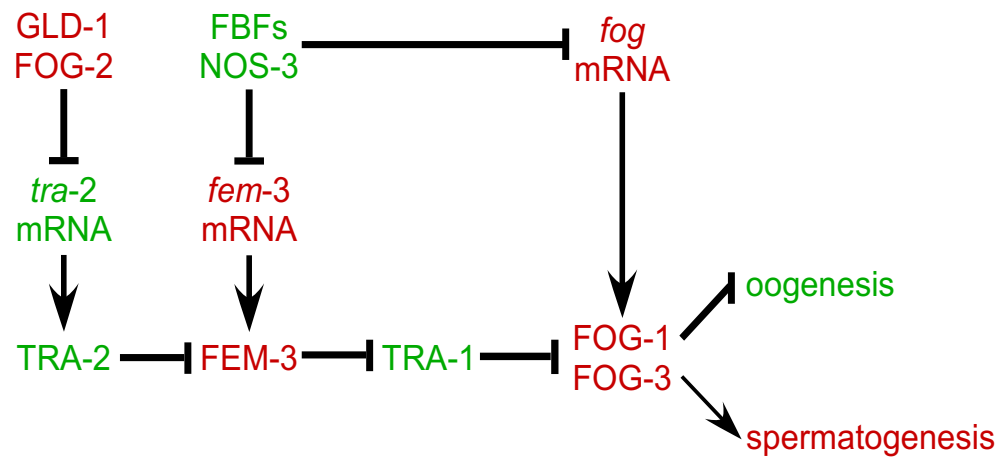


Figure 2.2. Germline sex-determination pathway in *C. elegans*. A diagram depicting the genetic pathway that controls germline sex-determination (GSD) in the hermaphroditic nematode *C. elegans*. Gene-regulation in the pathway is largely accomplished at the level of mRNA translation. A complex translational control network composed mostly of RNA-binding proteins culminates in the conditional synthesis of terminal sperm fate regulators FOG-1 and FOG-3. Genes shown in red are drivers of the spermatogenic fate, and genes shown in green are drivers of the oogenetic fate; mutations in these genes cause feminization of the germline (Fog) or masculinization of the germline (Mog) phenotypes respectively.

Results

IFE-3 promotes the sperm to oocyte switch and is important for oocyte development

The *ok191* allele deletes the promoter and first exon of *ife-3* (Fig. 2.3). Stably balancing this mutation proved difficult because the *ife-3* locus on the end of chromosome V is a hotspot for meiotic recombination [147]. Recombination may subsequently cause wild type reversion in mutation-balanced strains. It was therefore necessary to genotype worms from each generation for each experiment using a triple primer genomic-PCR to unambiguously confirm *ok191* (Fig. 2.3). Eggs from *ife-3*(+/-) mothers carry sufficient maternal IFE-3 for survival of some *ife-3*(-/-) offspring [147]. The escaping *ife-3*(-/-) hermaphrodites developed to adulthood but were sterile at 20°C. Their dissected gonads showed extensive sperm production and lack of oocytes, as evidenced by immunostaining for major sperm protein (MSP) and lack of LIN-41 (oocyte marker; Fig. 2.4C). Wild type hermaphrodite (Fig. 2.4A) and male germlines (Fig. 2.4B) are shown for comparison. These data demonstrate that *ife-3* mutant hermaphrodites have masculinized germlines (*Mog*) and fail to produce oocytes at 20°C. Surprisingly, at elevated temperatures (25°C) at least 21% of *ife-3*(-/-) hermaphrodites were able to initiate oogenesis (Fig. 2.4D, E). Oocytes were evident in the proximal gonad by their expression of LIN-41 but did not grow normally. These results indicate that IFE-3 has roles in oocyte development and growth beyond the sperm to oocyte switch. This phenotype differs strikingly from that of *ife-1*(*bn127*) worms, which displays arrested spermatocyte differentiation and produces oocytes that mature slowly [25, 48].

Figure 2.3

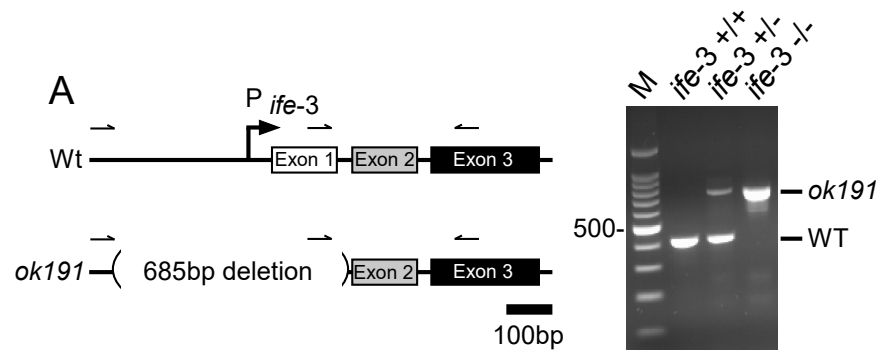


Figure 2.3. The *ife-3(ok191)* allele. A) The *ok191* lesion (685 bp) removes the promotor and exon 1 of *ife-3*, indicating that it is a putative null allele. Agarose gel shows whole worm genomic PCR at the *ife-3* locus from wild type (+/+), heterozygous (+/-), and homozygous (-/-) *ife-3* animals primers used for PCR shown as arrows for reference.

We determined the prevalence of *ife-3(ok191)* induced sterility at 20°C and 25°C. No heterozygous *ife-3(+/-)* hermaphrodites were sterile at 20°C (n= 61) or 25°C (n= 49) (Fig. 2.4E). However, 100% of *ife-3(-/-)* hermaphrodites were sterile (n=60) at 20°C, due to *Mog* phenotype. Interestingly, only 79% of *ife-3(-/-)* hermaphrodites were sterile (n=62) at 25°C, indicating a ~20% rescue of oocyte fate (Fig. 2.4E). The *ife-3(-/-)* mutants had smaller brood sizes (84 ± 7 , n=4) compared to wild type animals (221 ± 10 , n=3) at 25°C (data not shown), and the embryos from *ife-3(-/-)* mothers never hatched (Fig. 2.4E). This observation supports our previous finding using *ife-3(RNAi)* that showed substantial embryonic lethality [44]. Curiously, the distal gonads of *ife-3(-/-)* hermaphrodites were occasionally bifurcated at 20°C (8%, n=60) and at 25°C (5%, n= 62) (data not shown). These phenotypes are unique to *ife-3* among the eIF4E isoforms, since neither the *Mog* phenotype nor the aberrant gonad morphology was ever observed in *ife-1(bn127)*, *ife-2(ok306)*, *ife-4(ok320)*, or *ife-5(ok1934)* animals [48-50, 52]. Interestingly, *ifet-1* (IFE-3 4EIP) mutants also have defects in GSD and show bifurcated gonad morphology in a small percentage of animals [53].

ife-3* is dispensable for spermatogenesis unlike *ife-1

Lastly, we addressed whether IFE-3 was required for male-derived sperm to fertilize oocytes upon mating with *fog-2(q71)* females, as was previously found for IFE-1 [48]. Soaking males in SYTO17 labels their sperm for detection after mating (Fig. 2.5A-D). We used this strategy as a way to track competent sperm production in males, and by tracking offspring production in their mated females we were able to determine the competence of the male derived sperm for fertilization. Although *ife-3(-/-)* males had somewhat decreased mating efficiency at 25°C, their sperm were able to fertilize eggs

in 100% of *fog-2(q71)* females at both 20°C and 25°C (Fig. 2.5B, C, E). This is in stark contrast to what was found for *ife-1(bn127)* mutant males. Males lacking IFE-1 produce very few sperm at 20°C [48], but still fertilized eggs in 100% of *fog-2(q71)* females, indicating their functionality (Fig. 2.5E). By comparison, *ife-1(-/-)* males produced no sperm at 25°C, resulting in no fertilization events (Fig. 2.5D, E). These data establish that IFE-3 is dispensable for producing functional sperm regardless of temperature, in contrast to IFE-1, which is required for robust spermatogenesis at all temperatures, and for functional sperm production at 25°C.

Figure 2.4

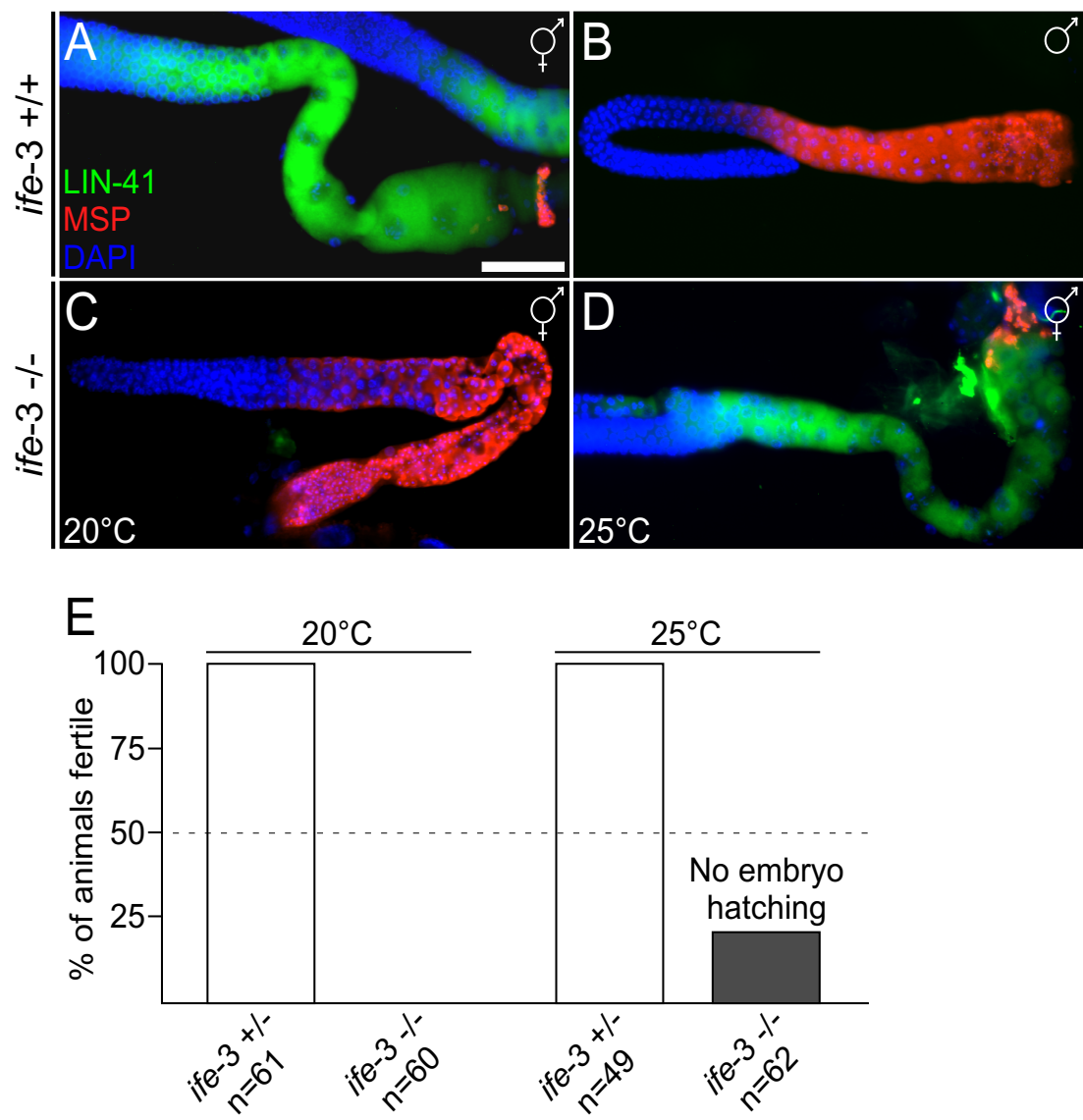


Figure 2.4. Characterization of *ife-3(ok191)* mutants. A) Wild type(+/+)

hermaphrodite gonad showing sperm in the spermatheca stained for major sperm protein (MSP; red), and oocytes, showing LIN-41 expression (green). B) Wild type(+/+) male gonad showing only MSP-stained sperm and no LIN-41-expressing oocytes. C) *ife-3(-/-)* hermaphrodite gonad showing masculinization phenotype (*Mog*) at 20°C, as indicated by overabundance of MSP-stained sperm throughout the gonad, and complete lack of LIN-41-expressing oocytes. D) *ife-3(-/-)* hermaphrodite gonad showing both MSP-stained sperm and small LIN-41-expressing oocytes at 25°C. E) Fertility and embryonic viability in *ife-3(+/-)* and *ife-3(-/-)* mothers at both 20°C and 25°C. Embryos from *ife-3(-/-)* mothers at 25°C never hatched. Genotype of each individual was confirmed by genomic PCR (see figure 2.3). Germlines shown (A-D) carry *lin-41(tn1541[gfp::tev::s::lin-41])* to visualize oocytes and were dissected, fixed, stained with anti-MSP antibody, and counterstained (DAPI, blue). Scale bar: 50 µm.

Figure 2.5

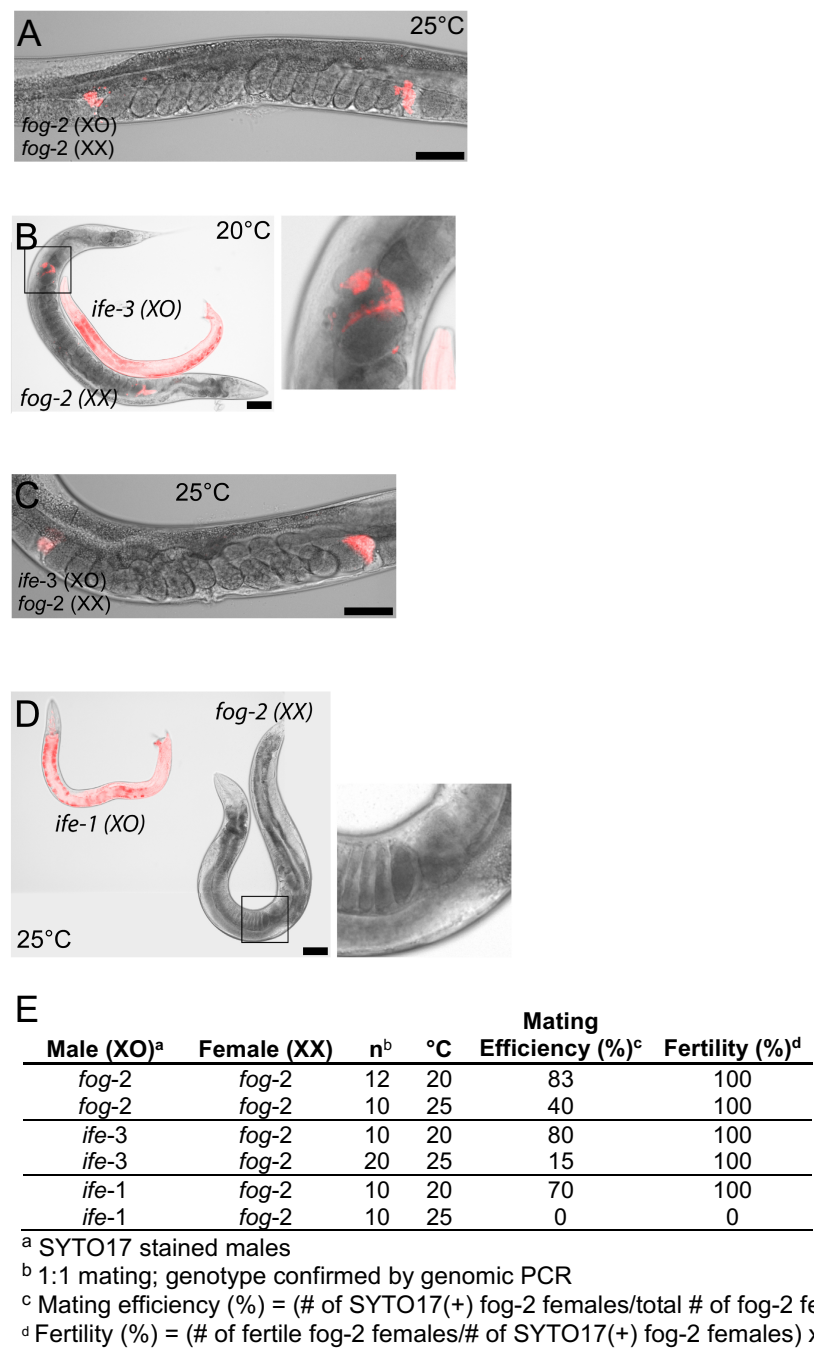


Figure 2.5. SYTO17 staining of mutant males. A) *fog-2(q71)* female mated with SYTO17 stained *fog-2(q71)* male at 25°C showing efficient mating, as indicated by SYTO17(+) sperm in the spermatheca, and functionality of those sperm, as indicated by presence of embryos in the uterus. B) *fog-2(q71)* female mated with SYTO17 stained *ife-3(-/-)* male at 20°C showing efficient mating, as indicated by SYTO17(+) sperm in the spermatheca, and functionality of those sperm, as indicated by presence of embryos in the uterus. C) *fog-2(q71)* female mated with SYTO17 stained *ife-3(-/-)* male at 25°C showing efficient mating, as indicated by SYTO17(+) sperm in the spermatheca, and functionality of those sperm, as indicated by presence of embryos in the uterus. D) *fog-2(q71)* female mated with SYTO17 stained *ife-1(-/-)* male at 25°C showing inability of *ife-1(-/-)* males to produce sperm at elevated temperatures, as indicated by lack of SYTO17(+) sperm in the spermatheca and stacked oocytes in the absence of fertilization events. E) Table showing mating efficiency and fertility for mutant breeding pairs (*n*) at 20 and 25°C.

***ife-3* promotes oocyte fate with *fbfs* in the GSD pathway**

Many mutations that alter the sexual fates of germ cells have been identified in *C. elegans* [155, 156]. Most of the corresponding GSD genes encode RBPs or mRNAs within a translational control network that culminates in the conditional synthesis of FOG-1/-3, which drives sperm fate (Fig. 2.2). To understand where *ife-3* might function in this network, we conducted epistatic analyses with mutations in components of the GSD pathway in conjunction with IFE-3 depletion. By evaluating terminal phenotypes in these mutant backgrounds subjected to IFE-3 loss, we were able to determine where *ife-3* functions within the pathway relative to the other GSD genes investigated. Briefly, if IFE-3 loss could reverse feminized phenotypes, then it would be considered downstream of the feminizing gene in question. Conversely, if IFE-3 loss failed to revert a feminized phenotype, then it would be considered upstream of that gene. We chose to use *ife-3*(RNAi) for these experiments due to the embryonic lethality of *ife-3(ok191)* and the instability of the balanced mutation, both of which complicate the derivation of double mutant strains, which is commonplace for these types of experiments. Following *ife-3*(RNAi) treatment, 22% (n=157) of wild type hermaphrodites failed to produce oocytes and developed the *Mog* phenotype, evident by extensive MSP staining and apparent lack of oocytes (Fig. 2.6A, C). Interestingly, the penetrance of *ife-3*(RNAi)-induced sterility is lower than that of the null allele (22% vs. 79% at 25°C respectively) (see discussion). By comparison, 100% (n= 129) of control(RNAi)-treated wild type hermaphrodites showed both sperm and oocytes (Fig. 2.6A, B). To rule out the possibility of weak *ife-3*(RNAi) efficacy, we also performed *ife-3*(RNAi) in an RNAi-

sensitized strain *eri-1(mg366)*, but obtained similar frequencies of *Mog* phenotype compared to wild type animals (20%, n=86; Fig. 2.6A).

Figure 2.6

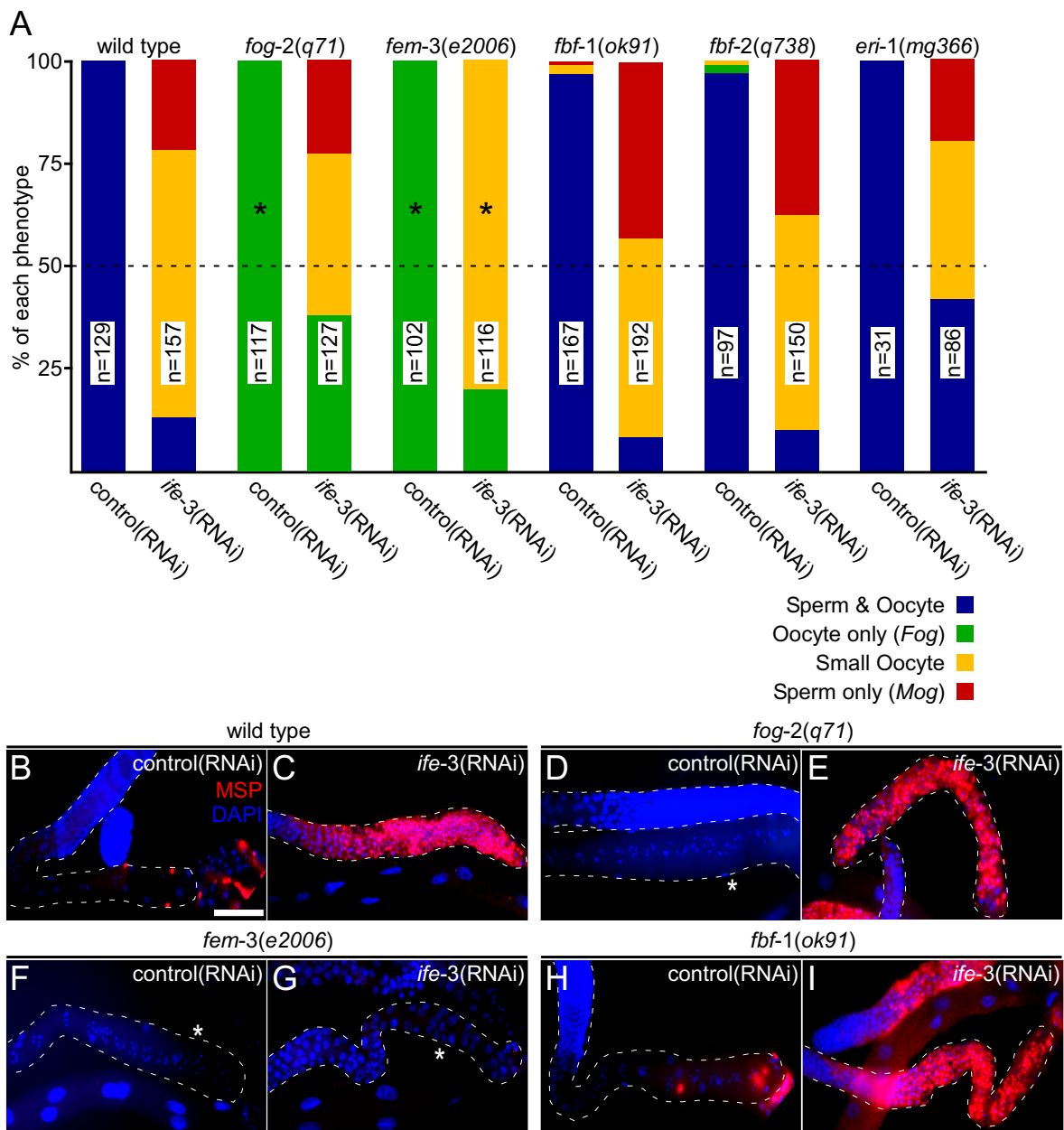


Figure 2.6. Epistatic analysis of *ife-3* and other regulators of GSD. A) Epistatic outcomes from *ife-3*(RNAi) in mutant backgrounds depicted in a bar graph that shows the distributions of phenotypes for each group. B) Control(RNAi)-treated wild type hermaphrodite gonad showing sperm (MSP, red) and oocytes as identified by nuclear morphology (DAPI, blue). C) *ife-3*(RNAi)-treated wild type hermaphrodite gonad showing masculinization phenotype (*Mog*) as indicated by an overabundance of sperm and lack of oocytes. D) Control(RNAi)-treated *fog-2(q71)* gonad showing feminization phenotype (*Fog*) as indicated by lack of MSP-stained sperm, and presence of stacked oocytes. E) *ife-3*(RNAi)-treated *fog-2(q71)* gonad showing *Mog* phenotype, demonstrating reversion of *fog-2(q71)*-induced feminization. F) Control(RNAi)-treated *fem-3(e2006)* gonad showing *Fog* phenotype. G) *ife-3*(RNAi)-treated *fem-3(e2006)* gonad showing *Fog* phenotype with small germ cells. No sperm were ever observed in *fem-3(e2006)* worms demonstrating failure to revert the feminization phenotype. The resulting small germ cells were later found to be LIN-41-expressing oocytes (Fig. 4A). H) Control(RNAi)-treated *fbf-1(ok91)* gonad showing sperm and oocytes. I) *ife-3*(RNAi)-treated *fbf-1(ok91)* gonad showing *Mog* phenotype. *fbf-1(ok91)* mutants treated with *ife-3*(RNAi) displayed enhanced penetrance of *Mog* phenotype relative to wild type animals. Germlines of the indicated genotype were dissected, fixed, stained with for MSP to visualize sperm, and counterstained (DAPI, blue). Gonads are outlined for clarity. *Fog* phenotype is denoted by (*) in (A) and (B-I). Scale bar: 50 μ m.

To place *ife-3* within the GSD pathway, we looked for reversion of known feminizing mutations or enhancement of *Mog* phenotype by *ife-3*(RNAi). The *fog-2(q71)* mutation results in feminized hermaphrodite germlines (*Fog* phenotype) that never produce sperm [155]; FOG-2 is an upstream factor in the GSD pathway where it represses oocyte promoting *tra-2* translation (Fig. 2.2). Consistent with this, *Fog* gonads were observed in 100% (n= 117) of control(RNAi)-treated *fog-2(q71)* hermaphrodites, evident by the absence of MSP staining and presence of large stacked oocytes (Fig. 2.6A, D). By contrast, 23% (n=127) of *ife-3*(RNAi)-treated *fog-2(q71)* hermaphrodite gonads developed as *Mog*, showing extensive MSP staining and no oocytes (Fig. 2.6A, E). IFE-3 depletion was thus able to revert the *fog-2(q71)* feminizing mutation at a similar frequency to wild type animals, indicating that *ife-3* promotes oocyte fate downstream of *fog-2*. Another feminizing mutation, *fem-3(e2006)*, also causes hermaphrodites to develop *Fog* at 25°C but not 20°C [157]. As expected, all of the control(RNAi)-treated *fem-3(e2006)* hermaphrodites were *Fog* at 25°C (100%, n= 102; Fig. 2.6A, F). Interestingly, none of the *ife-3*(RNAi)-treated *fem-3(e2006)* worms (0%, n=116) developed as *Mog* at 25°C, as shown by the lack of MSP staining (Fig. 2.6A, G). IFE-3 depletion was therefore unable to revert the *fem-3(e2006)* feminizing mutation, indicating that *ife-3* promotes oocyte fate at a step genetically upstream or at the level of *fem-3*, consistent with the findings of Mangio, *et al.* [147].

FBF-1 and FBF-2 (*fem-3* binding factors) are homologous RBPs that repress translation of *fem-3* and *fog-1/-3* mRNAs to promote oocyte cell fate [153, 154]. *fbf-1(ok91)* mutants are slightly masculinized (1% *Mog*), and *fbf-2(q738)* mutants are slightly feminized (1% *Fog*), for reasons that remain unclear [158, 159]. *ife-3*(RNAi)

masculinized 43% (n=192) of *fbf-1(ok91)* hermaphrodites (Fig. 2.6A, I), whereas only 1% displayed *Mog* phenotype upon control(RNAi) (Fig. 2.6A, H). Therefore, IFE-3 depletion enhanced *Mog* penetrance two-fold in *fbf-1* mutants relative to *ife-3*(RNAi)-treated wild type animals. Similarly, enhanced *Mog* penetrance was seen in *fbf-2(q738)* hermaphrodites relative to *ife-3*(RNAi)-treated wild type animals (Fig. 2.6A). These data show that *ife-3* and *fbfs* act synergistically to promote oocyte fate, suggesting that IFE-3 works with FBFs in translational control.

A substantial number of *ife-3*(RNAi) hermaphrodites were not *Mog*, but developed small, stunted oocytes that failed to develop normally (Fig. 2.7A). This was first observed in some *ife-3(-/-)* null worms grown at 25°C (Fig. 2.4D) but was more consistent upon *ife-3*(RNAi). Small oocytes were observed in 39-80% of IFE-3-depleted worms, depending on the genetic background, and was most prevalent when masculinization could not be achieved (Fig. 2.6A, G; e.g. *fem-3*). These small cells were negative for MSP staining and were confirmed to be oocytes by LIN-41 expression (Fig. 2.7A). DAPI staining confirmed aberrant nuclear morphology indicating defects in oocyte meiotic progression when IFE-3 is depleted (Fig. 2.7A, insets). Both the RNAi and null mutation phenotypes indicate that IFE-3 must have roles beyond GSD in oocyte growth and completion of meiosis.

IFE-3 regulates the translation of some GSD mRNAs

Because eIF4E isoforms bind to mRNA caps and catalyze translation initiation, we postulated that IFE-3 loss might adversely affect the translation of GSD pathway mRNAs that contribute to oocyte cell fate. We directly analyzed translational efficiency of several GSD mRNAs in control and *ife-3*(RNAi)-treated wild type animals using high

resolution polysome profiling [160]. Our previous studies used the identical methodology to analyze mRNAs that respond to IFE-1, IFE-2, and IFE-4 deficiencies *in vivo* [25, 48-50]. Each IFE isoform previously investigated positively regulates a unique subset of mRNAs. Importantly, no GSD mRNAs have been shown to respond to loss of any of the aforementioned IFEs in these studies.

Large scale *ife-3*(RNAi) over a single generation provided sufficient material for sucrose fractionation of whole worm populations and allowed mRNAs to be resolved by their polysome loading (Fig. 2.7A). RNAi treatment induced *Mog* phenotype in a minority (22%) of the worms, whereas most (78%, Fig. 2.7A) produced primarily oocytes. IFE-3 depletion was confirmed under these conditions via fluorescent imaging and western blot analysis (Fig. S2.1A-C). Overall polysome content relative to 80S was modestly decreased by *ife-3*(RNAi), indicating that IFE-3 depletion does not dramatically alter bulk protein synthesis (Fig. 2.7A). Individual mRNAs were quantified across gradient fractions (Fig. 2.7B-H). The translation of *fem-3*, *fog-1*, *fbf-1*, *gld-1*, and *tra-2* were altered upon *ife-3*(RNAi) treatment when compared to control(RNAi)-treated animals (Fig. 2.7B-F). Unexpectedly, most of the GSD mRNAs assayed translated more efficiently after IFE-3 depletion. A substantial fraction of non-translating *fem-3* mRNA (fractions 1-4) in control(RNAi) worms became shifted into light polysomes (fractions 5-8) upon *ife-3*(RNAi), indicating that IFE-3 deficiency allowed for more efficient initiation of *fem-3* mRNA (Fig. 2.7B). Strikingly, the bulk of *fog-1* mRNA also shifted from non-translating fractions into both light and heavy polysomes upon IFE-3 depletion (Fig. 2.7C). Polysomal loading of *fog-1* mRNA was therefore greatly enhanced, particularly in the heavy fractions (9-11) where many ribosomes translate simultaneously. The

synthetic output of FOG-1 is expected to be higher and might contribute to the *Mog* phenotype observed (Fig. 2.4 and 2.6). *fbf-1* and *gld-1* mRNAs experienced more modest increases in translational efficiency after IFE-3 depletion. For these, some non-translating mRNA (fractions 1-4) appeared to shift to light and heavy polysomes (Fig. 2.7D, E). Given the amount of *fbf-1* and *gld-1* mRNAs already translating in control(RNAi) worms, the output of these proteins may not be substantially changed. *tra-2* mRNA was less efficiently translated after IFE-3 depletion, as shown by a shift to light polysomes (Fig. 2.7F, fractions 5-8) from heavy polysomes (Fig. 2.7F, fractions 9-11). The effect is modest, but a positive dependence of *tra-2* translation on IFE-3 is consistent with IFE-3's role in promoting oocyte fate. Importantly, the translational efficiency of some mRNAs was unchanged upon *ife-3*(RNAi), including housekeeping mRNAs GAPDH (*gpd-3*; Fig. 2.7G) and β -tubulin (*tbb-2*; not shown). Neither showed preferential dependence on any single IFE previously [25, 49, 50]. More strikingly, *mex-1* mRNA translational efficiency was also unchanged (Fig. 2.7H). Our lab previously showed *mex-1* mRNA translation to be strongly IFE-1-dependent [48].

By summing mRNA content across gradients, relative to *gpd-3* mRNA, we found that with the exception of *fog-1* (2.5-fold increase), all germline mRNAs assayed were decreased ~25-50% in *ife-3*(RNAi)-treated animals (Fig. 2.7I). This is consistent with *ife-3*(RNAi)-treated germlines being smaller than their control(RNAi)-treated counterparts (Fig. 2.7A). Therefore, changes in the translational efficiency seen were not due to alterations in the abundance of the mRNAs. These data suggest that IFE-3 is involved in the translation of several GSD mRNAs, but not housekeeping mRNAs, nor those regulated by IFE-1 (*mex-1*). IFE-3 acts to repress *fem-3*, *fog-1*, *fbf-1*, and *gld-1* mRNA

translation, and possibly enhance *tra-2* mRNA translation. While a negative translational role for an initiation factor was wholly unexpected, it is interesting that the mRNAs repressed by IFE-3 are also well-defined FBF targets; FBFs are known to bind to the 3'-UTR of their targets and repress translation through a variety of mechanisms (see Discussion and [10, 161]).

Figure 2.7

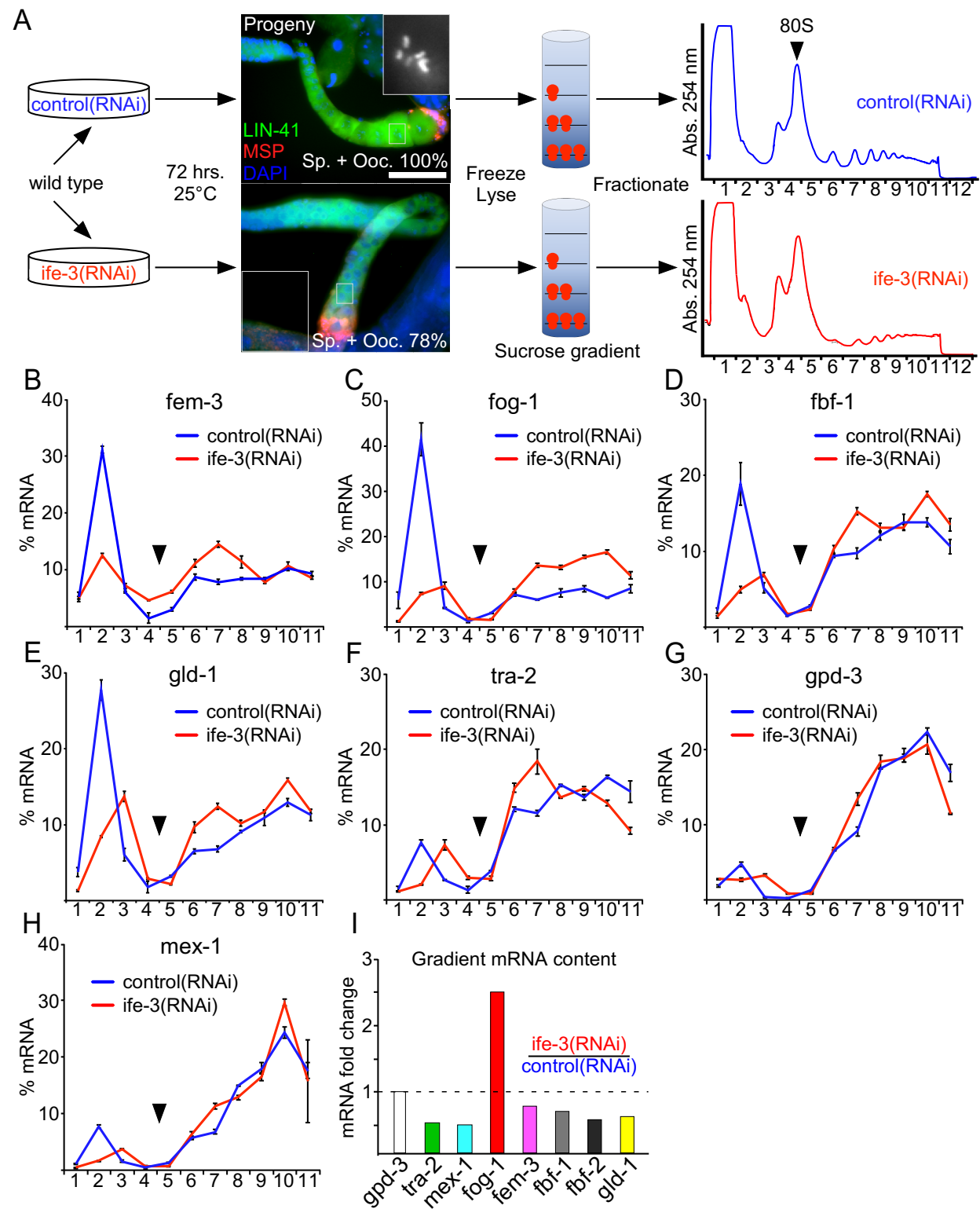


Figure 2.7. Translational efficiency of GSD mRNAs is altered by IFE-3 depletion.

A) Schematic of the experimental design for high-resolution polysome analysis. Wild type animals were grown on control(RNAi) (blue) or *ife-3*(RNAi) (red). Micrographs depict representative phenotypes of control(RNAi) or *ife-3*(RNAi) worms. As before, IFE-3 depletion caused a minority (22%) of *Mog* animals; the majority (78%) remained oogenetic. Absorbance (A_{254}) profiles for gradient fractionation of extracts from control(RNAi) (blue) and *ife-3*(RNAi)-treated (red) worms depicts the polysome content. The highest resolved peak is the monosome (80S, black arrow) and each peak to the right represents the addition of one ribosome (polysomes). Individual graphs depict normalized mRNA content across the gradient by qRT-PCR for B) *fem-3*, C) *fog-1*, D) *fbf-1*, E) *gld-1*, F) *tra-2*, G) *gpd-3*, and H) *mex-1* mRNAs. Normalization by total RNA content across the gradient corrects for slight differences in polysome yield, allowing direct comparison of the partitioning of each mRNA. Error bars indicate the standard deviation of triplicate qRT-PCR measurements. Similar profiles were obtained for *fem-3*, *fog-1*, and *gpd-3* mRNAs in 2-3 independent experiments. I) Relative mRNA abundance in each gradient (normalized to *gpd-3*) shows that in *ife-3*(RNAi)-treated animals *fog-1* is increased 2.5-fold, while all other mRNAs assayed (*tra-2*, *mex-1*, *fem-3*, *fbf-1*, *fbf-2*, and *gld-1*) were decreased by 25-50% in control(RNAi)-treated animals. Germlines shown (A) carry *lin-41(tn1541[gfp::tev::s::lin-41])* to visualize oocytes and were dissected, fixed, stained for MSP to visualize sperm, and counterstained (DAPI, blue). Scale bar: 50 μ m.

IFE-3 regulates the steady state levels of some GSD mRNAs

Of the mRNAs assayed translationally, only *fog-1* mRNA steady-state levels appeared to increase upon *ife-3*(RNAi) (Fig 2.7I). We wondered if these changes were due to IFE-3 loss *per se* or the 22% of *Mog* animals in the population (Fig. 2.7A). It is possible that the levels of sperm-promoting mRNAs (*fem-3* or *fog-1*) increase following complete loss of IFE-3. Indeed, some studies suggest that steady state *fem-3* mRNA levels increase in certain *Mog* backgrounds [162], while others demonstrated that *fog-1* transcription is enhanced under conditions that favors spermatogenesis [163]. We determined the relative *fem-3* and *fog-1* steady-state mRNAs levels relative to *tbb-2* mRNA in *ife-3*(*-/-*) L4 hermaphrodites and compared them to wild type animals. Early and late L4 hermaphrodites were analyzed in order to compare equivalent spermatogenic germlines (avoiding comparison of disparate oogenetic and *Mog* germlines). We found no statistically significant difference ($p=0.328$) in *fem-3* mRNA levels between *ife-3*(*-/-*) and wild type early L4 hermaphrodites (Fig. 2.8A). However, a large and statistically significant increase (9-fold; $p=0.007$) in *fog-1* mRNA levels was found in early L4 *ife-3*(*-/-*) hermaphrodites compared to wild type animals (Fig. 2.8B). We also examined late L4 worms to determine if the changes in *fem-3* and *fog-1* mRNAs levels were maintained through the sperm to oocyte switch, since these animals are expected to have initiated oogenesis. The *fem-3* mRNA levels were decreased by half in *ife-3*(*-/-*) worms relative to wild type ($p=0.018$, Fig. 2.8A), again consistent with having smaller germlines. However, *fog-1* mRNA levels remained 6-fold higher in *ife-3*(*-/-*) worms ($p=0.019$, Fig. 2.8B). These data suggest that IFE-3 loss resulted in enhanced transcription/ stabilization of *fog-1* mRNA even prior to the sperm

to oocyte switch (see Chapter IV); *fem-3* mRNA levels do not change prior to the sperm to oocyte switch, but drop at later stages, likely due to the size of the gonad relative to the soma.

Figure 2.8

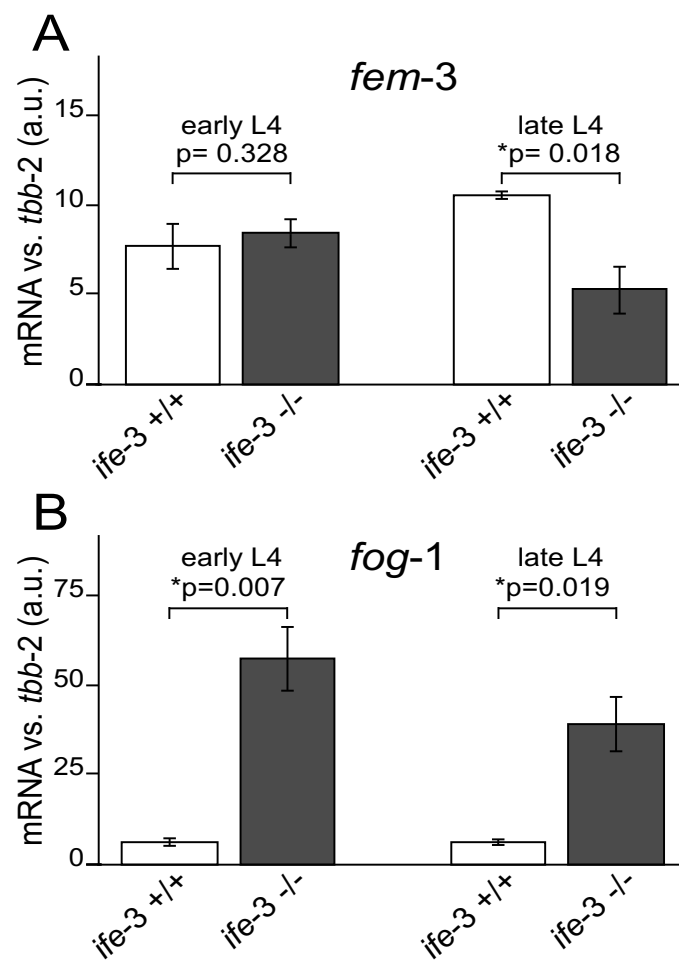


Figure 2.8. Steady state levels of *fem-3* and *fog-1* mRNAs in *ife-3(ok191)*

hermaphrodites. A) Relative *fem-3* mRNA expression in early L4 hermaphrodites shows no statistically significant change ($p=0.328$) between *ife-3(-/-)* (grey) and wild type (white) animals. Relative *fem-3* mRNA expression in older L4 hermaphrodites shows a statistically significant ($p=0.018$) two-fold decrease in *ife-3(-/-)* animals. B) Relative *fog-1* mRNA expression in early L4 hermaphrodites shows a 9-fold increase ($p=0.007$) in *ife-3(-/-)* animals (grey) compared to wild type (white). Relative *fog-1* mRNA expression in older L4 hermaphrodites shows a similar 6-fold increase ($p=0.019$) in *ife-3(-/-)* animals. Animals were synchronized and staging of L4 larvae was confirmed via vulva morphology. Triplicate qRT-PCR measurements from three independent biological replicates are shown with error bars indicating standard deviation. Quantifications were normalized to tubulin (*tbb-2*) mRNA. Student's t-test was used to determine statistical significance between wild type and mutant animals, with a p-value cutoff of 0.05.

IFE-3 and IFET-1 regulate the same GSD mRNAs

The cognate 4EIP for IFE-3 is IFET-1; they have been shown to bind *in vitro* and have been isolated from mRNP complexes *in vivo* [80, 164]. Our own biochemical data indicates that IFE-3 and IFET-1 are strong interactors (see Chapter III and IV). IFET-1 is the worm ortholog of mammalian eIF4E transporter protein, which facilitates nucleoplasmic shuttling of eIF4E [165]. Furthermore, IFET-1 has been shown to be a “broad scale translational repressor” in *C. elegans* [53]. We hypothesized that the mRNAs that were de-repressed translationally upon IFE-3 loss (Fig. 2.7) would be similarly de-repressed upon IFET-1 loss. If IFE-3 genuinely represses these GSD transcripts directly, it likely does so through its association with its cognate 4EIP, IFET-1 to sequester mRNAs away from translating complexes.

To address this directly, we compared the polysomal profiles of germline mRNAs following separate depletion of IFE-3 or IFET-1 via RNAi. We quantified the total contribution of *Mog* phenotype in each population, and found that ~25% of *ife-3*(RNAi)-treated animals were masculinized, whereas in *ifet-1*(RNAi)-treated populations the prevalence of *Mog* was only ~5%. Going forward it was important to assess this parameter to determine if changes in translation were an effect of phenotype or loss of IFE-3/IFET-1 *per se* (see discussion). Overall polysome content relative to 80S was similarly decreased by both *ife-3*(RNAi) and by *ifet-1*(RNAi) indicating a modest decrease in bulk protein synthesis (Fig. 2.9A). Remarkably, the de-repression of *fog-1* and *fem-3* mRNAs was identical upon depletion of either IFE-3 or IFET-1, where a substantial portion of each untranslated mRNA shifted into heavy polysomes (Fig. 2.9B, C). The translation of *daz-1* mRNA in the germline has been shown by others to be

repressed by IFET-1 [53]. This repression relies on sequences in the 3'-UTR of *daz-1*. IFET-1 does not bind RNA directly, therefore this repression complex must rely on other 3'-UTR RBPs. We were able to show that loss of either IFE-3 or IFET-1 caused identical *daz-1* de-repression (Fig. 2.9D), suggesting that IFE-3 is also involved in this repression complex. Translation of IFE-1-dependent *mex-1* mRNA and housekeeping *gpd-3* mRNA were largely unaffected by loss of IFET-1 or IFE-3 (Fig. 2.9E, F).

The three overlaid polysome profiles for each mRNA indicate an identical requirement for both subunits of the IFE-3:IFET-1 complex to exert translational regulation in certain GSD mRNAs. Notably, IFE-3 is the nematode ortholog of canonical eIF4E-1, and would be expected to exert positive translational activity on some population of mRNAs, consistent with diminished polysome content following RNAi. Intriguingly, our polysome data also infers that IFET-1 (the nematode ortholog of human eIF4E-nuclear transport protein), too assists in that positive role of maintaining robust polysomes. Identifying which mRNAs they might positively regulate requires genomic approaches similar to what we used previously to identify all IFE-1, -2, and -4 responding mRNAs [25, 48-50]. However, our recent proteomics data argues somewhat against this possibility, as it revealed that IFE-3 associates robustly with many trans-acting factors with described roles in translational repression (see Chapter IV and V).

Figure 2.9

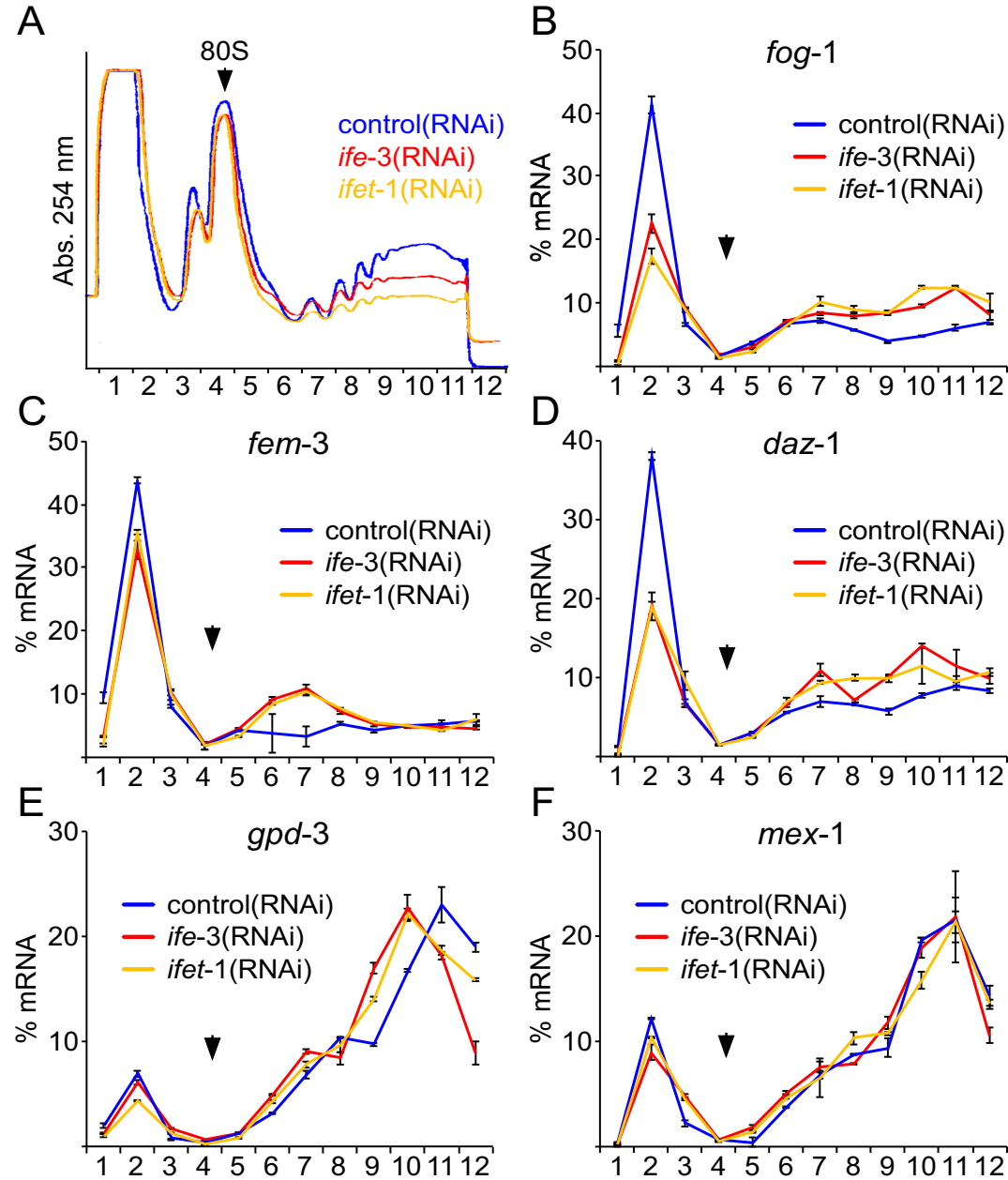


Figure 2.9. Loss of IFE-3 or IFET-1 causes identical changes in GSD mRNA

translation. A) Absorbance (A_{254}) profiles for matched gradient fractionation of extracts from control(RNAi) (blue), *ife-3*(RNAi) (red), and *ifet-1*(RNAi)-treated (yellow) worms depicts the polysome content. The highest resolved peak is the monosome (80S, black arrow) and each peak to the right represents the addition of one ribosome (polysomes). Individual graphs depict normalized mRNA content across three matched gradients as measured by qRT-PCR for B) *fog-1*, C) *fem-3*, D) *daz-1*, E) *gpd-3*, and F) *mex-1* mRNAs. Normalization by total RNA content across the gradient corrects for slight differences in polysome yield, allowing direct comparison of the partitioning of each mRNA in polysomal and non-polysomal complexes. Error bars indicate the standard deviation of triplicate qRT-PCR measurements.

Discussion

Unique roles for multiple eIF4E isoforms in *C. elegans* germline

In this study we observed unique germline phenotypes, genetic interactions, and translational functions of eIF4E isoforms IFE-1 and IFE-3, as well as for IFET-1, the cognate 4EIP that functions with IFE-3. We previously characterized the unique functions of IFE-1 to promote spermatogenesis and oocyte maturation [25, 48]. Epistasis experiments placed *ife-1* downstream of the terminal regulator of GSD, *fog-1*, indicating that *ife-1* is not involved in GSD *per se* but rather promotes the final stages of gametogenesis [166]. This is in contrast to *ife-3* which functions in GSD upstream of *fog-1* (Fig. 2.6). Spermatocytes lacking IFE-1 arrest just prior to spermatid budding resulting in multinucleated spermatocytes that possibly undergo apoptosis; oocytes are deficient in Ephrin (VAB-1) signaling [48, 82]. mRNAs under IFE-1's control in oocytes have been identified and include *pos-1*, *pal-1*, *oma-1*, *vab-1*, and *mex-1* [25, 48]. For example, IFE-1 recruits *vab-1* mRNAs to heavy polysomes, linking its translational activity to one of its major phenotypes: defective oocyte maturation. Differential polysome array analysis between wild type and *ife-1(bn127)* worms identified all IFE-1-dependent mRNAs [25]. We employed similar approaches to identify IFE-2 and IFE-4-dependent mRNAs [49, 50].

Initial studies on eIF4E isoform 3 (IFE-3) showed it was essential for both embryogenesis and the sperm to oocyte switch in hermaphrodites [44, 147]. Here we extend the genetic characterization of IFE-3 and find that it has roles beyond GSD (Fig. 2.4 and 2.6). IFE-3 depletion by RNAi and elevated temperature in *ife-3(ok191)* mutants allowed bypass of the sperm-oocyte switch but still resulted in embryonic lethality,

possibly due to defects in oocyte meiosis and growth (Fig. 2.4). Thus, major roles for IFE-3 are to promote the sperm to oocyte switch, oocyte meiosis, and embryogenesis, which is a clear distinction from roles of IFE-1 in sperm cytokinesis and oocyte maturation. For instance, IFE-3 is fully dispensable for spermatogenesis, whereas IFE-1 is required (Fig. 2.5). Remarkably, there appear to be non-redundant functional roles for three eIF4E isoforms in the germline, where each regulates its own unique subset of mRNAs.

The mechanism by which eIF4E isoforms discriminate among their mRNA targets is unlikely to be due to their m7G cap binding domains, as these structures are highly homologous [44]. The structure of each mRNA cap-binding protein allows for recognition of only 2-3 nucleotides beyond the 5' m7G cap [167]. Although IFE-1 and IFE-3 differ somewhat in binding to m7G and m2,2,7G caps [168], the modest affinity differences are also unlikely to account for the selective mRNA translational control we observe [25, 49, 50]. In addition, genes subject to trans-splicing give rise to heterogeneity of m7G- and m2,2,7G-capped mRNAs, with the exception of downstream operon mRNAs [169]. We suggest that mRNA selectivity is based on association with unique cognate 4EIPs, namely PGL-1 for IFE-1 and IFET-1 for IFE-3. Other accessory factors (RBPs, miRNA, helicases, *etc.*) resident in IFE-1 or IFE-3 mRNP complexes are also likely to contribute to the functional differences we see in translation. The fact that identical translational regulation of IFE-3-responsive mRNAs was observed upon loss of IFET-1 lends strong support to this mechanistic model (see Chapter III and IV).

Roles for IFE-3 and IFET-1 in translational control of GSD mRNAs

Many of the GSD-disrupting mutations have been mapped to regulatory elements within the 3'-UTR of several genes (e.g. *fem-3*gf and *tra-2*gf alleles) [150, 170], or were null mutations in RBP genes (*fbfs*, *fog-1*, *gld-1*) [148, 153, 156]. Each case pointed to mRNA repression events mediated by sequence-specific RBPs. Genetic characterization indicated a strong requirement for *ife-3* during hermaphrodite GSD (Fig. 2.4). Our epistatic analysis placed *ife-3*'s role downstream of *fog-2*, upstream or at the level of *fem-3*, and implicated synergistic involvement of *fbfs* to promote oocyte fate (Fig. 2.6). These studies further define the genetic requirements for *ife-3* in GSD pathway; it is likely that *ife-3* regulates gene expression at multiple levels within the central part of this translational control network.

Using polysome analysis, we identify mRNAs that IFE-3 and IFET-1 regulate in the GSD pathway, and show that their role appears to include co-repression and/or activation of translation. Loss of IFE-3 led to enhanced translation initiation of repressed *fem-3*, *fog-1*, *fbf-1/2*, and *gld-1* mRNAs (Fig. 2.7 and 2.9) The *fem-3* and *fog-1* mRNAs show the most dramatic de-repression, which may contribute to IFE-3's involvement in the sperm to oocyte switch as these genes provide a strong sperm fate signal. We found no evidence that IFE-3 depletion altered translational efficiency of other GSD mRNAs involved in oocyte fate (*tra-1*, *nos-3*, *mog-1*; data not shown). Importantly, translation of IFE-3-responsive GSD mRNAs was not substantially altered by loss of IFE-1, indicating that translational control of these GSD mRNAs is an IFE-3 exclusive function [25]. They were however, de-repressed identically by depletion of IFE-3 or IFET-1 (Fig. 2.9).

The similarity of translational outcomes and developmental defects in GSD strongly suggest a unified function for the IFE-3:IFET-1 mRNP complex in repression to activation switches of certain mRNAs (see Chapters III and IV). Loss of IFE-3 also induced substantial new expression of *fog-1* mRNA in larval germlines even before the sperm to oocyte switch occurred (Fig. 2.8). Additionally, *tra-2* mRNA translational efficiency was partly dependent upon IFE-3 (Fig. 2.7F). However, it is also possible that enhanced *gld-1* mRNA translation (Fig 2.7E) may contribute to decreased translational efficiency of *tra-2*, as GLD-1 is a 3'-UTR-binding protein that is known to repress *tra-2* mRNA directly [148]. The translational efficiency of a known IFE-1-dependent mRNA, *mex-1*, was unperturbed by loss of IFE-3 or IFET-1 (Fig. 2.7 and 2.9), providing further evidence that each eIF4E works selectively with its own mRNA targets. Housekeeping mRNAs *gpd-3* and *tbb-2* appear to be impartial to any single isoform (Fig. 2.7 and 2.9 and [25, 49, 50]. Interestingly, the mRNAs de-repressed upon IFE-3 or IFET-1 loss are also direct FBF targets [161]. Combined with our genetic data, this suggests that IFE-3:IFET-1 may take part in FBF mRNP-mediated repression of some GSD mRNAs. Alternatively, IFE-3 may positively regulate an unknown factor that inhibits FBF expression. Our polysome analysis showed that translation of both *fbf-1* (Fig. 2.7) and *fbf-2* mRNAs (data not shown) were de-repressed upon IFE-3 loss, supporting the latter interpretation. However, any interpretation is complicated by the finding that FBFs autoregulate their own translation [158]. If an IFE-3-FBF cooperation exists, it must exclude germline stem cell maintenance mRNAs, because we found no role for IFE-3 in regulating mitotic activity in the germline (Fig. S2.2) as FBFs are known to do [171].

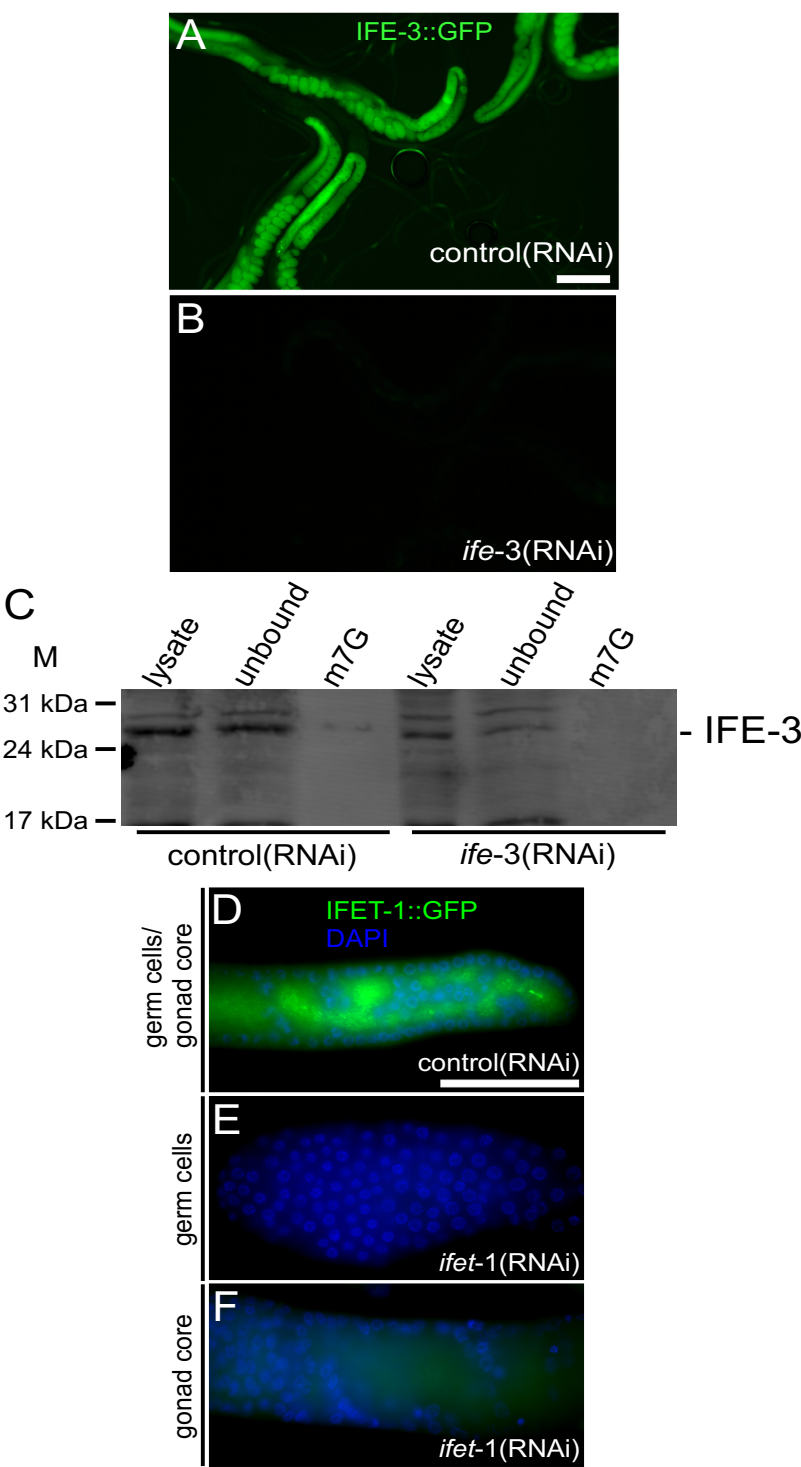
Acknowledgements

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Funding

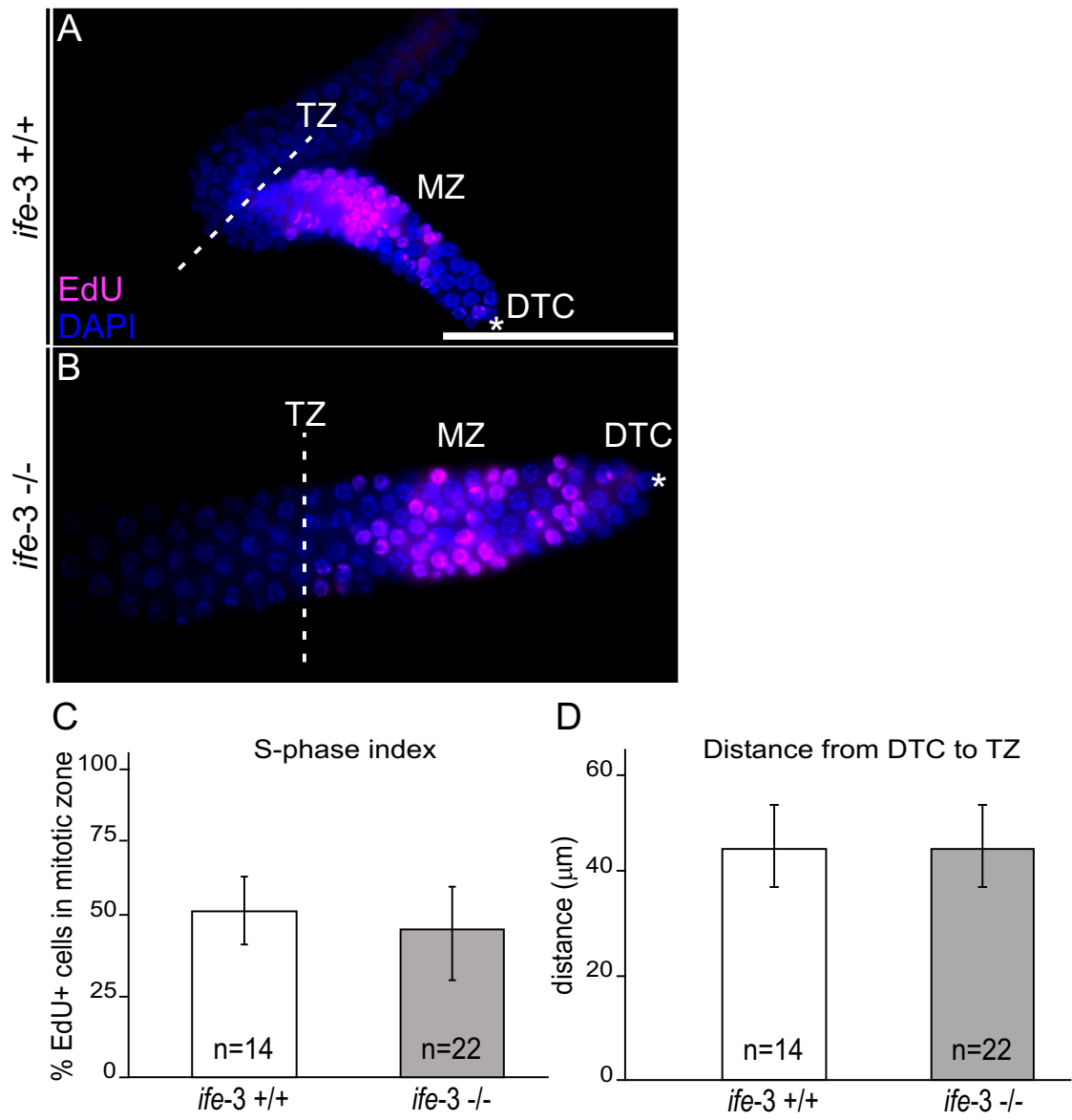
This work was supported by the NSF [MCB-1714264 and MCB-0842475 to B.D.K] and the NIH [AG060373 to M.H.L].

Supplemental Figure 2.1



Supplemental Figure 2.1. IFE-3 and IFET-1 depletion via RNAi. A) control(RNAi)-treated worms carrying *ife-3(eu21[gfp::tev::3xflag::ife-3])V* showing IFE-3 expression throughout the germline and in embryos. B) *ife-3*(RNAi)-treated worms carrying *ife-3(eu21[gfp::tev::3xflag::ife-3])V* showing IFE-3 depletion, as indicated by undetectable levels of GFP::IFE-3. C) Western blot analysis of wild type control(RNAi) and *ife-3*(RNAi)-treated worms showing IFE-3 depletion in the m⁷G bound fraction of the *ife-3*(RNAi) lysate but not in the control(RNAi) lysate. IFE-3 was detected with primary antibody CA82. D) control(RNAi)-treated gonads carrying the *ifet-1::gfp::3xflag* transgene showing IFET-1 expression throughout the germline. E) *ifet-1*(RNAi)-treated gonad carrying *ifet-1::gfp::3xflag* showing loss of IFET-1::GFP from germ cells. F) *ifet-1*(RNAi)-treated gonad carrying *ifet-1::gfp::3xflag* showing IFET-1::GFP depletion in the rachis. These results confirm robust and specific IFE-3 and IFET-1 depletion upon *ife-3*(RNAi) and *ifet-1*(RNAi) treatments, respectively.

Supplemental Figure 2.2



Supplemental Figure 2.2. S-phase index and mitotic zone length in *ife-3(ok191)* animals. A) Wild type hermaphrodite gonad showing EdU (magenta) incorporation in germ cell that underwent S- phase. A) *ife-3(-/-)* hermaphrodite gonad showing EdU incorporation in germ cell that underwent S-phase, similar to what was seen for wild type. C) Quantification of S-phase indices and mitotic zone (MZ) length as measured from distal tip cell (DTC) to transition zone (TZ) in wild type (white) and *ife-3(-/-)* (grey) animals. No statically significant changes were seen in S-phase index or mitotic zone length in *ife-3(-/-)* animals compared to wildtype. Germlines of the indicated genotype were dissected, fixed, and counterstained with DAPI (blue) to visualize nuclear morphology. TZ was defined as the plane where at least two crescent shaped meiotic nuclei could be seen. Scale bar: 50 μ m.

CHAPTER III

DIFFERENTIAL EXPRESSION OF eIF4E ISOFORMS DURING *C. elegans* GERMLINE DEVELOPMENT

The contents of this chapter are adapted from Huggins et. al., Journal of Cell Science 2020, doi: 10.1242/jcs.237990, Published 30 March 2020.

Summary

Here we describe the unique expression patterns and subcellular localization of two translation initiation factor eIF4E isoforms (IFE-1 and IFE-3) during *C. elegans* germline development. Although both isoforms are highly enriched in the germline, IFE-1 is predominantly expressed in late spermatocytes, whereas IFE-3 is expressed most robustly in oocytes. These are times during germline development where each eIF4E is expected to function based on their respective mutant phenotypes. Interestingly, both isoforms localize to perinuclear germ granules. However, IFE-1 and IFE-3 granules are distinct, yet adjacent to one another. Each eIF4E is localized to its respective granule by its cognate eIF4E-interacting protein (IFE-1:PGL-1 and IFE-3:IFET-1), which is expected to inhibit translation for bound mRNAs. Distinct 4EIP dependent *in vivo* localization, and differential mRNA translation suggest independent repression and activation periods for each eIF4E isoform in the germline.

Introduction

Post-transcriptional control of gene expression is paramount in developing germline tissues, and is largely accomplished at the level of translational repression/activation switches [1, 58]. The “decision” between translational repression or activation ultimately determines synthesis of the gene product, and has the potential to quench intrinsic signals or produce novel ones driving cell fate and function (Fig. 1.1). In the germ lineage newly transcribed mRNAs exit the nucleus and come under regulation by germ granules, which are thought to act broadly to control the formation and usage of regulated mRNPs by localizing sequence specific RBPs and interacting with the small-RNA pathways to induce translational/transcriptional silencing (see Chapter I) [58]. We hypothesize that they may also regulate mRNP usage by sequestration of eIF4Es via their cognate 4EIPs, ultimately controlling access to the ribosome (Fig. 1.1 and 2.9). An attractive model is one where: eIF4E:4EIP repression complexes are formed in germ granules after nuclear export, then upon translational activation the mRNP is shuttled to the cytoplasm where eIF4G displaces the resident 4EIP. This leads to ribosome recruitment and functional gene expression. Alternatively, mRNPs that mediate repression after transit into the cytoplasm may be shuttled back to granules for appropriate translational control. Failure to induce proper shuttling and repression may result in ectopic transcript stabilization or translation (Fig. 2.8 and 2.9)

In *C. elegans*, like many other organisms, germ granules (P granules) were first identified as electron dense, non-membrane bound organelles that segregated specifically to germline precursors during embryogenesis [172]. P granules house a large cohort of proteins known to regulate mRNAs post-transcriptionally [73, 173, 174].

Additionally, P granules localize to nuclear pore complexes (NPC) and are thus uniquely poised to regulate translation and stability of mRNAs exiting the nucleus (Fig. 1.1) [175]. We now know that they are highly dynamic RNA-protein condensates that rapidly exchange components with the surrounding cytoplasm during germ cell development, gametogenesis, and embryo cleavage and regulate many aspects of mRNP metabolism (see Chapter I). Interestingly, P granules appear distinct from other hubs of cytoplasmic mRNP regulation such as P bodies and stress granules [59, 60]. However, this observation is likely to be species dependent as chromatoid bodies in mouse spermatogonia were shown to be more closely related to P bodies [61].

Whether or not different germ granules are functionally similar is still in question. Furthermore, are these granules similar in the different cell types of the germline? *i.e.* are P granules in mitotically dividing stem cells the same as P granules in differentiating spermatocytes? At a deeper level, are translational repression/activation switches of different cell types subject to the same modes of regulation by translation initiation machinery? Interestingly, recent evidence has shed some light on this subject by identifying additional granules such as *Mutator* foci and Z granules which are adjacent yet distinct from P granules in pachytene cells [62]. Each of these newly identified granules has functions in transcript silencing via siRNA pathways. Z granules specifically regulate transgenerational epigenetic inheritance [62]. Evidence presented here supports the notion that all perinuclear foci are not identical in regards to translational regulation.

Members of the eIF4 group undoubtedly play an important role in controlling these translational repression/activation switches (see Chapter I). To add a layer of

complexity, it is the norm among the diverse phyla of plants and animals to express multiple isoforms of proteins within this group (eIF4E, eIF4G, and eIF4A) during germline development [176]. Expression of multiple eIF4 group isoforms in the same tissue leads to the possibility of each adopting specialized translational functions over evolutionary time [145]. In fact, our evidence shows that isoforms of eIF4E and eIF4G have adopted specialized function in regard to controlling translation repression/activation switches on distinct populations of mRNA in nematodes [26, 29, 48, 50, 177]. IFE-1 and IFE-3 are the predominant eIF4Es expressed in the germline [45]. Here we show that IFE-1 and IFE-3 localize to distinct perinuclear germ granules in early germ cell and gametes. Additionally, IFE-3 localizes to unique mRNP structures in the shared cytoplasm of the rachis (gonad core). These two eIF4E isoforms exhibit unique dynamics throughout germline development that rely on their cognate 4EIPs, PGL-1 and IFET-1 respectively. IFE-1 binds directly to PGL-1 [52], a core component of P granules that sequesters germ cell mRNAs *in vivo*. PGL-1 is a combined 4EIP-RNA-binding protein that is required for normal fertility, especially at higher temperatures similar to IFE-1 (Fig. 2.5) [54]. Likewise, IFE-3 binds directly to IFET-1 and both are responsible for translational repression of the same GSD mRNAs (Fig. 2.9). We hypothesize that each eIF4E isoform resides in unique germline granules and is responsible for regulating translational repression/activation switches on distinct populations of mRNAs each contributing to different physiological processes.

Results

eIF4E isoforms IFE-1 and IFE-3 localize differently in the germline

Comparing and contrasting differential eIF4E isoform expression might provide insight into their divergent functions and the distinct phenotypes of each null mutant. To obtain a reliable expression pattern for each isoform, we edited endogenous *ife-1* and *ife-3* genes using CRISPR/Cas9 to generate N-terminally fluorescent-tagged mKate2::IFE-1 and GFP::IFE-3, respectively [178] (see Chapter VI). Strains homozygous for each fusion were fertile and superficially wild type, indicating that each was functional as a translation factor (data not shown). Both eIF4Es were robustly expressed in the gonad throughout development, but expression and localization patterns were distinct.

Both IFE-1 and IFE-3 localized to perinuclear germ granules but were also soluble in the cytoplasm of germ cells in the distal gonad (mitotic to early pachytene region; Fig. 3.1A-F). A previous study found IFE-1 in embryonic P granules [52]. Intriguingly, we observed separate perinuclear localization of IFE-1 and IFE-3 in a strain expressing both IFE fusions (Fig. 3.1A-C; insets, carats). IFE-1 granules were larger (570 ± 13 nm, $n=81$) than IFE-3 granules (366 ± 7 nm, $n=74$) and more numerous. Another striking difference between IFE-1 and IFE-3 was seen in the rachis (gonad core), where IFE-3 associated with complex lattice-like structures (Fig. 3.1D-F). IFE-1 did not form similar structures (Fig. 3.1E). In the distal gonad, the soluble fractions of IFE-1 and IFE-3 were overlapping, unlike their respective granules (Fig. 1A and D; merged images, yellow).

In spermatocytes and oocytes, IFE-1 and IFE-3 localization patterns became even more divergent. Using nuclear morphology to stage spermatocytes (Fig. 3.2A-D), we observed that IFE-1 expression was upregulated in 1° spermatocytes, where it formed perinuclear granules, then became largely soluble in 2° spermatocytes. After spermatid budding, IFE-1 was deposited into residual bodies (Fig. 3.2B). Conversely, IFE-3 was soluble throughout spermatogenesis and was diminished early in 2° spermatocytes (Fig. 3.2C). Similarly, in oocytes IFE-3 was strongly expressed and fully soluble, whereas IFE-1 formed granules (Fig. 3.2E-H, carats). Our observations suggest that each eIF4E isoform forms distinct granules in early germ cells, but then each becomes soluble in the respective gametes where they predominate; IFE-1 in spermatocytes and IFE-3 in oocytes. It may be that localization to these granules at the expense of cytoplasmic localization and *vice versa* may be important for translational regulation of mRNAs in these particular cell types (see discussion).

Figure 3.1

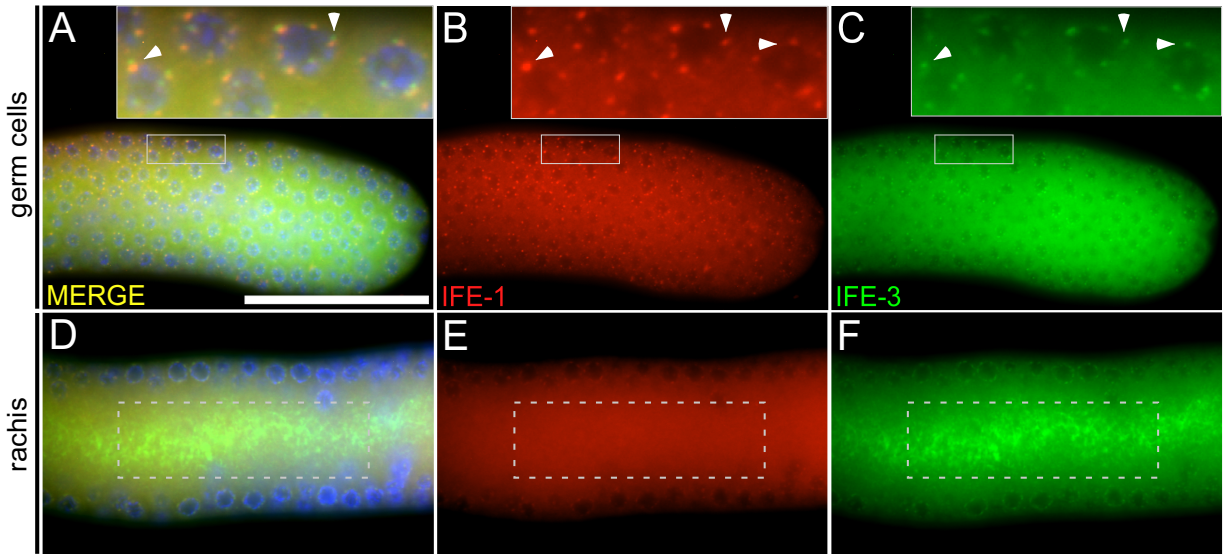


Figure 3.1. IFE-1 and IFE-3 localization in early germ cells. A-C) IFE-1 (red) and IFE-3 (green) co-expression in distal germ cells shows that they localize to adjacent yet distinct perinuclear foci (insets; carats). Cytosolic IFE-1 and IFE-3 overlap substantially (yellow) throughout distal germ cells. D-F) Co-expression in the gonad core (rachis; dashed boxes) shows that IFE-1 is soluble and localized diffusely, whereas IFE-3 associates with extensive lattice-like structures. CRISPR/Cas9 fluorescently-tagged mKate2::TEV::3xmyc::IFE-1 and GFP::TEV::3xflag::IFE-3 were used to evaluate expression of endogenous genes. Gonads were dissected, fixed, and counterstained (DAPI, blue) for nuclear morphology. Scale bar: 50 μ m.

Figure 3.2

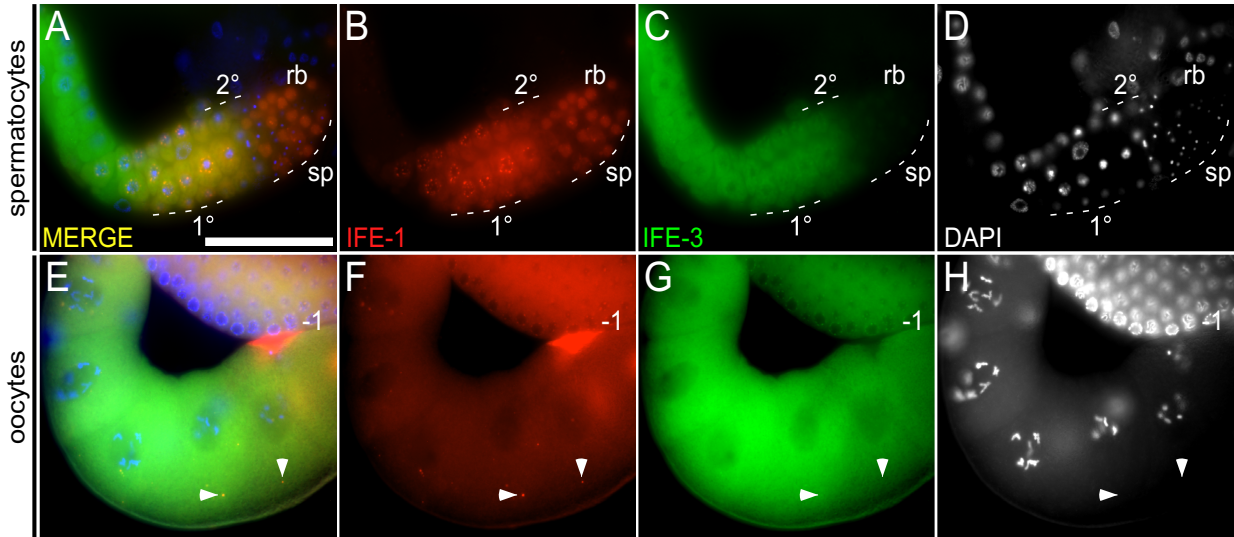


Figure 3.2. IFE-1 and IFE-3 localization in spermatocytes and oocytes of the adult hermaphrodite. A-D) Co-expression in spermatocytes shows that IFE-1 is upregulated and becomes enriched in perinuclear foci in 1° spermatocytes, but then becomes largely soluble in 2° spermatocytes. IFE-1 is deposited into residual bodies (rb) after spermatid budding. IFE-3 does not form perinuclear foci, but instead forms faint cytoplasmic ones, and is diminished during the latter stages of spermatogenesis. E-H) Co-expression in oocytes shows that IFE-1 forms perinuclear foci that move away from the nucleus as oocytes approach the spermatheca, whereas IFE-3 is completely soluble and diffuse. CRISPR/Cas9 fluorescently-tagged mKate2::TEV::3xmyc::IFE-1 and GFP::TEV::3xflag::IFE-3 were used to evaluate expression of endogenous genes. Gonads were dissected, fixed, and counterstained (DAPI, blue) for nuclear morphology. Scale bar: 50 μ m.

IFE-3 localizes to perinuclear granules adjacent to IFE-1:PGL-1 granules

We confirmed IFE-1 residence in P granules of distal germ cells by co-localization with a PGL-1::GFP CRISPR fusion confirming our previous findings [52]. We observed a high degree of overlap for IFE-1 and PGL-1 in early germ cells (Fig. 3.3A-C; insets, carats). We wondered if the perinuclear IFE-3 granules in distal germ cells also co-localized with PGL-1, considering IFE-1 and IFE-3 granules appeared distinct (Fig. 3.1A-C). Unlike IFE-1 foci, the IFE-3 foci we observed were adjacent but clearly separate from PGL-1 (Fig. 3.3A vs. D; insets, carats). We often observed multiple IFE-3 foci decorating a singular PGL-1 granule (Fig. 3.3D-F, inset; top carat). This was never observed for IFE-1 regardless of cell type (Fig. 3.3 and 3.4). These data confirm that IFE-1 is the major P granule associated eIF4E isoform while IFE-3 associates with accessory granules whose function may be related to mutator foci or Z granules (see Chapter IV and discussion).

IFE-1 expression was notably upregulated in 1° spermatocytes compared to early germ cells from L4 hermaphrodites where it was enriched in PGL-1 granules (Fig. 3.4A-C). Importantly, IFE-1 left PGL-1 granules and became soluble when PGL-1 disappeared from 2° spermatocytes (Fig. 3.4B and C, dashed line). These data indicate that PGL-1 likely stabilizes IFE-1 in P granules and when it is degraded IFE-1 released. Similarly, IFE-1 was present in PGL-1 granules that moved toward the cortex as oocytes approached the spermatheca (Fig. 3.4D-F; carats). These data link IFE-1 tightly to PGL-1 in P granule dynamics during gametogenesis in the hermaphrodite. Furthermore, IFE-1 becomes completely soluble during late spermatogenesis, at a time and place during development that matches the defect in *ife-1(bn127)* mutants [48, 52].

Also, in contrast to IFE-1, IFE-3 expression levels were not obviously upregulated during spermatogenesis relative to early germ cells but remained constant (Fig. 3.4G-I vs. 3.4B) and largely soluble rather than enriched in granules (Fig. 3.4H vs. B). IFE-3 was strongly expressed in large oocytes where it also appeared to be completely soluble rather than enriched in granules like IFE-1 (Fig. 3.4J-L vs. D-F, carats). Considering that oocyte growth and embryogenesis have a greater requirement for IFE-3 (Fig. 2.4) than IFE-1 [48], soluble IFE-3 may participate in protein synthetic events that contribute to these physiological processes. In healthy oocytes and spermatocytes IFE-1 localization is tightly linked to its cognate 4EIP, PGL-1, whereas IFE-3 localization appears to be PGL-1 independent in these cell types. Finally, the major soluble eIF4E isoform in spermatocytes is IFE-1, while IFE-3 is the abundant soluble isoform in oocytes the respective gametes where their protein synthetic functions are required for fertility.

Figure 3.3

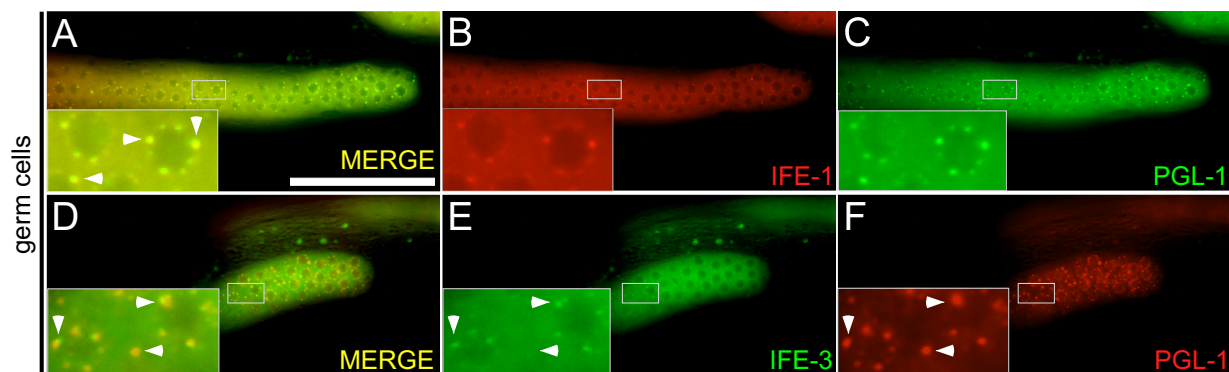


Figure 3.3. IFE-1 and IFE-3 localization in early germ cells relative to PGL-1. A-C) IFE-1 (red) and PGL-1 (green) co-expression in distal germ cells shows that IFE-1 localizes to P granules (insets; white carats) with perfect overlap. D-F) IFE-3 (green) and PGL-1 (red) co-expression in distal germ cells shows that IFE-3 perinuclear granules are adjacent yet distinct from PGL-1 granules (insets; white carats). Multiple IFE-3 foci surrounding a singular PGL-1 focus was frequently observed (D-F; top carat). CRISPR/Cas9 fluorescently-tagged mKate2::TEV::3xmyc::IFE-1 and GFP::TEV::3xflag::IFE-3 were used to evaluate expression. CRISPR-tagged PGL-1 of the reciprocal color was used to track expression of endogenous PGL-1 (also seen in Fig. 3.4-3.6). Gonads were imaged in immobilized live animals. Scale bar: 50 μ m.

Figure 3.4

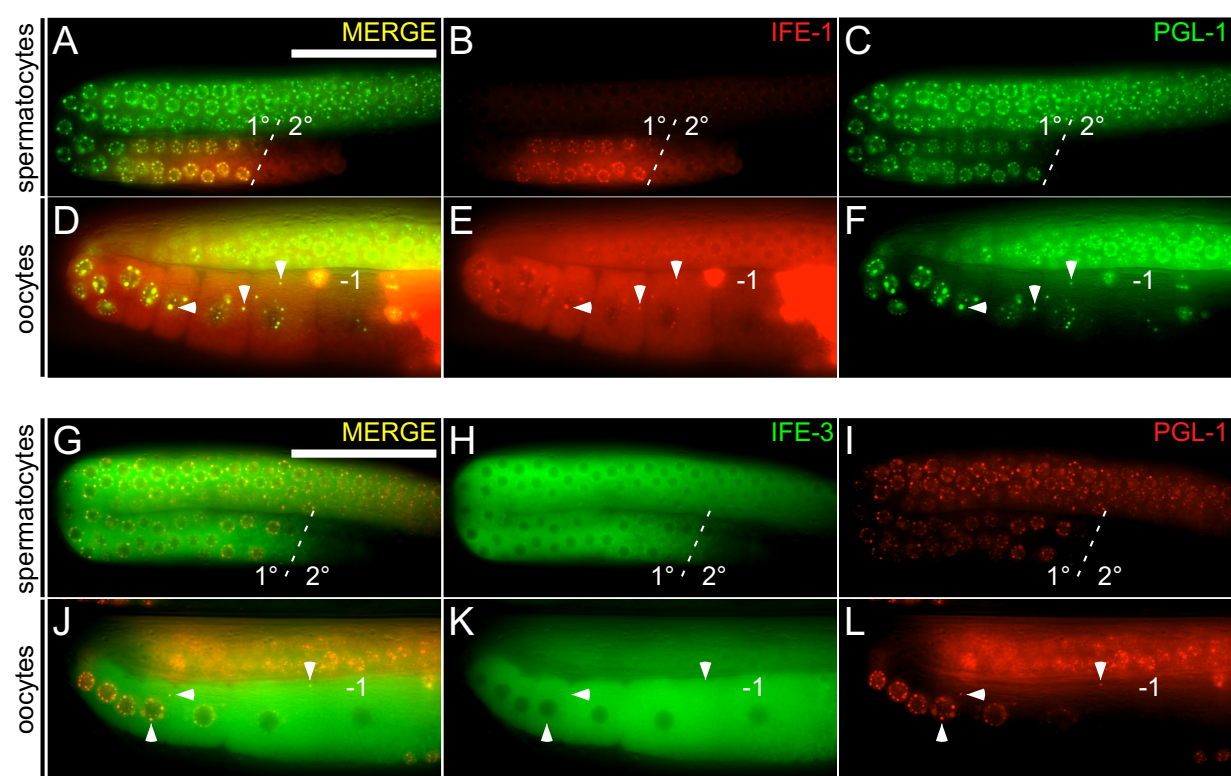


Figure 3.4. IFE-1 and IFE-3 localization in spermatocytes and oocytes relative to PGL-1. A-C) Co-expression in an L4 hermaphrodite gonad shows that IFE-1 (red) is upregulated during spermatogenesis and becomes enriched in PGL-1 granules (green) in primary spermatocytes (1°). Upon PGL-1 disappearance from secondary spermatocytes (2°), IFE-1 is lost from granules and becomes soluble. D-F) Co-expression in an adult hermaphrodite gonad shows that IFE-1 localizes to PGL-1 granules that move toward the cortex as oocytes approach the spermatheca (white carats). G-I) Co-expression in an L4 hermaphrodite shows that IFE-3 (green) expression is relatively constant throughout spermatogenesis and not enriched in perinuclear PGL-1 granules (red). However, IFE-3 does appear to form some faint cytoplasmic granules. J-L) Co-expression in an adult hermaphrodite gonad shows that IFE-3 is completely soluble in oocytes, not localized to PGL-1 granules (white carats). CRISPR/Cas9 fluorescently-tagged mKate2::TEV::3xmyc::IFE-1 and GFP::TEV::3xflag::IFE-3 were used to evaluate expression. Gonads were imaged in immobilized live animals. Scale bar: 50 μ m.

IFET-1 controls IFE-3 localization

IFET-1 is the worm ortholog of mammalian eIF4E transporter protein, which facilitates nucleoplasmic shuttling of eIF4E [165]. IFE-3 and IFET-1 are found in OMA-1 mRNP complexes *in vivo* [80]. Others have shown that recombinant IFET-1 is able to bind each of the recombinant IFEs except IFE-4 [164]. Although IFET-1 shows the potential to bind several IFEs *in vitro*, isolation of native mRNP complexes provides evidence of the IFE-3:IFET-1 interaction only ([80], and Chapter IV). Thus, IFET-1 is thought to represent a cognate 4EIP for IFE-3. We hypothesized that loss of IFET-1 would disrupt IFE-3 localization, while having no effect on IFE-1 localization.

We depleted IFET-1 by RNAi and re-examined localization patterns. IFET-1 depletion was confirmed by loss of IFET-1::GFP expression (Fig. S2.1D-F). In our hands, *ifet-1*(RNAi)-treated animals had similar phenotypes to those reported for *ifet-1(tm2944)*, including poor oocyte growth, occasional *Mog*, and bifurcated distal gonads [53]. Importantly, these are similar phenotypes to those of *ife-3(ok191)* (Fig. 2.4). Depletion of IFET-1 disrupted IFE-3 localization to perinuclear granules in distal germ cells (Fig. 3.5A vs. B; insets, carats) without disrupting perinuclear PGL-1 granules (Fig. S3.1A vs. B; insets). Likewise, *ifet-1*(RNAi) prevented IFE-3 localization to lattice-like structures in the gonad core (Fig. 3.5C vs. D, insets). However, the inverse was not the case. IFET-1 localization to perinuclear granules (Fig. 3.5E vs. F) and lattice-like structures in the rachis (Fig. 3.5G vs. H) were unaffected by *ife-3*(RNAi). These data provide evidence of hierarchical binding of IFE-3 to IFET-1 similar to that found for IFE-1:PGL-1 [52]. Therefore, localization of IFE-3 to perinuclear granules and rachis structures depends on its cognate 4EIP, IFET-1 similar to IFE-1:PGL (Fig. 3.3 and 3.4).

IFE-3 and IFET-1 expression patterns were very similar throughout the germline (Fig. 3.5A, C vs. E, G).

In contrast to IFE-3, IFE-1 localization to perinuclear PGL-1 granules was maintained following depletion of IFET-1 (Fig. 3.5I, J vs. S3.1E, F; insets, carats). However, upon *ifet-1*(RNAi) aberrant aggregates formed in the rachis that contained IFE-1 and PGL-1 (Fig. 3.5L and Fig. S3.1H; insets). These aggregates appeared quite dissimilar to normal IFE-3 or IFET-1 lattice-like structures and are likely unrelated. Similar PGL-1 mis-localization was observed by others in *ifet-1(tm2944)* germlines [53], which likely causes the IFE-1 mislocalization seen here. It is evident from these results that each IFE is localized by its cognate 4EIP. IFET-1 localizes IFE-3 both to perinuclear granules and to structures in the gonad core, but does not contribute to normal IFE-1 localization.

Figure 3.5

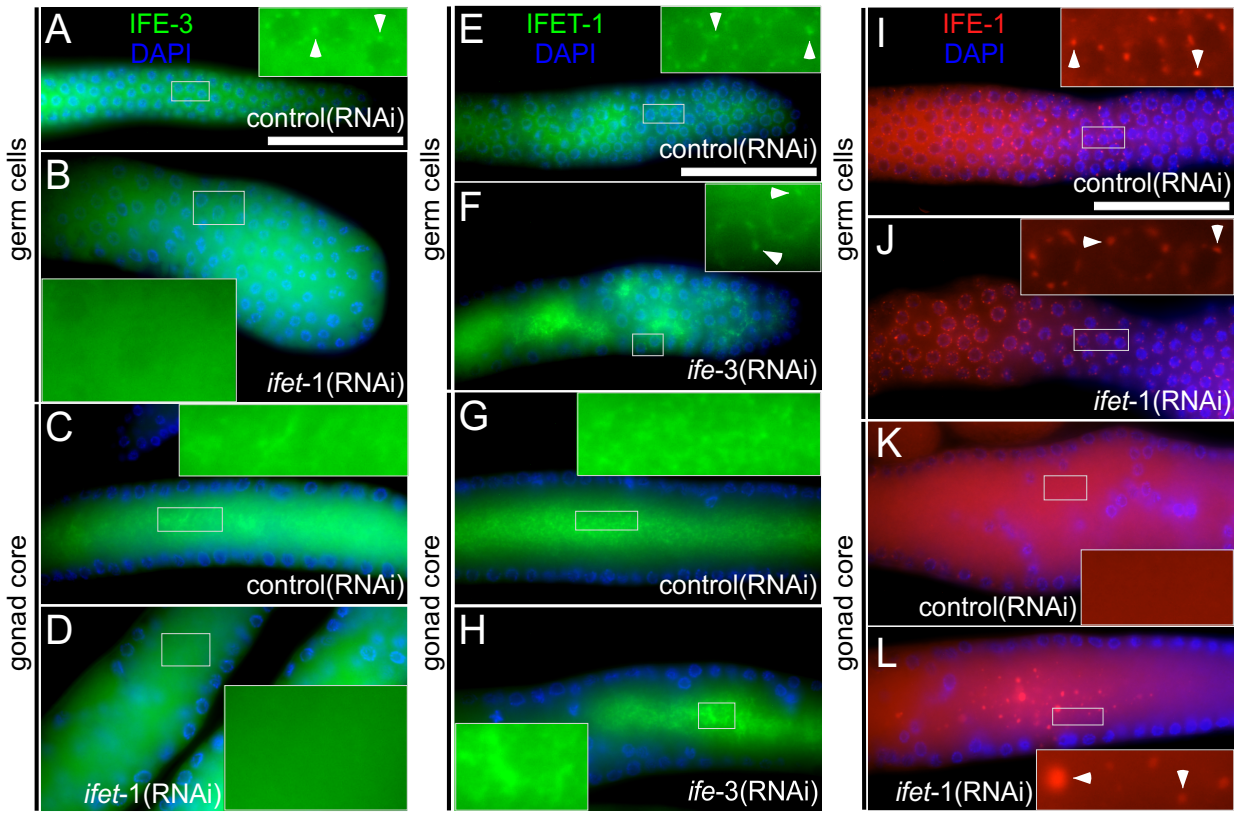


Figure 3.5. IFE-3:IFET-1 localization following depletion of each subunit. A) IFE-3 (green) expression in animals treated with control(RNAi) shows that IFE-3 forms perinuclear granules in distal germ cells (inset; carats). B) IFE-3 expression in animals treated with *ifet-1*(RNAi) shows that perinuclear IFE-3 granules are disrupted (inset). C) IFE-3 expression in animals treated with control(RNAi) shows that IFE-3 also forms lattice-like structures in the gonad core (inset). D) IFE-3 expression in animals treated with *ifet-1*(RNAi) shows that IFE-3 lattice-like structures in the gonad core are also disrupted (inset). E) IFET-1 (green) expression in animals treated with control(RNAi) shows that IFET-1 forms perinuclear granules in distal germ cells (inset; carats). F) IFET-1 expression in animals treated with *ife-3*(RNAi) shows that perinuclear IFET-1 granules remain intact (inset; carats). G) IFET-1 expression in animals treated with control(RNAi) shows that IFET-1 also forms lattice-like structures in the gonad core (inset). H) IFET-1 expression in animals treated with *ife-3*(RNAi) shows that IFET-1 lattice-like structures remain intact (inset). I) IFE-1 (red) expression in animals treated with control(RNAi) shows that IFE-1 forms perinuclear granules in distal germ cells (inset; carats). J) IFE-1 expression in animals treated with *ifet-1*(RNAi) shows that perinuclear IFE-1 granules remain intact (inset). K) IFE-1 expression in animals treated with control(RNAi) shows that IFE-1 does not form structures in the gonad core (inset). L) IFE-1 expression in animals treated with *ifet-1*(RNAi) shows that IFE-1 accumulates into aggregates in the gonad core (inset) (see Fig. S3.1). CRISPR/Cas9 fluorescently-tagged mKate2::TEV::3xmyc::IFE-1 and GFP::TEV::3xflag::IFE-3 were used to evaluate expression. Gonads were dissected, fixed, and counterstained (DAPI, blue). Scale bar: 50 μ m.

IFE-1 and IFE-3 localization responds to oocyte maturation

Serendipitously, we noticed that a large portion of the soluble IFE-3 in oocytes condensed and formed large foci in old hermaphrodites depleted of sperm. Ageing or feminized worms (*e.g. fog-1* mutations/depletion) lack sperm which causes oocytes to arrest in diakinesis [179]. Meiotic arrest in oocytes is common among animals (albeit at different stages depending on species) and responds to hormone signaling *e.g.* progesterone. Hermaphroditic nematode oocytes have evolved to respond to hormone signals (major sperm protein; MSP) derived from sperm stored in the spermatheca. Without MSP to activate oocyte Ephrine receptors these oocytes arrest and stack in the gonad. Other translational regulators like CAR-1 and CGH-1 have been found to condense into large mRNP aggregates in these arrested oocytes [180]. Interestingly, these proteins localize to lattice like structures in the rachis similar to IFE-3 [53].

To investigate IFE-1 and IFE-3 granule dynamics in arrested oocytes, we used *fog-1*(RNAi) to feminize worms and tracked their localization relative to PGL-1. IFE-1 and PGL-1 formed coincident foci in the midplane of *fog-1*(RNAi) arrested oocytes (Fig. 3.6D-F; insets, carats), these granules were also abundant at the cortex (Fig. 3.6G-I; insets, carats). In control(RNAi)-treated animals, IFE-1:PGL-1 foci detached from the nuclear periphery, but never localized substantially to the cortex (Fig. 3.6A-C; insets, carats). These data indicate that IFE-1:PGL-1 granules respond to oocyte arrest by moving to the cortex. In control(RNAi)-treated animals, IFE-3 did not form granules but was diffusely localized throughout the oocyte cytoplasm as in Fig 3.2G. It had no obvious association with PGL-1 granules (Fig. 3.6J-L; insets, carats). Interestingly, soluble IFE-3 condensed into large mRNP aggregates in arrested *fog-1*(RNAi) oocytes,

visible in the midplane (Fig. 3.6M-O) and at the cortex (Fig. 3.6P-R); similar to what was observed in older hermaphrodites that had become depleted of sperm. These newly formed IFE-3 aggregates were sometimes coincident with PGL-1 foci (Fig. 3.6M; inset, top carat). At other times they were obviously distinct (Fig. 3.6M; inset, bottom carat). Clearly, however, the IFE-3 aggregates had a distinct morphology from that of IFE-1:PGL-1 granules (Fig. 3.6G vs. P; insets, carats). IFE-3 condensates were much larger and seemed to surround PGL-1 in a larger complex (Fig. 3.6P-R; insets, carats). What functional significance these IFE-3 mRNP aggregates have in oocytes is unclear, however, these structures have been hypothesized to protect maternal transcripts in preparation of fertilization and maintain mRNP homeostasis (see discussion). Interestingly, our data here suggest that IFE-3 may regulate these mRNAs post-transcriptionally in a manner distinct from IFE-1:PGL-1 mRNPs.

Figure 3.6

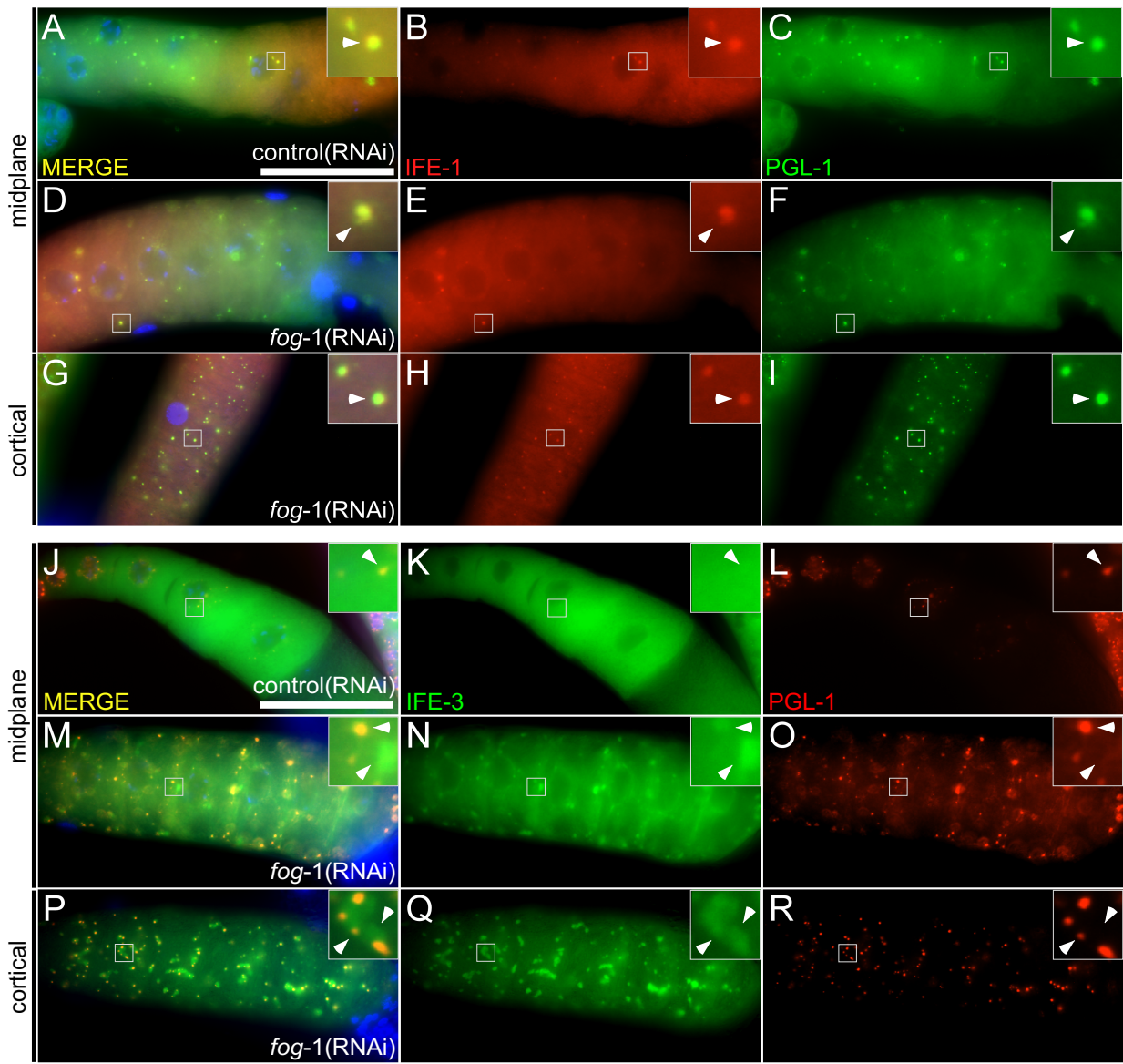


Figure 3.6. IFE-1 and IFE-3 localization in meiotically arrested oocytes. A-C) IFE-1 (red) and PGL-1 (green) co-expression in an adult hermaphrodite gonad treated with control(RNAi) shows that IFE-1 localizes to PGL-1 granules that move toward the cortex as oocytes approach the spermatheca (insets; white carats). D-F) IFE-1 and PGL-1 co-expression in an adult hermaphrodite gonad treated with *fog-1*(RNAi) shows coincident IFE-1:PGL-1 granules in the midplane of arrested oocytes (insets; white carats). G-I) IFE-1 and PGL-1 co-expression in an adult hermaphrodite gonad treated with *fog-1*(RNAi) shows that coincident IFE-1:PGL-1 granules accumulate at the cortex of arrested oocytes (insets; white carats). J-L) IFE-3 (green) and PGL-1 (red) co-expression in an adult hermaphrodite gonad treated with control(RNAi) shows that IFE-3 is completely diffuse throughout the cytoplasm and does not associate with PGL-1 granules (insets; white carats). M-O) IFE-3 and PGL-1 co-expression in an adult hermaphrodite gonad treated with *fog-1*(RNAi) shows that IFE-3 condenses into large aggregates that are sometimes associated with PGL-1 granules in the midplane of arrested oocytes, but their size and morphology are distinct (insets; white carats). P-R) IFE-3 and PGL-1 co-expression in an adult hermaphrodite gonad treated with *fog-1*(RNAi) shows that IFE-3 aggregates and PGL-1 granules accumulate at the cortex of arrested oocytes, but their size and morphology are distinct (insets; white carats). CRISPR/Cas9 fluorescently-tagged mKate2::TEV::3xmyc::IFE-1 and GFP::TEV::3xflag::IFE-3 were used to evaluate expression of endogenous IFEs. Gonads were dissected, fixed, and counterstained (DAPI, blue) to visualize nuclear morphology. Scale bar: 50 μ m.

Discussion

IFE-1 and IFE-3 localization is controlled by their cognate 4EIPs

How each IFE regulates a subset of mRNAs for unique germline roles may be best explained by subcellular localization patterns. For example, these eIF4E isoforms could localize differentially to RNA-granules within the germline and regulate different aspects of mRNA metabolism. Indeed, mammalian eIF4E isoforms were previously shown to differentially localize to P bodies and stress granules, suggesting they differentially regulate mRNA trafficking, translation, and/or turnover [181, 182]. Our studies begin to put *C. elegans* eIF4E isoform-specific regulation into the context of temporal and spatial mRNP complexes and how they are repressed or activated for translation (Fig. 3.7). Here we characterized the two major germline expressed eIF4E isoforms using CRISPR/Cas9 to fluorescently tag endogenous *ife* genes, and tracked localization patterns in germ cells and gametes for the first time.

We found that, although each IFE localized to perinuclear germ granules, the granules were clearly distinct (Fig. 3.1 and 3.2). Our early transgenic studies showed that IFE-1 localized to P granules in embryos via a direct protein-protein interaction with its cognate 4EIP, PGL-1 [52]. CRISPR-tagged IFE-1 likewise localized tightly to PGL-1 granules in early germ cells, primary spermatocytes, oocytes, and embryos confirming our previous observations (Fig. 3.3 and 3.4). In fact, we never observed any IFE-1 foci that were not coincident with PGL-1 throughout development indicating their association is robust.

Figure 3.7

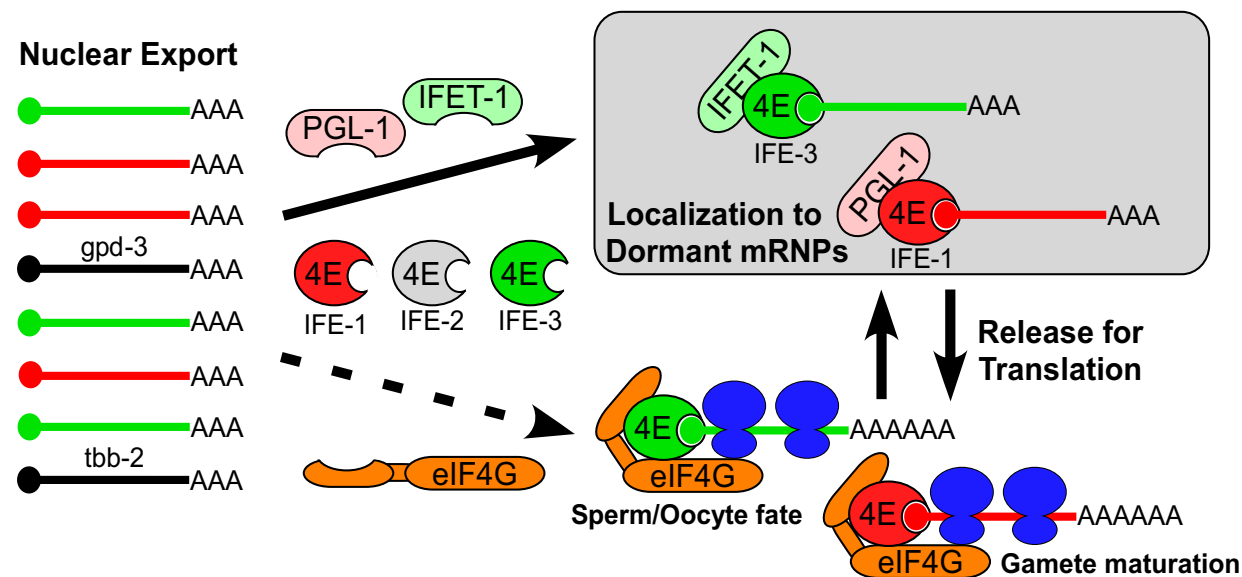


Figure 3.7. Model for eIF4E:4EIP complexes and their roles in translational regulation of specific mRNPs. Differential translational regulation of mRNAs involved in gamete maturation (red), sperm/oocyte fate (green) and housekeeping mRNAs (black) by germline eIF4E isoforms (IFE-1, 2, and -3). Mutually exclusive binding partners for eIF4Es (4EIPs and eIF4G) regulate mRNA selection by forming a repression to activation node after nuclear export. The model, based on this and other studies, is that IFE-1 and IFE-3 use distinct 4EIPs (PGL-1 and IFET-1 respectively) for differential spatial and temporal localization in germ granule mRNPs that prevent translation of their cargo mRNAs. During germ cell development each mRNPs is remodeled independently. IFE-bound mRNAs are released and recruited by eIF4G to actively translating ribosomal complexes. In this model, direct recruitment by IFE-1 or IFE-3 following nuclear export is unlikely (dotted arrow). Consequently, the IFEs and their 4EIPs play cooperative roles in both mRNA repression and activation. IFE-2 is a fully soluble germ cell eIF4E not found in granules, and it recruits a different subset of mRNAs [50].

Previously we described the roles of IFE-1 in regulating translation of maternal P granule associated mRNAs, and being required for spermatocyte cytokinesis [48]. Here we found that IFE-1 left P granules in secondary spermatocytes upon degradation of PGL-1, a time and place during spermatogenesis in which IFE-1's translational function is expected to be required [48]. This suggests that IFE-1 leaves P granules to activate translation of mRNAs critical for spermatocyte cytokinesis. Clearly, both the translational function and subcellular localization of IFE-1 are intricately tied to PGL-1. Surprisingly, CRISPR-tagged IFE-3 also resided in germ granules, but they were distinct from IFE-1:PGL-1 granules (Fig. 3.1-3.6). IFE-3's cognate 4EIP, IFET-1 has important functions in embryogenesis, gonad morphology, and GSD similar to IFE-3 [53]. IFET-1 was also required for translational repression of the same GSD mRNAs (*fog-1*, *fem-3*, *daz-1*) that IFE-3 represses suggesting a unified function for the pair (Fig. 2.9). IFET-1 depletion caused dissociation of IFE-3 from both perinuclear granules and rachis structures, but IFE-3 depletion did not disrupt association of IFET-1 with those same structures (Fig. 3.5A-D vs. 3.5E-H). This suggests a binding hierarchy in which IFET-1 directs IFE-3 localization to both structures where they prevent translation of their bound mRNAs (like GSD mRNAs) in localized mRNPs, and that their functions are tightly linked, similar to IFE-1:PGL-1. Yet localization and translational roles for IFE-3:IFET-1 appear to be distinct from IFE-1:PGL-1.

Control of eIF4E localization and translational function by non-canonical 4EIPs is also apparent from other systems, a *Drosophila* 4EIP called Cup was similarly shown to regulate the localization and inhibit function of an oocyte eIF4E isoform, as well as localization and translational repression of certain germline mRNAs (*nos*, *osk*, *etc.*; see

Chapter I) [75, 90]. While another called Mextli enhances eIF4E-1 activity in ovarian germ cells of the fly [23]. Our recent proteomics shows that nematode Mextli binds to both IFE-1 and IFE-3 in the germline yet PGL-1 binds uniquely to IFE-1, and IFET-1 binds uniquely to IFE-3 (see Chapter IV). These data suggests that eIF4E isoforms many share some 4EIPs while also having unique binding partners. By controlling expression or activity of these 4EIPs at different times during development it would allow for control of which eIF4Es are available to active translation of repressed transcripts at any given time. This represents a potentially powerful mechanism for controlling post-transcriptional gene regulation on a diverse array of germline transcripts through mRNP complexes containing unique eIF4E and 4EIP pairs.

Separate roles for IFE-1 and IFE-3 in oocytes

Interestingly, we found that soluble IFE-3 condensed into large aggregates in the meiotically arrested oocytes of infertile worms (Fig. 3.6). Similar “large P body like mRNPs” have been previously described, and are thought to regulate maternal mRNA turnover and storage as it relates to maturation and fertilization, however, their exact functions are not well understood [180]. Other negative regulators of translation like CAR-1 (Lsm RNA-binding protein), DCP-2 (de-capping enzyme), and CGH-1 (DDX6) localize to these structures upon meiotic arrest, indicating the capacity for these mRNPs to function similar to P bodies and degrade transcripts that are not required in arrested oocytes [180]. Some of these P body related proteins have also been described in structures in the rachis similar to IFE-3 and IFET-1, lending to the possibility that the IFE-3:IFET-1 lattice-like structures described here are functioning as P bodies [53]. Importantly, these IFE-3 aggregates appeared distinct from IFE-1:PGL-1 granules,

having different morphology and distribution (Fig. 3.6G vs. P; insets, carats). IFE-3 condensates were much larger and seemed to surround IFE-1:PGL-1 foci in a larger mRNP complex (Fig. 3.6P-R; insets, carats). What functional significance these IFE-3 mRNP aggregates have in oocytes is unclear, however, biochemical isolation of *in vivo* OMA-1 mRNPs containing both IFE-3 and IFET-1 also copurify with poly(A)-polymerase (GLD-2) and the Ccr4/NotI deadenylase machinery [80, 164]. OMA-1 mRNPs have well described roles in oocyte maturation and meiotic progression [80]. Such complexes are likely important for translational regulation by IFE-3 in these oocytes and may control mRNA polyadenylation or turnover of mRNAs important for maturation or the oocyte-to-embryo transition. Curiously, the *oma-1* mRNA relies heavily on IFE-1 for efficient translation [48], suggesting separable roles for the two eIF4Es that ensure normal translational regulation of oocyte development.

Translational repression/activation switches controlled by eIF4E:4EIP complexes

Where IFE-1 and IFE-3 are localized in separate granules, they likely sequester their preferred mRNAs from active translation in repressed mRNPs. Where both are soluble in the germ cell cytoplasm, they likely associate with eIF4G (IFG-1) to participate in mRNA translation initiation [26, 44]. Each IFE moves to the soluble fraction in a different cell type – IFE-1 in late spermatocytes (Fig. 3.4B) and IFE-3 in large oocytes (Fig. 3.4K). In each case, the relevant IFE becomes soluble at a time when its positive translation activity is expected, based on phenotype. It is well known that 4EBPs and some 4EIPs inhibit translationally productive eIF4E:eIF4G interactions [183, 184]. We propose a model in which IFE-1 and IFE-3 have active and dormant periods that are controlled by their regional expression and cognate 4EIPs, potentially

explaining how each eIF4E isoform regulates its own subset of mRNAs (Fig. 3.7). Dormant periods are those when each eIF4E:4EIP is associated with a unique germ granule and acts as a translational repressor. In active periods, each eIF4E leaves the germ granule and the 4EIP to exert positive initiation activity on its cargo mRNAs, recruiting them to ribosomes for translation. We do not yet have direct demonstration of each regulated mRNA that resides in an IFE-containing mRNP. Improved granule isolation techniques and biochemistry is beginning to elucidate the components of each IFE-containing mRNP (see Chapter IV). The mechanisms by which 4EIPs control eIF4E repression and activation in germ cells may also involve eIF4G competition (like canonical 4EBPs) or cooperation, as well as mRNP localization, shuttling, or remodeling. Our current findings are novel in characterizing two distinct eIF4E:4EIP mRNP complexes in *C. elegans* that have independent dynamics and lead to differential translational control of germline mRNAs.

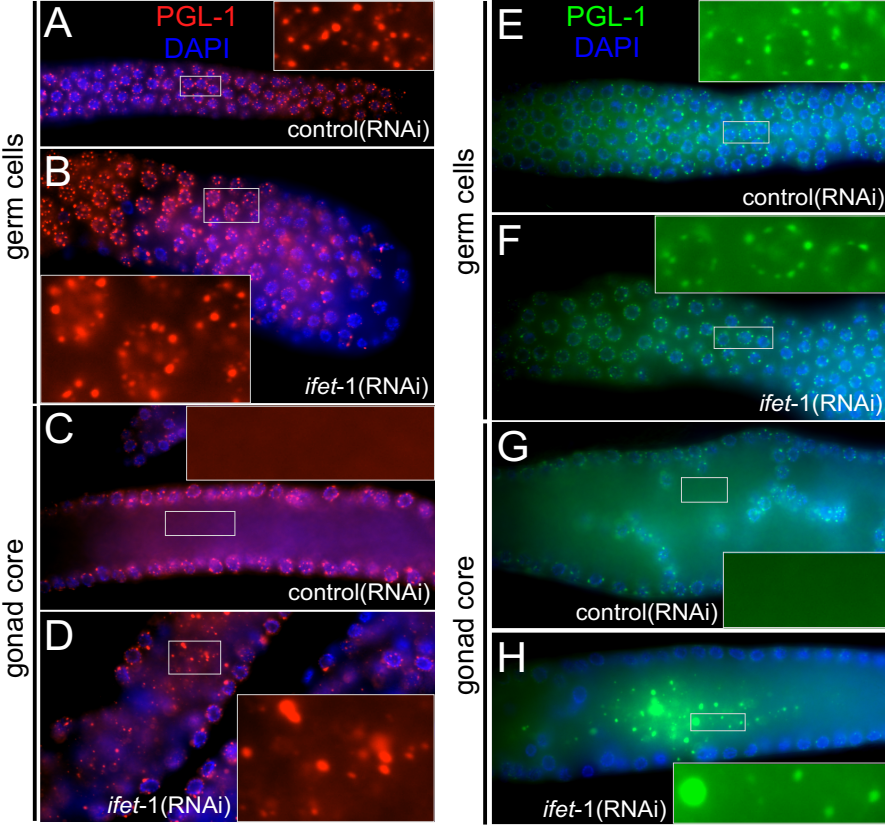
Acknowledgements

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Supplemental Figure 3.1



Supplemental Figure 3.1. PGL-1 localization following IFET-1 depletion. This figure depicts the same gonads as seen in Fig. 3.5 A-D and I-L. A) PGL-1 (red) expression in animals treated with control(RNAi) shows that PGL-1 forms perinuclear granules in distal germ cells (inset, carats). B) PGL-1 expression in animals treated with ifet-1(RNAi) shows that perinuclear PGL-1 granules remain intact (inset). C) PGL-1 expression in animals treated with control(RNAi) shows that PGL-1 does not form structures in the gonad core (inset). D) PGL-1 expression in animals treated with ifet-1(RNAi) shows that PGL-1 accumulates into aggregates in the gonad core (inset). E) PGL-1 (green) expression in animals treated with control(RNAi) shows that PGL-1 forms perinuclear granules in distal germ cells (inset, carats). F) PGL-1 expression in animals treated with ifet-1(RNAi) shows that perinuclear PGL-1 granules remain intact (inset). G) PGL-1 expression in animals treated with control(RNAi) shows that PGL-1 does not form structures in the gonad core (inset). H) PGL-1 expression in animals treated with ifet-1(RNAi) shows that PGL-1 accumulates into aggregates in the gonad core (inset). These PGL-1 aggregates were found to be coincident with IFE-1 aggregates in the gonad core (see Fig. 3.5L). CRISPR/Cas9 fluorescently-tagged PGL-1 was used to track expression of endogenous PGL-1. Gonads were dissected, fixed, and counterstained (DAPI, blue) to visualize nuclear morphology. Scale bar: 50 μ m.

CHAPTER IV

ISOLATION AND CHARACTERIZATION OF IFE-1 AND IFE-3 COMPLEXES

Summary

How each eIF4E isoform differentially regulates translation of subsets of mRNAs in the germline of *C. elegans* is unknown. A plausible explanation is that each eIF4E isoform resides in biochemically separable mRNP complexes that regulate different subpopulations of mRNAs. Cis-acting sequences in germline transcripts and trans-acting factors in the complex likely contribute to the overall mRNA metabolism for each regulated mRNA, similar to what is seen in other developmental models (see Chapter I). Here we find that the major germline eIF4E isoforms, IFE-1 and IFE-3, reside in discrete complexes along with their cognate 4EIPs, PGL-1 and IFET-1 respectively. The factors within each mRNP complex may explain how IFEs differentially regulate the translation of mRNAs in the same germ cells, and why their loss causes very different reproductive phenotypes. We present evidence that IFE-1 associates with proteins that have described roles in P granules and the CSR-1 siRNA pathway, whereas, IFE-3 associates with proteins with roles in P bodies, piRNA biogenesis, snRNP homeostasis, and polyadenylation/deadenylation. Interestingly, there is some overlap in the factors that reside in IFE-1 and IFE-3 complexes. We hypothesize that translationally regulated mRNPs have a preferred IFE, whereas others are indiscriminate. The non-redundant functions of each IFE within these mRNPs likely contributes to their unique roles in germline translation.

Introduction

It is beginning to be appreciated that a common functional component in regulated germline mRNPs is the eIF4E:4EIP cap-binding complex (see Chapter I). This complex regulates cap-dependent translation repression/activation, transcript transport, and mRNA turnover in multiple systems. Our localization and translation data suggest that the major germline eIF4E isoforms, IFE-1 and IFE-3, and their cognate 4EIPs, PGL-1 and IFET-1 respectively, participate in discrete mRNPs in order to differentially control translation and stability of their target mRNAs (see Chapters II and III). Other positive or negative trans-acting factors (RNA-binding proteins, helicases, Argonats, translation factors, *etc.*) are likely resident in these mRNP complexes, acting to control certain aspects of transcript fate *e.g.* repression, transport, decay, or activation. They could associate by either directly interacting with the cap-binding complex itself or indirectly by binding to the mRNA or other trans-acting factors.

Translational activation is critically important for the introduction of novel function in germ cells and relies on the activity of eIF4 members like eIF4E and eIF4G [44-47]. We hypothesize that IFE-1 and IFE-3 form discrete and biochemically separable mRNPs and associate with their own unique set of trans-acting factors which leads to the differential translation and mRNA metabolism we see in maternal and GSD mRNAs respectively (Fig. 2.7 and 2.9). Historically, attempts to isolate tagged, transgenic IFEs and characterize the trans-acting factors within each complex resulted in failure, likely due to the low expression levels of exogenous transgenes (our unpublished data). Here, we describe the biochemical isolation and characterization of CRISPR-tagged IFE complexes from mixed whole worm populations. We found that IFE-1 and IFE-3

associate robustly with only a few shared factors, suggesting that they form mRNP entities distinct from one another. There is however, some overlap in their shared components. We hypothesize that certain developmentally important mRNPs have a preferred IFE, whereas others are indiscriminate, and that the non-redundant function of each IFE within these complexes contributes to their translational functions and the unique phenotypes we see [26, 29, 48-50, 52]. Importantly, we find that IFE-1 associates with a number of unique trans-acting factors that have described roles in P granules, the CSR-1 22G siRNA pathway, and activation of translation. The CSR-1 22G RNAi pathway is one branch of a bipartite secondary siRNA pathway in *C. elegans* that regulates diverse processes including, chromosome integrity, histone mRNA processing, and fertility. The other half of the 22G pathway is controlled by WAGOs [77, 185]. Whereas IFE-3 associates robustly with trans-acting factors that have roles in P body function, polyadenylation/deadenylation, piRNA biogenesis, small-nuclear ribonucleoprotein (snRNP) complex homeostasis, and translational repression.

When investigating the translational consequences of IFE-1 loss we found that certain maternal mRNAs (*mex-1*, *vab-1*, *gld-1*, *oma-1*, *pos-1*, *glp-1*, *etc.*) translated poorly [26, 48]. Several of these mRNAs encode proteins critical for oocyte maturation, and localize to P granules. Importantly, *ife-1(bn127)* mutant hermaphrodites also show deficits in oocyte maturation, linking IFE-1 translational function to phenotype. Additionally, *ife-1(bn127)* mutants display major temperature sensitive defects in sperm cytokinesis [48, 52]. The sperm mRNAs that rely on IFE-1 for this process have not been identified definitively. Others have provided evidence that major sperm protein (MSP) mRNAs and Glc7-like protein phosphatase, GSP-3, mRNAs rely on IFE-1 for

translation [82]. Importantly, GSP-3 RNAi phenocopied the *ife-1(ok1978)* mutant used in these studies. IFE-1 binds directly to PGL-1, a core component of P granules that sequesters germ cell mRNAs *in vivo* [52]. PGL-1 is a combined 4EIP-RNA-binding protein that is required for normal fertility [54]. Clearly, IFE-1 is linked to P granules and their regulation of germline transcripts, however, what other trans-acting factors are resident in IFE-1:PGL-1 complexes are unknown.

Others have implicated IFE-1 in the positive translational regulation of sperm transcripts in a epigenetic feed-forward loop regulated by Argonats CSR-1 (22G binding) and ALG-3/-4 (26G binding) [77, 78]. Interestingly, ALG-3/-4 and CSR-1 are important for thermotolerance during spermatogenesis like IFE-1, and worms carrying mutations in these genes have similar, albeit distinct, phenotypes to that of *ife-1(bn127)* mutants [48, 52]. Additionally, ALG-3/-4 show similar expression patterns to those described here for IFE-1 (Fig. 3.2 and 3.4). There appears to be some disagreement about the exact role of P granule component CSR-1 during spermatogenesis, where different labs have put forth conflicting models for CSR-1 regulation of sperm transcripts [77, 185]. However, it is clear that CSR-1 is involved in regulating the overall fate of these spermatogenic mRNAs, yet the exact mechanisms of discrimination in male and female germlines is unclear. Thus, the aforementioned Argonaut proteins represent potential candidates in IFE-1:PGL-1 mRNP complexes. Using biochemical approaches to isolate and characterize IFE-1 associated mRNPs and accessory proteins in the complex could provide insights into how IFE-1 regulates its mRNAs and contributes to oocyte maturation and sperm development. Furthermore it may help to clarify IFE-1's

role in the ALG-3/-4/CSR-1 feed-forward loop that control sperm transcript accumulation and usage in the germline.

We found that IFE-3 and IFET-1 identically repressed translation of the same GSD mRNAs (*fog-1*, *fem-3*, *daz-1*, *etc.*) (Fig. 2.7 and 2.9). Finding that IFE-3 had a strong repressive role on these GSD mRNAs was unexpected considering it is homologous to human eIF4E-1, the canonical eIF4E isoform known to catalyze translation [145, 186]. We also found that *fog-1* transcripts accumulated to inappropriate levels in both L4s and adult *ife-3(ok191)* mutant hermaphrodites, suggesting that IFE-3 is directly or indirectly responsible for *fog-1* turnover and/or transcriptional repression (Fig. 2.8). Furthermore, IFET-1 was responsible for IFE-3 localization to perinuclear germline granules distinct from P granules and in structures in the rachis (Fig. 3.5). Importantly, these structures have been found to contain P body components like CAR-1 and CGH-1 [53]. IFE-3 may also be involved in the RNAi pathway; the penetrance of *ife-3*(RNAi)-induced sterility is lower than that of the null allele (22% vs. 79% at 25°C respectively) (Fig. 2.4E and 2.6A). These data cumulatively support a model where these IFE-3:IFET-1 granules are sites of translational repression and possible mRNA turnover. It may be that IFET-1 sequesters IFE-3 from eIF4G or directs IFE-3 to mRNP complexes with translational repression functions. Recent studies found IFE-3 associated with a novel mRNP called PETISCO that has functions in piRNA biogenesis and is critical during embryogenesis [55]. Importantly, *ife-3* null mutations are also embryonic lethal (Fig. 2.4). Others have isolated LIN-41 and OMA-1 mRNP complexes that contained IFE-3, IFET-1, and components of the polyadenylation and deadenylation machinery (GLD-2 and Ccr4-Not1) [80, 187, 188]. LIN-41 and OMA-1

have well-described roles in regulating oocyte maturation and translational repression/activation complexes during the maternal-zygotic transition [188]. Here we described that IFE-3 has roles in oocyte growth and meiotic progression, and forms large RNP foci that are distinct from IFE-1:PGL-1 during meiotic arrest caused by *fog-2* RNAi (Fig. 3.6). Thus, it is likely that OMA-1 and LIN-41 participate in mRNPs with IFE-3:IFE-1 and act to broadly regulate oocyte meiotic progression and maintaining maternal mRNA homeostasis by regulating transcript turnover and translational repression/activation switches. However, it is unknown what is necessary and sufficient for either translational activation or repression of these mRNPs, but the IFE-3:IFE-1 node surely plays a major role. Probing these mRNPs biochemically can provide further insight into how these complexes are remodeled to ensure appropriate post-transcriptional gene regulation and introduce novel proteins in oocytes and embryos.

Results

Validation of IFE-1 and IFE-3 immunoprecipitations

In order to characterize trans-acting factors in each eIF4E complex we immunoprecipitated mKate2::Myc::IFE-1 and GFP::Flg::IFE-3 individually from a CRISPR-engineered strain containing both endogenous translational-fusions using alpaca-derived nanobody traps targeting mKate2 and GFP, respectively. Identifying other factors in the complex is likely to provide insight into how each IFE differentially regulates translation in the germline (Fig. 2.7 and 2.9). Importantly, this strain containing both engineered *ife* genes showed no obvious developmental defects, and had wild type fecundity, indicating that each CRISPR-tagged eIF4E isoform was functional (our unpublished data). As non-specific binding controls for mKate2::Myc::IFE-1 and

GFP::Flg::IFE-3 we also immunoprecipitated mKate2::PH::Flg and GFP::PH::Flg from separate strains using the same nanotrap approach. This allowed us determine which factors were specific to the IFE complex, and not associated with the fluorescent tag alone. The PH (Pleckstrin homology) domain localizes mKate2 and GFP control to the membrane, Where they are likely to interact with complexes not involved in translational regulation, and could serve as a positive control as well.

Following immunoprecipitation of mKate2::Myc::IFE-1 and GFP::Flg::IFE-3 complexes and their respective controls, we performed SDS-PAGE in conjunction with western blot analysis and total protein staining via SYPRO™ Ruby on worm lysates (L), unbound fractions (U), and bound fractions (B) to determine the efficiency and specificity of each pull down (Fig. 4.1 and 4.2). For western blot analysis we used an antibody that recognizes mKate2 for the detection of mKate2::Myc::IFE-1 and its control. Likewise, we used an antibody that recognizes GFP for the detection of GFP::Flg::IFE-3 and its control. mKate2::Myc::IFE-1 and GFP::Flg::IFE-3 translational-fusions were found to be approximately 55 and 59 kDa in size respectively (Fig 4.1; red and green arrows). mKate2::PH::Flg and GFP::PH::Flg controls were found to be approximately 40 kDa (Fig 4.1; black and white arrows). Additional prominent bands at 55 kDa and 54 kDa can be seen when using the mKate2 antibody; we found these bands in multiple strains indicating that they are non-specific bands found generally in *C. elegans* lysates and were thus, ignored. It should be noted that the mKate2 derived antibody used for nano-trap immunoprecipitation was different from the one used for western blot analysis. As such, the nano-trap mKate2 antibody captured only one species (Fig. 4.1; red arrow). Importantly, we found that both mKate2::Myc::IFE-1 and

GFP::Flg::IFE-3 and their controls were captured efficiently, as seen by depletion of the targets in the unbound relative to the bound fractions (Fig. 4.1; U vs. B).

We also performed total protein staining using SYPRO™ Ruby to determine unique banding patterns and approximate yield for the proteins associated with each IFE complex and their respective controls (Fig. 4.2). Using this approach allowed us to identify any abundant proteins that were associated with the IFE complexes specifically, relative to their cognate fluorescent proteins. We found that the pattern in the GFP::Flg::IFE-3 bound fraction was unique and had a number of prominent bands that did not appear in the GFP::PH::Flg control, and that the yield for GFP-targeted pulldowns was quite high (Fig. 4.3). The total protein yield from the mKate2::Myc::IFE-1 bound fraction and its control were quite weak comparatively (Fig. 4.3). However, mKate2::Myc::IFE-1 can be seen in its bound fraction (Fig. 4.2; red arrow), as well as a band at 59 kDa. mKate2::PH::Flg can also be seen clearly in its respective bound fraction (Fig. 4.2; black arrow), along with bands at 59 and 27 kDa. Cumulatively from these results we determined that the GFP-targeted immunoprecipitations were both efficient and specific, making them suitable for downstream characterization via liquid chromatography and mass spectrometry (LC-MS/MS). The mKate2-targeted immunoprecipitations were relatively inefficient compared to GFP, and the total protein recovered was quite low based on SYPRO™ Ruby staining. However, LC-MS/MS is a very sensitive technique, thus we decided that these samples were also adequate for downstream characterization.

Figure 4.1

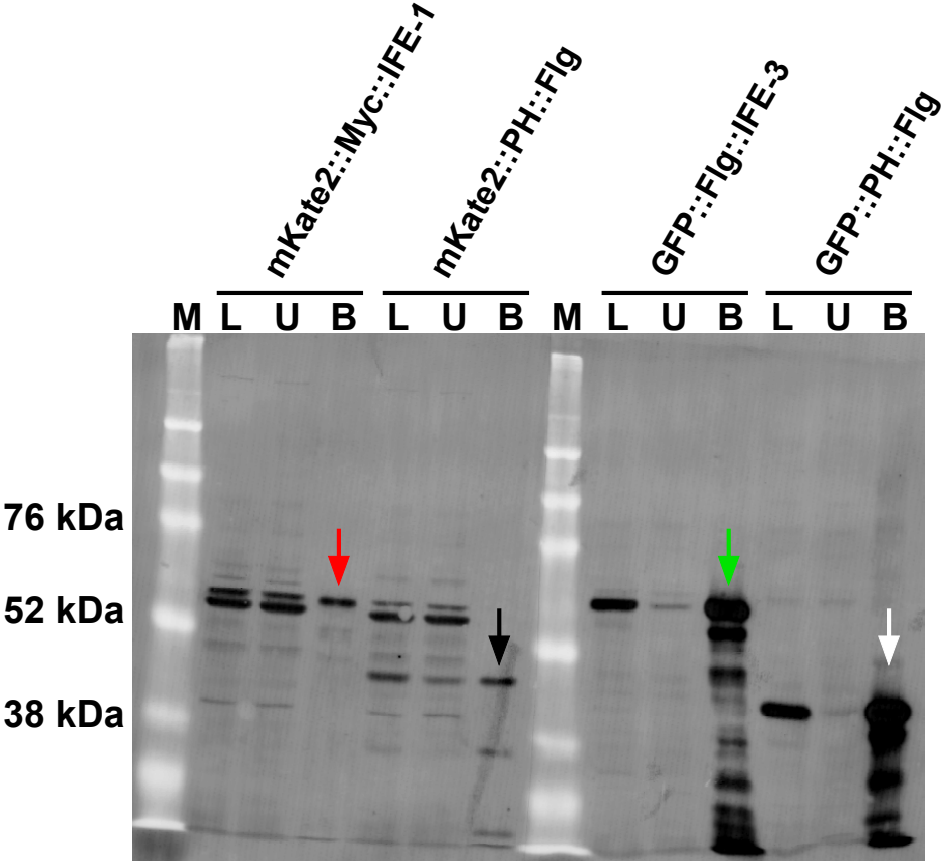


Figure 4.1. Western blot analysis of IFE-1 and IFE-3 immunoprecipitations.

Western blot showing the worm lysates (L), unbound fractions (U), and bound fractions (B) for mKate2::Myc::IFE-1, mKate2::PH::Flg, GFP::Flg::IFE-3, and GFP::PH::Flg immunoprecipitations. mKate2::Myc::IFE-1 (red arrow) and GFP::Flg::IFE-3 (green arrow) translational-fusions were found to be approximately 55 and 59 kDa in size respectively. mKate2::PH::Flg (black arrow) and GFP::PH::Flg (white arrow) controls were found to be approximately 40 kDa in size. The immunoprecipitations were efficient based on depletion of the target in U and enrichment in B. Lanes 1-7 were probed with an antibody that recognizes mKate2, and lanes 8-14 were probed with an antibody that recognizes GFP. Molecular weight markers in lane M are labeled at 38, 52, and 76 kDa for reference.

Figure 4.2

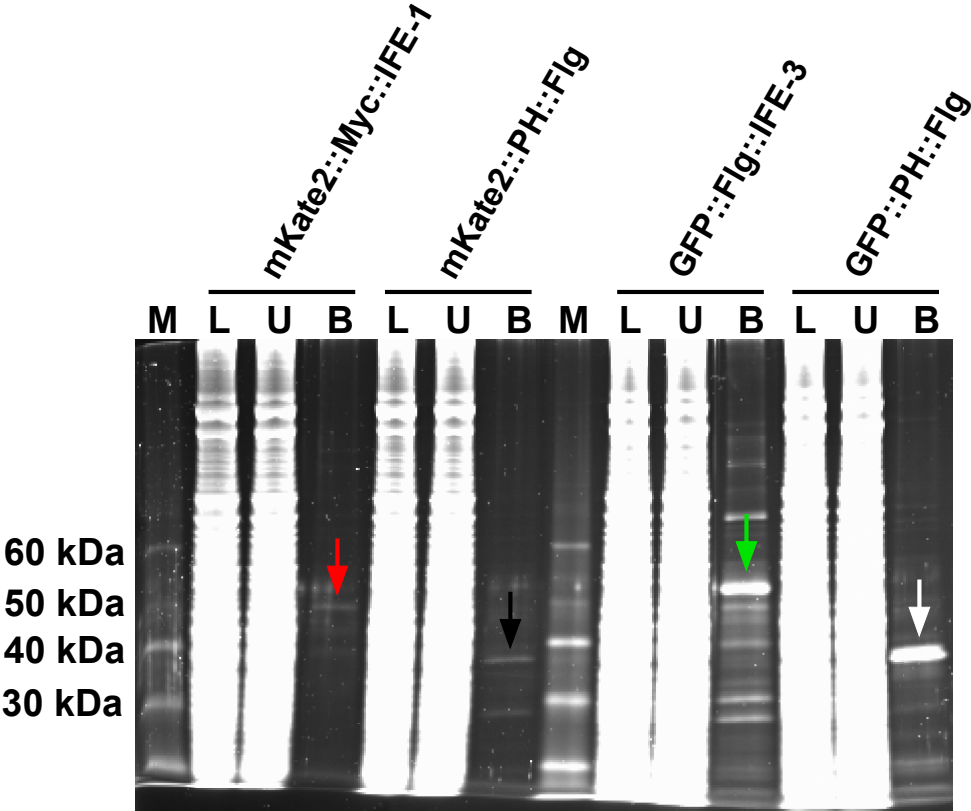


Figure 4.2. SYPRO™ Ruby staining of IFE-1 and IFE-3 immunoprecipitations.

SYPRO™ Ruby stained gel showing the worm lysates (L), unbound fractions (U), and bound fractions (B) for mKate2::Myc::IFE-1, mKate2::PH::Flg, GFP::Flg::IFE-3, and GFP::PH::Flg immunoprecipitations. Bound lanes for the mKate2::Myc::IFE-1 and mKate2::PH::Flg immunoprecipitations shows the target translational-fusions (red arrow and black arrow respectively), but showed little else in terms of prominent banding patterns. Bound lanes for the GFP::Flg::IFE-3 and GFP::PH::Flg immunoprecipitations shows the target translational-fusions (green arrow and white arrow respectively), and that the GFP::Flg::IFE-3 immunoprecipitation had a number of prominent bands that did not appear in the GFP::PH::Flg control. Molecular weight markers in lane M are labeled at 30, 40, 50, and 60 kDa for reference.

Comparative proteomic analysis of IFE-1 and IFE-3 complexes

The bound fractions of mKate2::Myc::IFE-1 and GFP::Flg::IFE-3 and their respective controls were subjected to on bead tryptic digest and analyzed in technical duplicate using LC-MS/MS. Coverage for mKate2::Myc::IFE-1 and its control were 46% and 32%, respectively, and coverage for GFP::Flg::IFE-3 and its control were 58% and 37%, respectively. We assessed the total spectral counts derived for each potential interactor as a semi-quantitative measure of the strength of association with each IFE complex [189]. From these data we generated two distinct lists of proteins that interact with IFE-1 and IFE-3 complexes. The first, a “low stringency” list was used to capture all potential interacting proteins even if the association was weak. These “low stringency” lists were populated by proteins that had at least an average spectral count of 1 in the two technical replicates, and had at least a two-fold enrichment in the IFE pulldown relative to its respective control. The second, a “high stringency” list was used to identify more robust interactions suited for downstream genetic and biochemical investigations. These “high stringency” lists were populated by proteins that had at least an average spectral count of 4 in the two technical replicates, and had at least a four-fold enrichment in the IFE pulldown relative to its respective control.

In the “low stringency” comparison we found that IFE-1 associated with 113 unique factors, whereas, IFE-3 associated with 278, and that IFE-1 and IFE-3 complexes had 37 common factors (Fig. 4.3). The somewhat high number of shared factors in this “low stringency” list suggests a level of relatedness between the two mRNP complexes. Possibly highlighting shared function the germline. In the “high stringency” comparison we found that IFE-1 associated with 18 unique factors, whereas

IFE-3 associated with 45, and that IFE-1 and IFE-3 complexes had only 3 common factors (Fig. 4.4). This indicated that although a few components are strongly shared by IFE-1 and IFE-3 complexes, they are quite dissimilar and likely represent discrete entities. We infer from these data that certain discrete mRNPs strongly prefer one IFE over another, whereas other mRNPs may be less discriminant. The three shared factors in IFE-1 and IFE-3 complexes were MEL-46 (DDX20 helicase homologue), MXT-1 (Mextli 4EIP), and GYF-1 a protein with unknown function (see discussion). Overall this “high stringency” list contained less noise (higher enrichment in IFE sample relative to its control), had only robustly interacting factors, and identified mostly proteins that have known roles in RNA regulation in the germline, allowing us to focus on the more relevant hits. We found that the factors that strongly associate with IFE-1 have defined roles in translational activation, P granule function, and the CSR-1 siRNA pathway, whereas the factors that associate with IFE-3 have defined roles in P body function, polyadenylation/deadenylation, piRNA biogenesis, small-nuclear ribonucleoprotein (snRNP) complex homeostasis, and translational repression (see below).

Figure 4.3

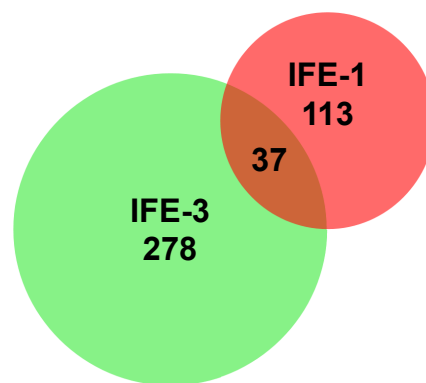


Figure 4.3. Low stringency comparison of IFE-1 and IFE-3 associated proteins.

Area proportional Venn diagram showing the number of proteins found associated with IFE-1 (113; red) and IFE-3 (278; green) immunoprecipitations, as well as, the 37 common proteins found associated in these two complexes by LC-MS/MS. These “low stringency” lists were populated by proteins that had at least an average spectral count of 1 in the two technical replicates, and had at least a two-fold enrichment in the IFE pulldown relative to its respective control.

Figure 4.4

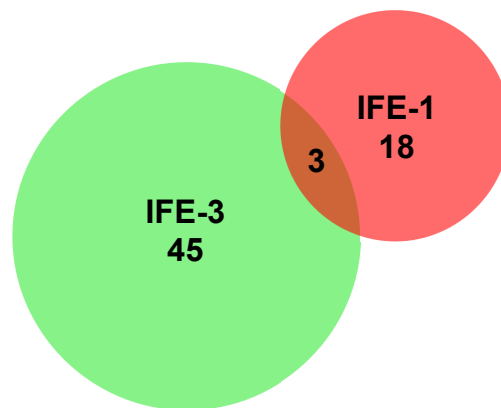


Figure 4.4. High stringency comparison of IFE-1 and IFE-3 associated proteins.

Area proportional Venn diagram showing the number of proteins found associated with IFE-1 (18; red) and IFE-3 (45; green) immunoprecipitations, as well as, the 3 common proteins found associated in these two complexes by LC-MS/MS. These “high stringency” lists were populated by proteins that had at least an average spectral count of 4 in the two technical replicates, and had at least a four-fold enrichment in the IFE pulldown relative to its respective control.

Additionally, we compared the 20 most robustly IFE-1 and IFE-3 associated proteins that had at least a 4-fold enrichment relative to controls (Tables 4.1 and 4.2 respectively). Among those that most strongly interact with IFE-1 we found IFG-1, the sole eIF4G in worms, as expected for an eIF4E isoform. Other noteworthy factors include the constitutive P granule components PGL-1, the combined RNA-binding protein and cognate IFE-1 4EIP, its closely related homologue PGL-3, and the DDX4/Vasa helicase orthologue GLH-1 (Table 4.1). Additionally, we found that the 22G siRNA pathway Argonaut protein CSR-1, and a protein named CID-1/CDE-1 that negatively regulates CSR-1 associated small-RNAs also associated with IFE-1 (Table 4.1) [190]. These data reinforce the idea that IFE-1 has functions in translational activation, and acts as the predominant P granule eIF4E isoform where it functions with PGL-1/GLH-1/CSR-1 in the regulation of germline transcripts, possibly those related to spermatogenesis (see discussion). Those proteins that associate most robustly with IFE-3 complexes include: known translational repressor and cognate IFE-3 4EIP, IFET-1, all components of the PETISCO complex (PID-1/-3, ERH-2, TOFU-6, and TOST-1) [55], P body related factors CAR-1 (LSM RNA-binding protein) and CGH-1 (DDX6 helicase), and snRNP related factors like SMN-1, GEMIN proteins, and the nuclear cap-binding complex component NCBP-1. Importantly, IFG-1 was not robustly associated with IFE-3 and only appeared in the “low stringency” list. This suggests that IFE-3 and IFG-1 may only interact transiently or not to the magnitude seen with IFE-1. These data combined with our translation data (see chapter II) strongly implicate IFE-3 as an eIF4E that has evolved to function in translational repression and P bodies.

We also found RNP-8, GLD-2, and GLD-4 associated with IFE-3 in the “high stringency” list (Fig. 4.4), implicating it in regulation of poly(A) tail length [191]. Importantly, the poly(A)-polymerase, GLD-2, is activated by RNP-8 and both were shown to be important for germline sex-determination and oocyte development similar to IFE-3 [115]. Additionally, in the “low stringency” lists, we found that another regulator of germline sex-determination, FBF-2, associated with IFE-3 (Fig. 4.3). This very likely explains the role of IFE-3 in translational repression of GSD mRNAs like *fog-1* and *fem-3*, as FBFs are RNA-binding protein known to repress translation of these transcripts directly [153, 161, 192]. These findings further distinguishing the roles of IFE-1 and IFE-3 in the germline by putting them in the context of very different mRNPs. Follow up investigations of how these complexes are remodeled in response to developmental stimuli are warranted, and should provide mechanistic insight to how eIF4Es are functioning uniquely to control translation of distinct populations of mRNAs in the germline.

Table 4.1. Robust IFE-1 interacting proteins

Protein name	Molecular weight	Enrichment ratio*	Total spectral count	Total unique peptide
CID-1 [#]	163 kDa	DIV/0 [†]	248	56
IFG-1	170 kDa	DIV/0	183	51
GYF-1	121 kDa	DIV/0	104	46
IFE-1[#]	24 kDa	DIV/0	58	4
PGL-1 [#]	83 kDa	54.00	27	18
MXT-1	57 kDa	DIV/0	27	11
F59A3.12	34 kDa	DIV/0	18	11
T26A5.2	96 kDa	DIV/0	17	13
TUFM-1	55 kDa	7.25	15	13
GLH-1 [#]	80 kDa	6.00	9	9
MEL-46	110 kDa	DIV/0	6	6
ROD-1	234 kDa	4.00	6	6
RPL-16	23 kDa	5.50	6	6
F58G11.3	74 kDa	DIV/0	8	7
NSPD-5	7 kDa	10.00	5	2
PGL-3 [#]	75 kDa	DIV/0	5	5
CCT-4	58 kDa	4.50	5	5
F08F3.4	40 kDa	4.00	4	4
CSR-1 [#]	116 kDa	9.00	5	5
C30G12.2	31 kDa	4.00	4	4

[†] Not found in the control sample (mKate2::PH::Flg)

* Total spectral count in target sample (mKate2::Myc::IFE-1) / control sample (mKate2::PH::Flg)

[#] Known P granule related proteins

Row in red denotes the immunoprecipitation target

Rows in cyan denote known physical interactors of IFE-1

Table 4.2. Robust IFE-3 interacting proteins

Protein name	Molecular weight	Enrichment ratio*	Total spectral count	Total unique peptide
IFET-1 ^{#‡\$}	84 kDa	161.75	324	30
PID-3 [‡]	35 kDa	DIV/0 [†]	262	19
MEL-46 [‡]	110 kDa	DIV/0	195	62
TOFU-6 [‡]	41 kDa	DIV/0	174	17
T21B10.3	137 kDa	67.25	135	54
IFE-3 ^{‡\$}	28 kDa	75.67	114	10
GYF-1	121 kDa	21.63	87	48
ERH-2 [‡]	13 kDa	DIV/0	69	7
SMN-1 [‡]	23 kDa	117.00	59	7
TOST-1 [‡]	16 kDa	DIV/0	46	14
CAR-1 ^{#‡\$}	38 kDa	4.33	33	18
MXT-1	57 kDa	31.00	31	16
PID-1 [‡]	19 kDa	DIV/0	24	11
PYR-1	243 kDa	12.67	19	18
D1007.4 [‡]	19 kDa	DIV/0	15	10
F10E9.5 [‡]	24 kDa	DIV/0	14	9
T08G11.1	359 kDa	4.17	13	13
CGH-1 ^{#‡}	49 kDa	7.25	15	9
NCBP-1	93 kDa	DIV/0	11	10
T23E7.2	98 kDa	DIV/0	10	10

[†] Not found in the control sample (GFP::PH::Flg)

* Total spectral count in target sample (GFP::Flg::IFE-3) / control sample (GFP::PH::Flg)

Known P body related proteins

Row in red denotes the immunoprecipitation target

Rows in cyan denote known physical interactors of IFE-3

[‡] Found in IFE-3 immunoprecipitations [55]

^{\$}Found in IFET-1 immunoprecipitations (personal communication, P. Boag)

Discussion

IFE-1 and IFE-3 reside in discrete mRNPs with their cognate 4EIPs

Unique phenotypes, differential translation, and discrete localization patterns suggests that eIF4E isoforms, IFE-1 and IFE-3, reside in separable mRNPs in the germline of *C. elegans* (see Chapters I and II). Here we sought to gain a deeper understanding of these mRNP complexes by biochemically isolating CRISPR-tagged IFE-1 and IFE-3. Broadly, we found that IFE-1 and IFE-3 robustly associated with unique factors with well-described roles in P granules and P bodies respectively. There was some overlap in IFE-1 and IFE-3 associated proteins (including the IFEs themselves), especially when “low stringency” parameters are used to cull potential hits (Fig. 4.3). We envision two possibilities that could explain this phenomenon: 1) Germline eIF4E isoforms form similar mRNP complexes with some shared trans-acting factors. 2) Biochemical isolation of one eIF4E complex inadvertently isolated the other, albeit weakly, due to proximity *i.e.* bridging effect. It is our hypothesis that there may be limited mobility of eIF4Es in some mRNP complexes, and that other mRNPs strongly “prefer” one IFE over the other for reasons not clear. This “preference” leads to the non-redundant functions we see in translation. Increasing the stringency of the culling parameters showed us that only three factors associated strongly with both IFE-1 and IFE-3. The three strongly shared factors were MEL-46 (DDX20 helicase homologue), MXT-1 (Mextli 4EIP), and GYF-1 a protein with unknown function (Fig. 4.4). MEL-46 was shown to be important for normal oocyte development and embryogenesis [193]. Additionally, genetic interactions indicate a level of feminizing activity for MEL-46 in the germline [193]. IFE-1 and IFE-3 participate in these development processes but appear

to have non-redundant roles in each [29]. This suggests that distinct IFE-1 and IFE-3 mRNP complexes may both utilize this helicase. Structural studies show that *C. elegans* Mextli orthologue, MXT-1, competes with eIF4G for eIF4E binding, suggesting that IFE-1 and IFE-3 have a shared 4EIP in the germline [194]. How this shared 4EIP regulation is utilized to drive proper development warrants further investigation. GYF-1, a largely uncharacterized protein, was shown to interact in a miRNA translational repression complex with IFE-4 in cell free embryonic extracts (Thomas Duchaine, personal communication). Combined with our data here it suggests that GYF-1 participates in mRNP complexes with at least most of *C. elegans* eIF4E isoforms.

As expected, we found that IFE-1 associated strongly with the core components of P granules, namely PGL-1/-3, and GLH-1 (Fig. 4.3 and Table 4.1). Importantly, we found that PGL-1 was 54-fold enriched in IFE-1 immunoprecipitations, and only 2-fold enriched in IFE-3 immunoprecipitations (Table 4.1). Additionally, we did not detect GLH-1 in isolated IFE-3 complexes (Fig. 4.1). These data strongly suggests that IFE-1 resides in P granules, while IFE-3 does not. Similarly, IFET-1, the cognate 4EIP of IFE-3 was enriched 162-fold in IFE-3 complexes, and not detected in IFE-1 immunoprecipitations (Table 4.2). This combined with our localization data (see Chapter III) indicate that IFE-1 is the predominant eIF4E in P granules, and that IFE-3 forms its own distinct germ granules with IFET-1 that are separate from the core P granule.

Potential roles for IFE-1 the CSR-1 siRNA pathway

We also found that IFE-1 associated with CSR-1 and its regulator CID-1 (Table 4.1). The role of CSR-1 in the germline is the subject of some controversy. One study found that both P granules and CSR-1 suppress spermatogenic transcript accumulation in the germline [77]. Whereas, others found that ALG-3/-4 and CSR-1 operate in maintaining an epigenetic program for thermotolerant spermiogenesis by promoting robust spermatogenic gene expression [185, 195]. Importantly, IFE-1 is also important for spermatogenesis at elevated temperatures, whereas IFE-3 is not (Fig. 2.5). ALG-3/-4 was shown to be required for GSP-3 expression during spermatogenesis, similar to what others have suggested for IFE-1, indicating that they might positively regulate the same mRNAs [82]. The confusion about the role of CSR-1 in regulation of spermatogenic mRNAs might be explained by it having different roles in male and female germlines [77]. Additionally, it may be explained by two CSR-1 isoforms that are expressed in the germline (CSR-1A and CSR-1B). CSR-1A is expressed in spermatocytes exclusively, and is enriched for 22G siRNAs that target spermatogenic transcripts (Carolyn Philips, personal communication). Although CSR-1 possesses the catalytic residues required for endonucleolytic activity, it has only recently been shown to mediate this activity *in vivo* to degrade target transcripts to fine tune gene expression in the embryo [196]. Whether or not CSR-1A negatively or positively regulates spermatogenic transcripts is unknown but seems plausible.

Surprisingly, we did not identify ALG-3 or ALG-4 in our IFE-1 immunoprecipitations, but we did find CSR-1 is both IFE-1 and IFE-3 pulldowns (Fig. 4.3). One way that IFE-1 might communicate with the CSR-1 siRNA pathway is through

CID-1. CID-1, a terminal uridylyl transferase, was shown to prevent accumulation of siRNAs in the context of the CSR-1 pathway, and without CID-1, siRNA gene silencing was enhanced [190]. Interestingly, this enhanced gene silencing was not directly CSR-1-mediated, rather the authors suggest that siRNAs from the CSR-1 pathway might be detrimental when they enter into other germline RNAi pathways such as those controlled by other AGOs. Thus, CSR-1 associated siRNAs feeding into other AGO related repression pathways could explain their findings. Unexpectedly, CID-1 was the strongest interactor identified in our IFE-1 immunoprecipitations, and did not appear in IFE-3 complexes (Table 4.1). These data indicate the possibility that IFE-1 works with CID-1 to repress siRNA-mediated repression of spermatogenic mRNAs, and thus contributes to robust spermatogenesis. An attractive model is one where IFE-1 and CID-1 containing mRNPs act to inhibit CSR-1 siRNA pathway repression of spermatogenic mRNAs and activate them for translation. Investigating the effects of CSR-1 depletion during spermatogenesis in an *ife-1(bn127)* mutant background might help to elucidate its role in the pathway.

Roles for IFE-3 in P bodies, poly(A) tail regulation, piRNA biogenesis, snRNP homeostasis, and translational repression

Our immunoprecipitation of IFE-3 revealed that it strongly associates with many factors with described roles in translational repression and P body function further separating its function from IFE-1 and P granules (Fig. 4.4 and Table 4.2). For instance, we identified P body proteins CAR-1 and CGH-1 as strong interactors and PATR-1 and ALG-1 as weak interactors with IFE-3 (Fig. 4.2 and Table 4.2). Importantly, CAR-1 and PATR-1 were not detected in IFE-1 complexes, suggesting that IFE-3 alone functions in

P body related mRNP metabolism. We also found factors that regulate poly(A) tail length associated with IFE-3 but not IFE-1 (Fig. 4.1). GLD-2 is the predominant cytoplasmic poly(A)-polymerase in the germline, and GLD-4 is a poly(A)-polymerase that has distinct roles in polyribosome maintenance [191]. Both of these were found in IFE-3 immunoprecipitations (Fig. 4.3). Additionally, we found the Not1 orthologue, LET-711, also associates with IFE-3, implicating it in deadenylation as well (Fig. 4.3) [101]. These findings suggest that IFE-3 is involved with regulation of translation repression/activation switches by controlling poly(A) tail length, and support other investigations that found IFE-3 associated with OMA-1 mRNPs that also contained GLD-2 and deadenylase machinery [188]. Importantly, the IFE-3 related RNP foci we detected in meiotically arrested oocytes are reminiscent of ones suggested to be involved in regulating homeostasis of maternal mRNAs by controlling turnover and poly(A) tail length (Fig. 3.6) [180, 197].

We also identified all components of the PETISCO complex in our IFE-3 immunoprecipitations alone (Table 4.2). PETISCO is a recently discovered mRNP complex with dual functions in 21U piRNA biogenesis and embryogenesis driven by PID-1 or TOST-1 respectively [55]. These data support the essential role of IFE-3 during embryogenesis (Figure 2.4). In PETISCO IFE-3 binds directly to Tudor domain containing 4EIP TOFU-6, and might function in controlling de-capping and 5' processing of piRNA precursors [55]. We also found several factors related to snRNP homeostasis (SMN-1, several GEMIN proteins, and the nuclear cap-binding protein NCBP-1) associated with IFE-3 and not IFE-1. Interestingly, other snRNP related proteins MOG-1/-6 have strong feminizing activity in the germline similar to IFE-3 [192]. Apparently,

activity of snRNP complexes is critical for proper germline sex-determination in hermaphrodites; it seems likely to us that these snRNP activities lead to proper timing of post-transcriptional gene expression and rely on IFE-3. However, the exact mechanisms of this type of post-transcriptional regulation during development are poorly understood.

Importantly, although not robustly, we did detect FBF-2 in our IFE-3 complexes, and not in IFE-1 complexes (Fig. 4.3). Based on our genetic data that showed *ife-3* and the *fbfs* were synergistic in their control of oocyte fate, and our translation data that shows that loss of IFE-3 results in translational de-repression of several direct FBF target mRNAs, our initial hypothesis was that IFE-3 may physically interact with an FBF mRNP complex (see Chapter II). These proteomic data suggest that this may be true. The fact that FBF-2 was shown to be more closely related to P granules than FBF-1 in germline stem cells adds some confusion to this matter however [73]. An attractive model for the role of IFE-3 in GSD is that it functions in a closed loop repressive mRNP with the FBFs to mediate translational repression of transcripts like *fog-1* and *fem-3*, and without IFE-3 repression cannot be initiated or maintained, resulting in masculinization of the germline. Further pulldowns are required to determine the extent of these interactions.

The second strongest interactor of IFE-1 was IFG-1, suggesting that IFE-1 either 1) participates in a substantial amount of cap-dependent translation or 2) resides in translationally stalled complexes (Table 4.1). Intriguingly, robust interactions between IFE-3 and IFG-1 were not detected in our experiments (average spectral count of 2 in IFE-3 pulldowns vs. 183 in IFE-1 pulldowns). Our current postulations to explain this

phenomenon include a combination of the following: 1) IFE-3 interacts only transiently with IFG-1 but still initiates substantial translation 2) IFE-3 does not participate in meaningful levels of cap-dependent translation 3) competition from factors, like IFET-1 or other 4EIPs, prevent IFE-3:IFG-1 interactions from predominating in the germline. IFET-1 was the strongest IFE-3 interactor, supporting the latter hypothesis (Table 4.2). Our translation data showing similar IFE-3 and IFET-1-dependent translational repression of GSD mRNAs also support this notion (Fig. 2.9). Future studies should be conducted to explore if IFE-3 represents a eIF4E with dual roles in repression and activation or solely as repressive factor.

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CHAPTER V

CONCLUSIONS AND GENERAL DISCUSSION

Significance

The work outlined in this dissertation sheds new light on how germ cells modulate fate determining gene expression at the level of translation through the activity of translation factors and their interacting proteins. Such knowledge is critical for our broader understanding of how cellular systems utilize biological information contained in volatile mRNA intermediates for functional gene expression which drives fate. Deeper understanding of these regulatory systems is important for developing therapeutics for diseases of aberrant cellular fate in humans, such as malignant cancer, congenital birth defects, neuronal impairment (see below), and infertility. Complex mRNPs regulate many aspects of a transcript's fate. One bipartite complex that is intimately tied to the regulation of developmentally regulated mRNAs is the eIF4E:4EIP cap-binding complex. Here, we discuss divergent function of two isoforms of the mRNA cap-binding, eIF4E, and their cognate 4EIPs in the germline of *C. elegans*. It has been known for some time that canonical 4EBPs represses the ability of eIF4E to initiate translation by competing with eIF4G [198]. However, it has only recently begun to be appreciated that distinct eIF4E isoforms form mRNP complexes with cognate non-canonical 4EIPs to control the translation, localization, and stability of subpopulations of mRNAs. This more focused view of selective protein synthesis via distinct eIF4E:4EIP mRNP complexes has made great strides in our understanding of spatial and temporal protein synthesis in developing animals.

Perhaps the most prominent example of human pathology related to eIF4E:4EIP mRNP complex dysfunction is seen in Fragile X syndrome (FXS). Patients suffering from this disease show severe developmental defects including learning disabilities and cognitive impairment [199]. Genetically, FXS is characterized by mutations and loss of function in the FMR1 (fragile X mental retardation 1) gene [200]. Fragile X mental retardation protein (FMRP) is an RNA-binding protein and well-characterized translational repressor that is estimated to bind 4% of mRNAs in brain tissue [201]. FMRP binds to a non-canonical 4EIP called cytoplasmic FMRP-interacting protein 1 (CYFIP1), which sequesters eIF4E from eIF4G in order to repress cap-dependent translation [202]. It is speculated that without FMRP, CYFIP1 cannot adequately sequester eIF4E and repress translation of brain specific mRNAs, however the exact details of the mechanisms of repression are currently vague. Small molecule inhibition of eIF4E:eIF4G interactions restores protein synthesis balance and alleviates some neuronal symptoms in mice, indicating that ectopic cap-dependent translation is a causative factor in the manifestation of the disease [201]. Thus, disruption of this repressive eIF4E:4EIP mRNP complex can modulate gene expression in subpopulations of brain transcripts in ways that lead to a human neurological development disorder. Using simpler invertebrate animal models to study similar eIF4E:4EIP complexes and how they selectively control protein synthesis to drive cellular fate and function will aid in developing a more mechanistic understanding of this type of gene regulation and possibly lead to novel therapeutics.

Our data from *C. elegans* suggests that distinct eIF4E:4EIP complexes are selectively controlling translational repression and activation of certain fate determining

mRNAs, and that their dysfunction leads to developmental defects in discrete aspects of reproduction [29]. Historically, emphasis has been put on individual mRNP factors, especially RNA-binding proteins. However, for a more complete understanding of how these complexes are utilized to activate and repress translation to drive cellular fate the entire mRNP must be considered, as they are likely the functional units of regulation. Through a combination of reverse genetics, CRISPR/Cas9 genomic editing, polysome profiling, *in vivo* localization, and biochemical isolation of distinct mRNPs we have contributed significantly to the understanding of how distinct eIF4E isoforms function in germ cells with their cognate 4EIPs to control the translation of important fate determining mRNAs.

Importance of the eIF4E:4EIP node in controlling translation repression/activation switches

Germ cells and embryos must modulate gene expression to promote proper progression through development and ensure the survival of critical lineages of cells. While transcriptional regulation undoubtedly plays a role, there is a necessity in germ cells and neurons to control when and where mRNAs are repressed, degraded, or activated for translation [2, 3]. In these cell types the limitations that temporal and spatial constraints impose on gene expression must be appreciated. For instance, the long cell projects of neurons impose a great distance between the nucleus, where transcription occurs, and dendrites where translation must occur. Similarly, in germ cell control of gene expression must occur with precise timing to ensure proper development. We have learned that the functional units of this translational control appear to be multicomponent mRNPs, often containing RNA-binding proteins (RBPs),

small-RNAs and their interacting proteins Argonats, eIF4E isoforms, and 4EIPs [58]. However, what is not well understood is how the “decision” to activate or repress a particular mRNP in response to developmental stimuli is made. This question is relevant in all dynamic developmental systems (e.g. learning neurons, stem cells, germ cells, embryos) responding to intrinsic and extrinsic signals by turning genes “on” and “off” post-transcriptionally. What resident mRNP factors are necessary and sufficient for either repression or activation are the subject of intensive research.

Much has been learned about individual mRNP factors that control individual repression/activation switches, with a large emphasis on repression by RNA-binding proteins and small-RNA pathways. This is perhaps most evident in seminal work on PUF-protein (Pumilio and FBF) binding landscapes within the linear germline of *C. elegans*. Here stem cell proliferation, meiotic entry, and differentiation are all controlled by a remarkably small number of regionally expressed RBPs that bind 3'-UTRs of a subset of mRNAs to repress their translation [203]. Yet this does not fully explain how mRNAs are turned “on” when they are outside of these repressive regions. Evidence points to repressed mRNPs being remodeled for activation. Activation for translation is arguably the more important step for introducing new activities into cells. At the heart of activation is the rate-limiting step of initiation catalyzed by eIF4E, eIF4G, and eIF4A, which recruit mRNAs to the ribosome for cap-dependent translation [176]. One critical determinant of whether or not a mRNP is repressed or activated, and thus the functional gene product made, is the highly regulated eIF4E:4EIP node. Developing systems express a number of non-canonical 4EIPs which have much broader roles in post-transcriptional gene regulation (see Chapter I). It has recently become appreciated that

these eIF4E:4EIP cap-binding complexes are involved in most aspects of a transcript's life including: repression/activation, transcript stability, polyadenylation/deadenylation and transport, thus more thorough investigations of this complex and its regulation are warranted. Prevailing models suggests that germline mRNPs form closed-loop complexes via eIF4E:4EIP interacting with repressive RBPs in 3'-UTRs, and are remodeled for activation in response to intrinsic or extrinsic signals (see Chapter I). Here we investigate two distinct eIF4E isoforms in the germline of *C. elegans*, IFE-1 and IFE-3, and provide evidence that each has unique development roles, participates in distinct mRNP complexes along with their cognate 4EIPs (PGL-1 and IFET-1 respectively), and exerts control over the translation of unique subpopulations of mRNAs critical for germ cell fate (Fig. 5.1).

Divergent developmental and translational requirements for germline eIF4E isoforms in *C. elegans*

The regulated activation of mRNPs by the translational initiation machinery has been especially understudied in germ cells. Remarkably, studies from nematodes, flies, and frogs all indicate that the eIF4 group factors are playing very specific and non-redundant developmental roles. Interestingly, a theme emerges from all animals and plants investigated, that multiple isoforms of each factor are expressed during development, providing the opportunity for each isoform to evolve a specialized function over evolutionary time [44-47]. Indeed, many have gained novel functions, as studies now show definitively that different isoforms of eIF4E and eIF4G have non-redundant developmental and biochemical roles in these systems and regulate distinct mRNA populations [29, 37, 44, 47-50, 204-207].

In *C. elegans* five distinct isoforms of eIF4E (IFE-1-5) are expressed; our earlier studies showed that two of these isoforms are primarily expressed in somatic tissues (IFE-2 and IFE-4), while IFE-1, IFE-3, and IFE-5 are germline enriched [52]. Here we show that the predominant germline isoforms, IFE-1 and IFE-3, have non-redundant roles in reproduction, differentially regulate the translation of developmentally relevant mRNAs, and show distinct germ granule localization. For instance, IFE-1 was previously found to be critical for the final cytokinesis of spermatogenesis, especially at elevated temperatures [48, 52]. Additionally, IFE-1 is required for normal progression of oocyte maturation, presumably through its positive action on the translation of certain maternal transcripts [25, 48]. Here we show that IFE-3 has no apparent role in spermatogenesis, distinguishing it from the major developmental role of IFE-1 (Fig. 2.5). Instead, IFE-3 has its own unique roles; we show that it is required for proper sex-determination of germ cells, embryogenesis, and meiotic progression of the oocyte (Fig. 2.4). Importantly, we have found no requirement for another IFE in the cell fate decision to make sperm or oocyte, or to be essential for embryogenesis [29, 48-50, 52].

IFE-3 strongly represses translation of some mRNAs that drive spermatogenesis (*fog-1* and *fem-3*), as well as, some mRNAs that drive oogenesis (*gld-1* and *fbf-1*) in the germline (Fig. 2.7 and 2.9). Importantly, each of these mRNAs is a direct FBF target [161]. Additionally, it is responsible for preventing *fog-1* mRNA accumulation, the terminal regulator of germline sex-determination (GSD) (Fig. 2.8). However, why *ife-3(ok191)* mutant phenotypes are alleviated at higher temperatures is puzzling considering IFE-1 shares an inverse relationship with temperature (Fig 2.4). GSD is complex and has many redundant layers of translational control (Fig. 2.2). Epistasis

experiments showed that *ife-3* works centrally in the pathway, downstream of *fog-2* and upstream or at the level of *fem-3* (Fig. 2.6). It stands to reason that loss of IFE-3 dysregulates overall translation of FBF targets and *fog-1* steady-state levels which allows spermatogenic fate to dominate (see below).

Distinct germ granule dynamics of IFE-1 and IFE-3 are controlled by their cognate 4EIPs and tie them to P granules and P bodies respectively

Present in most animals studied, germ granules serve as conserved hubs of mRNA metabolism and contain many diverse mRNPs that function to control mRNA fate. There is strong evidence that germ granules contain (or maintain) mRNA-repressive structures because many of their components have prescribed roles in translational repression, mRNA decay, and RNAi [58]. However, there is emerging evidence that both repression and activation may be pre-programmed within the germ granule, since they often contain not only repressive RBPs and small-RNA machinery, but also positive acting factors that contribute to mRNA stabilization and ribosome recruitment [29, 52, 208]. Indeed, germ granules must localize, protect, and store transcripts important for maintaining germline integrity [57, 68, 209, 210]. What remains poorly understood is: How does the selection of either repression or activation on an mRNA or group of mRNAs begin in the granule? Here, we sought to understand how different germline eIF4E isoforms localize with respect to germ granules and how this localization is regulated by their 4EIPs. Differential localization and sequestration of these translation factors by their binding partners could shed light on their divergent germline roles and explain how they regulate translation spatially and temporally.

Perinuclear germ granules, called P granules, have historically been a hotly studied subject in *C. elegans* since their discovery [211]. They regulate many aspects of post-transcriptional gene control and mRNP remodeling in the germline and are required for normal fertility and epigenetic inheritance [54]. Importantly, investigations have shown that P granules and P bodies are distinct entities in *C. elegans*, yet they cooperate to appropriately control gene expression [59]. Previous studies found that IFE-1 binds directly to PGL-1 a constitutive component of P granules [52]. Additionally, PGL-1 is required for appropriate IFE-1 localization to embryonic P granules [52]. Here, we used CRISPR/Cas9 genomic engineering to tag the endogenous genes encoding IFE-1 and IFE-3 (see Chapter III) and tracked their expression in germ cells and gametes (Fig. 3.1 and 3.2). Expectedly, we found that IFE-1 is resident in P granules throughout development, with the exception that IFE-1 becomes completely diffuse in the cytoplasm of secondary spermatocytes concurrent with the degradation of PGL-1 (Fig. 3.3 and 3.4). Importantly, this is the exact time when IFE-1 is most required for translation that leads to completion of spermatogenesis [48, 52]. We take this to mean that IFE-1 is released from the repressive environment of P granules in order to initiate translation of important spermatogenic mRNAs, possibly those related to cytokinesis or MSP production [82]. Additionally, PGL-1 appeared to control the dynamic localization of IFE-1 in oocyte P granules (Fig. 3.4). How this might contribute to IFE-1's translational regulation of maternal mRNAs like *vab-1* to encourage oocyte maturation is unknown. However, it seems likely that IFE-1 leaves the repressive environment of P granules with its cargo mRNAs and activates their translation in response to MSP signaling.

Future studies should be directed at investigating what other trans-acting factors contribute to the translational repression/activation of these IFE-1:PGL-1 complexes, especially those related to spermatogenesis. Our proteomics data suggest that IFE-1 might interact with the CSR-1 siRNA pathway via a complex containing CID-1 (Table 4.1). CSR-1 is known to regulate post-transcriptional control of spermatogenic transcripts, and CID-1 prevents ectopic CSR-1 associated siRNA gene silencing in the germline [190]. Additionally, the mammalian orthologue of CID-1, TUT7 was shown to regulate maternal transcriptome stability [212]. Thus, IFE-1 may function in a complex with CID-1 in order to repress siRNA mediated gene silencing of spermatogenic and maternal transcripts, eventually leading to their stabilization and translation (Fig. 5.1).

Unexpectedly, we found that like IFE-1, IFE-3 also localized to perinuclear germ granules (Fig. 3.3). However, upon further examination these IFE-3 granules appeared distinct yet adjacent to IFE-1:PGL-1 granules in germ cells and gametes (Fig. 3.3 and 3.4). We often saw multiple IFE-3 foci surrounding a singular PGL-1 focus, strongly suggesting that they reside in discrete mRNP complexes. We cannot, however, discount the possibility that IFE-3 interacts with P granules to regulate post-transcriptional control. In fact, our proteomics data suggests that they may interact to some limited degree (Fig 4.3). Interestingly, IFE-3 very obviously localized to large lattice-like structures in the gonad core (rachis), while IFE-1 did not (Fig. 3.1). Similar to the PGL-1 exerted control of IFE-1, we found that IFET-1 controlled the localization of IFE-3 to perinuclear granules and to these structures in the gonad core (Fig. 3.5). Conversely, IFET-1 depletion did not affect IFE-1:PGL-1 granule formation (Fig. 3.5 and S3.1). The observed lattice-like structures have been previously found to contain CGH-1

and CAR-1, proteins with known P body functions [53, 213]. Additionally, our IFE-3 immunoprecipitations identified these proteins, along with other P body components, further suggesting a potential role for IFE-3 in P bodies. (Fig. 4.3 and table 4.4). We did however, fail to identify another major P body protein DCP-1 (de-capping enzyme) in these pulldowns.

During normal progression of oogenesis IFE-3 is completely diffuse in the cytoplasm, however, upon meiotic arrest induced by *fog-1* RNAi, IFE-3 forms large mRNP aggregates (Fig. 3.6). Interestingly, IFE-1 containing P granules in these arrested oocytes were separate from IFE-3 granules and seemed to be surrounded by them (Fig. 3.6). Other have provided evidence that mRNP aggregates induced by meiotic arrest regulate maternal transcript homeostasis and stability, as they contain P body components [180, 197]. Importantly, we found multiple components of both polyadenylation (GLD-2/-4) and deadenylation (LET-711/CNOT1) machinery in our IFE-3 but not in IFE-1 immunoprecipitations (Fig. 4.4). These are additional clues that IFE-3 may be related to P body function and mRNA stability, especially during oogenesis. Because they reside in unique yet possibly interacting structures, IFE-1 and IFE-3, may be regulating different aspects of maternal transcript metabolism. Cumulatively, these data strongly suggest that the granule dynamics of germline eIF4E isoforms are uniquely controlled by their cognate 4EIPs, and that IFE-1 is the predominant P granule eIF4E isoform, whereas IFE-3 likely has roles in P body mRNA metabolism (Fig. 5.1).

Roles for IFE-3:IFE-1 complexes in translational repression

eIF4E was initially characterized biochemically as an exclusively positive acting catalytic translation initiation factor [214, 215]. As such, we previously found that IFE-1

was required for the efficient translation of certain maternal mRNAs [25, 48]. Our evidence presented here suggests that IFE-1 is released from P granules at specific times during development in order to activate translation. Likewise, our original investigations of IFE-2 and IFE-4 found them have positive translational roles on mRNAs involved in meiotic crossover (*msh-4* and *msh-5*) and muscle function related to egg-laying (*egl-3* and *egl-15*) respectively [49, 50]. However, eIF4E is found to reside in repressive (with 4EIPs) and active (with eIF4G) mRNP complexes, suggesting that mRNA cap-binding proteins indeed have roles as both an activator and repressor of translation depending on cellular and sequence-specific contexts. Indeed, whether any given eIF4E isoform acts positively or negatively may be mRNA-dependent, or more likely due to the composition of the larger mRNP complex in which it resides. This could allow germ cell to selectively control spatial and temporal gene expression by regulating which eIF4E:4EIP pairs are either active or repressed.

Here, we found that IFE-3 has major roles in repressing translation of GSD mRNAs (*fog-1*, *fem-3*, *gld-1*, *fbf-1/-2*, and *daz-1*), likewise, IFET-1 inhibited translation of many these mRNAs, strongly indicating a shared role in a eIF4E:4EIP repression complex (Fig. 2.9). Whether or not IFE-3 mediated translational repression relies on the IFET-1 interaction is unknown yet remains likely. The first identification of canonical 4EBPs showed that they sequestered eIF4E from eIF4G, thereby blocking initiating activity [198, 216]. Recent investigations have led to the discovery of many classes of non-canonical 4EBPs (also called eIF4E-interacting proteins; 4EIPs) that form large mRNPs, of which, both PGL-1 and IFET-1 belong [23, 42, 53, 81, 98]. Other studies have shown that the eIF4E2 (4EHP) isoform was shown to interact with the 4EIP,

Bicoid, to facilitate translational repression of *cad* mRNA during fly development [71]. Similarly, in *H. sapiens*, Hoxb4 translational repression relies on the 4EHP isoform [217]. However, phylogenetic analysis shows that IFE-4 and not IFE-3 represents the sole 4EHP homologue in worms [145]. Interestingly, we show that IFE-1 strongly interacts with *C. elegans* eIF4G orthologue, IFG-1, whereas IFE-3 does so only weakly (Fig. 4.4 and Tables 4.1 and 4.2). In our experiments, the strongest IFE-3 interactor was IFET-1 (Table 4.2). The capacity of IFET-1 to inhibit eIF4E:eIF4G interactions is unknown but seems likely based on what is known about orthologues, 4E-T and Cup, from other systems and what we for GSD translation here [42, 75, 90, 165, 182, 218]. It may be that IFE-3 is mostly sequestered from translation complexes by IFET-1. This could explain why similar translational de-repression outcomes are seen for GSD mRNAs with IFE-3 or IFET-1 loss (Fig. 2.9). All the mRNAs strongly repressed by IFE-3 are also direct FBF targets [161]. FBFs are known to regulate GSD by mediating translational repression on their target transcripts [153, 154]. Interestingly, FBF-2 was found exclusively in our IFE-3 immunoprecipitations, possibly providing an explanation for how IFE-3 is selective in regulating GSD (Fig. 4.3). An attractive model is one where an IFE-3:IFET-1 moiety physically participates in a closed-loop FBF-2-mediated translational repression complex that regulates some GSD mRNAs (Fig. 5.1). Follow up pulldowns should be conducted to test the strength of these interactions.

IFE-3 participates in complexes that regulate piRNA biogenesis and snRNP homeostasis

Our biochemical isolation of IFE-3 also revealed that some of its strongest interactors were components of a previously discovered complex called PETISCO (PID-

1/-3, ERH-2, TOFU-6, and TOST-1) that has separate roles in 21U piRNA biogenesis and embryonic development mediated by PID-1 and TOST-1 respectively [55]. Importantly, none of these factors were found in IFE-1 related complexes (Fig. 4.3). The role of IFE-3 in piRNA biogenesis was unexpected, yet fascinating. piRNAs precursors are enzymatically processed at their 5' end to produce a mature 21U piRNA [219]. IFE-3 binds directly to the 4EIP, TOFU-6, in the PETISCO complex and might regulate the de-capping and subsequent maturation of piRNA precursors [55]. piRNA precursors exclusively contain monomethyl 5'-caps [219]. IFE-3 binds to monomethyl 5'-caps but not to trimethylated ones, possibly explain the unique role of IFE-3:TOFU-6 in this piRNA generating complex. IFE-3 is the only *C. elegans* eIF4E essential for embryogenesis [44]. Additionally, our studies of *ife-3(ok191)* heterozygotes showed that it is the maternal contribution of IFE-3 that functions in the embryo (Fig. 2.4). The embryonic role of PETISCO is thought to be in maintaining SL1 *trans*-splicing snRNPs which rely on the SMN complex [220]. We found several factors with known snRNP functions in our IFE-3 immunoprecipitations including: SMN-1, GEMIN proteins, and SNR proteins (Fig. 4.3 and Table 4.2). How IFE-3 might be regulating snRNP homeostasis is unknown, but mutations in genes of other snRNP related proteins (*mog-1/-6*) show the exact same GSD defects as *ife-3(ok191)* mutants [192]. Importantly, MOG proteins are implicated in the translational repression of *fem-3* [192]. Taken together, maternally derived IFE-3 functions in PETISCO with TOST-1 to ensure embryonic survival through maintenance of snRNP homeostasis. Additionally, the role of IFE-3 in GSD may be explained by its function in snRNP mediated control of appropriate gene expression of sperm fate mRNAs.

Figure 5.1

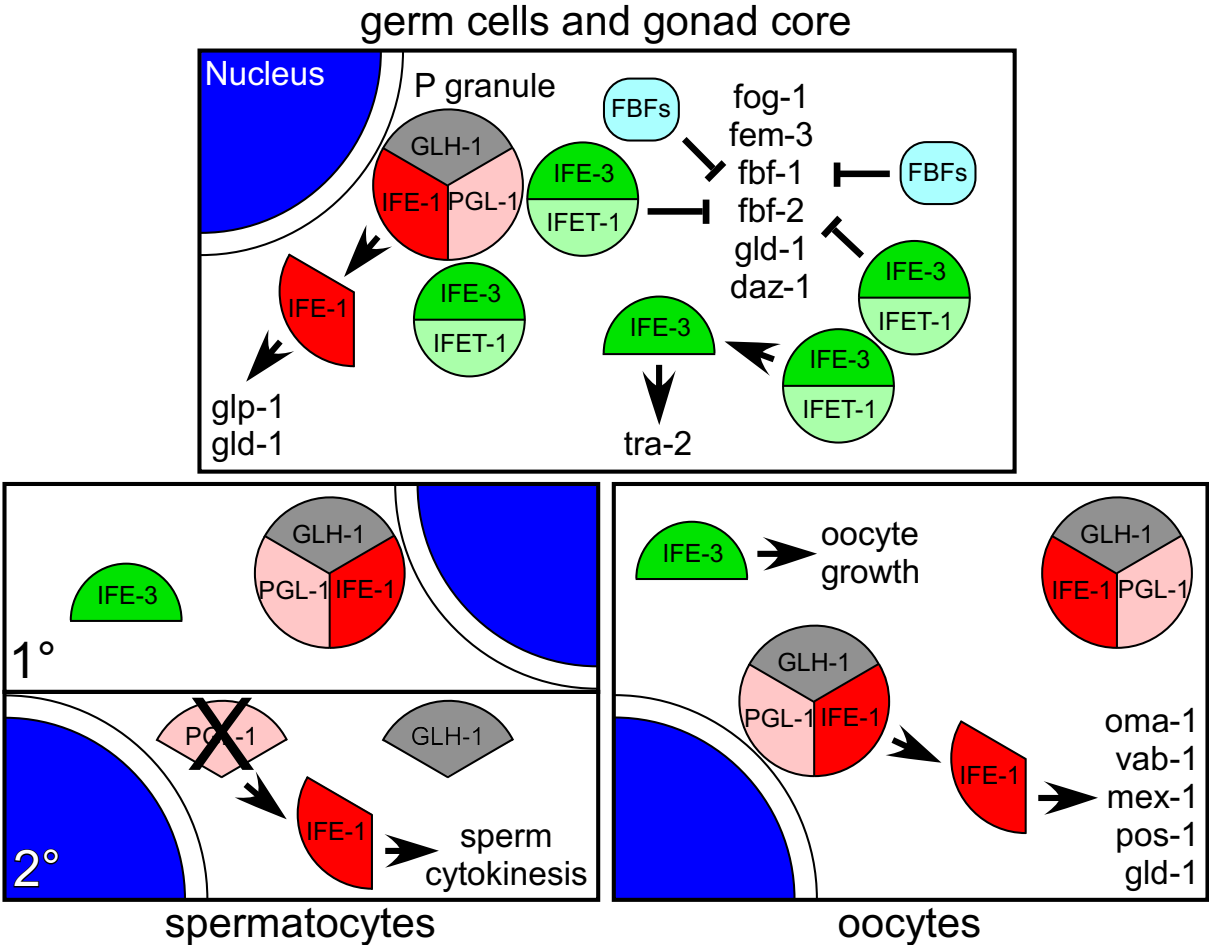


Figure 5.1. Model for differential spatial and temporal function of distinct eIF4E:4EIP complexes in the germline. Our findings demonstrate that loss of IFE-1 or IFE-3 cause very different germline phenotypes in *C. elegans*. These two eIF4E isoforms show different localization patterns, have different cognate 4EIPs, and translationally regulate different mRNAs. The model, based on this and other studies, is that IFE-1 and IFE-3 use their cognate 4EIPs to localize into unique germ granule mRNPs that presumably carry cargo mRNAs for translational repression. During germ cell development the IFE:4EIP mRNPs are remodeled to enter actively translating ribosomal complexes. IFE-1 binds its 4EIP, PGL-1, directly and is the predominant P granule eIF4E. It promotes spermatocyte cytokinesis after leaving P granules in the latter stages of spermatogenesis and also positively regulates the translation of some maternal mRNAs which are also contained in P granules. In contrast, IFE-3 is dispensable for spermatogenesis, and its major functions are in germline sex-determination (GSD), oocyte meiotic development and growth, and embryogenesis. IFE-3 also localizes to germ granules in the gonad, but they appear distinct from IFE-1:PGL-1 granules. IFET-1 binds IFE-3 directly, and controls its localization. We also provide evidence for hierarchical binding of IFET-1 to IFE-3, by showing that loss of IFE-3 has no effect on IFET-1 localization. IFE-3 ostensibly promotes oocyte fate by preventing translation of some GSD mRNAs (*fog-1*, *fem-3*, *fbf-1*, *fbf-2*, *gld-1*, *daz-1*) in conjunction with FBF proteins that are known to bind and repress GSD mRNAs. The role of IFE-3:IFET-1 may be to sequester these mRNAs to germ granules adjacent to P granules. The soluble portion of IFE-3 may activate the translation of *tra-2* mRNA and other oocyte growth mRNAs consistent with its role in promoting oocyte fate. We

propose that distinct eIF4E:4EIP-containing mRNPs in the germline are remodeled in response to developmental stimuli, which regulates the repression, activation, and degradation of mRNAs spatially and temporally throughout development.

Future directions

Future studies should address what other proteins are necessary to push a repressed mRNP into an active translation complex in response to genetic or developmental stimuli. This new understanding of mRNP regulatory dynamics will be important in providing a complete picture of how germ cells regulate gene expression by translational control. By understanding what is necessary and sufficient for repression and activation of translation given these new modular eIF4E:4EIP mRNPs, we may gain to ability to sufficiently manipulate gene expression to drive desired cell fate outcomes in other systems relevant to human disease. Technical advances that allow biochemical dissection of repressed vs. activated mRNPs isolated from cells in a variety of conditions (genetic mutants, developmental stage, intrinsic and extrinsic signaling, *etc.*) will be incredibly useful. One such RNA-proximity labeling technique, called APEX-Seq, can be used to determine not only spatial information about RNAs, but also their enrichment or depletion near RBPs of interest under varying conditions [221]. APEX-Seq was used to elucidate how stress granules change in their RNA composition with different stressors, and the organization of 43S PIC complexes. Such methodology will contribute greatly to our understanding of spatial and temporal post-transcriptional gene expression. Our comprehension of germ cell mRNA metabolism has made great strides in the past several decades, uncovering the roles of individual RBPs in mRNPs that inhibit translation. However, we must now begin to treat the whole mRNP as a discreet regulatory unit with both negative and positive roles, and probe deeper into what signals cause subsequent translational activation.

Here, we uncovered the unique developmental roles and translational functions of discrete eIF4E:4EIP complexes in the germline of *C. elegans*. We also began to probe at additional protein components in these complexes further delineating them and shedding light on their unique functions. Moving forward, efforts should be focused on a few phenomenon: 1) the potential role of IFE-1 in regulation of the CSR-1 siRNA pathway via its interactions with CID-1 in the context of spermatogenesis and regulation of the maternal transcriptome prior to embryogenesis 2) elucidating why IFE-1 robustly interacts with IFG-1, whereas IFE-3 does not (resident time of IFE-1 in the eIF4E:eIF4G complex must be longer compared to IFE-3). Probing IFET-1 in this facet could prove useful as it was the strongest IFE-3 interactor and might prevent gross IFE-3:IFG-1 association in the germline. 3) determining if IFE-3 has canonical translation initiation factor activity by discovering mRNAs that it positively regulates with genomic approaches similar to what was used for IFE-1, IFE-2, and IFE-4. 4) clarifying the role of IFE-3 in the negative regulation of GSD mRNA by maintaining snRNP homeostasis and FBF-mediated translational repression via genetics and *in vivo* biochemistry. 5) eIF4E:4EIP cap-binding complex mediated regulation of diverse small-RNA pathways. These associations were unexpected but interesting. It appears that eIF4E:4EIP complexes are interacting with small-RNA pathways extensively. Studies such as these will aid in completing our understanding of the individual roles of eIF4Es and their cognate 4EIPs during *C. elegans* development, as well as in other systems. This broader view will advance our understanding of post-transcriptional genetic switches and the unique cells that use them so extensively.

CHAPTER VI

MATERIALS AND METHODS

Strains and Maintenance

C. elegans Bristol var. N2 was used as the wild type strain in all experiments unless otherwise noted. DUP71 *pgl-1(sam33[pgl-1::gfp::3xflag])IV* and DUP 121 *glh-1(sam24[glh-1::gfp::3xflag])I*; *pgl-1(sam52[pgl-1::mTagRFP::3xflag])IV* were obtained from Dr. Dustin Updike (MDI Biological Laboratory, Bar Harbor, ME). Strains LP306 *cpIs53[mex-5p::gfp-C1::PLC(delta)-PH::2xflg::tbb-2 3'UTR + unc-119 (+)]II* and LP307 *cpIs54[mex-5p::mKate2-C1::PLC(delta)-PH::2xflg::tbb-2 3'UTR + unc-119 (+)]II* were obtained from Dr. Daniel Dickinson (Univ. Texas at Austin). Strains JK2958 *nT1[qIs51](IV;V)/dpy-11(e224) unc-42(e270)V*, CGC20 *dpy-18(e364)/eT1 III*; *unc-46(e177)/eT1[umnlS9]V*, JK574 *fog-2(q71)V*, CB3844 *fem-3(e2006)IV*, JK3022 *fbf-1(ok91)II*, JK3101 *fbf-2(q738)II*, GR1373 *eri-1(mg366)IV*, and DG3913 *lin-41(tn1541[gfp::tev::s::lin-41])I* were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota) which is funded by NIH Office of Research Infrastructure Programs [grant number P40 OD010440]. The *ife-3(ok191)* allele was obtained from the *C. elegans* Knockout Consortium (OMRF), outcrossed ten times to N2 worms, and balanced with *unc-34(e566)* to make KX10 *ife-3(ok191)/unc-34(e566)V*. The *ife-3* allele was then crossed into strain JK2958 to more stably balance it with *nT1[qIs51]* to make KX98 *ife-3(ok191)/nT1[qIs51]V*, and with *eT1[umnlS9]* to make KX140 *ife-3(ok191)/eT1[umnlS9]V*. Breakpoint of *ife-3(ok191)* deletion was determined by subcloning and sequencing of the genomic deletion PCR product, and found to be

taattttcatattttccgct/tatcta/ttatcgatttttccagatg. KX143 *ife-1(eu20[mkate2::tev::3xmyc::ife-1])*III, and KX152 *ife-3(eu21[gfp::tev::3xflag::ife-3])*V, were made using CRISPR/Cas9-driven homologous recombination to make in-frame insertions at the respective endogenous gene loci in N2 worms. KX149 *ife-1(eu20[mkate2::tev::3xmyc::ife-1])*III, *pgl-1(sam33[pgl-1::gfp::3xflag])*IV was made by crossing KX143 and DUP75. KX155 *ife-1(eu20[mkate2::tev::3xmyc::ife-1])*III, *ife-3(eu21[gfp::tev::3xflag::ife-3])*V was made by crossing KX143 and KX152. KX157 *ife-3(eu21[gfp::tev::3xflag::ife-3])*V, *pgl-1(sam52[pgl-1::mTagRFPT::3xflag])*IV was made by crossing KX152 and DUP121 and outcrossing *glh-1(sam24)*. KX159 *ife-3(ok191)/nT1[qIs51]*V, *lin-41(tn1541[gfp::tev::s::lin-41])*I was made by crossing KX98 and DG3913. Strain PRB171 *qfIs012*(FL-IFET-1 :*ifet-1(tm2944):GFP::3XFLAG + unc-119(+)*) was created by biolistic transformation of *unc-119(ed3)* worms [222] with recombineered fosmid DNA generated by the *C. elegans* TransgeneOmics Project, Max Planck Institute of Molecular Cell Biology and Genetics Dresden, Germany. All strains were maintained on normal growth medium (NGM) plates at 20°C unless otherwise indicated, with *E. coli* strain OP50 as described [223].

Mating and fertility experiments

Fertility of *ife-1* and *ife-3* mutant worms was determined at two different temperatures: permissive temperature (20°C) or restrictive temperature (25°C). To this end, *ife-1(bn127)*, *ife-3(ok191)*, and *fog-2(q71)* (control) mutant strains were grown from embryo stage at either 20°C or 25°C. L4 males were transferred to new NGM plates and incubated for 24 h either at 20°C or 25°C. Adult males were soaked in M9 solution with 50 µM SYTO17 red fluorescent dye (Molecular Probes) for 3 h and each male was transferred to a OP50 seeded NGM with a single *fog-2* female mutant worm. 24 h later,

mating efficiency was determined by counting the number of *fog-2* female worms with SYTO17(+) sperm in their spermatheca among *fog-2* worms tested. Fertility was determined by counting the number of *fog-2* females with SYTO17(+) sperm and embryos in the uterus.

Characterizing *ife-3(ok191)* temperature sensitive fertility rescue

To unambiguously determine the frequency of the temperature sensitive rescue of *ife-3(ok191)* on fertility and separate poorly balanced *ife-3* genetic recombinants from true *ife-3(-/-)* animals at 25°C, we generated synchronized populations of KX98 *ife-3(ok191)/nT1[q/s51]V* worms by using a solution of NaClO (1.3%) and NaOH (0.5M) on gravid hermaphrodites, hatching F₁ embryos overnight in M9 at 20°C, and seeding L1 larva onto NGM plates with OP50 to be grown at 20°C or 25°C. 24 h after L4 molting, animals were dissected with a 30-gauge needle under a stereomicroscope to determine if fertile (embryos) or sterile (no embryos), then individual worms were collected for genomic PCR using the following triple primer set: forward 1: 5'-TATGTGCATGTTGTGGAGGCTG-3', reverse: 5'-TGTAGTCTCCGTACTCGTCG-3', forward 2: 5'-CAGAATCGCTGGGCTCTC-3'; which yields a single 417bp product in *ife-3(+/+)* worms, a single 756bp product in *ife-3(-/-)* worms, and both products in *ife-3(+/-)* worms. Results were pooled from 3 individual experiments.

RNA interference

Gene-specific RNA interference (RNAi) was performed by feeding *C. elegans* with double-stranded RNA (dsRNA) expressing *E. coli* (HT115) as described with minor modifications [224, 225]. Briefly, HT115 was transformed with either L4440 vector or gene-specific constructs were grown overnight at 37°C in 2xYT media containing 50

µg/µl carbenicillin. Cultures were concentrated 5-fold by centrifugation, seeded onto Nematode Growth Medium (NGM) plates containing 1mM IPTG and 50 µg/µl carbenicillin, then induced overnight at room temperature before plating worms. dsRNA was extracted from bacterial plates after induction and run on denaturing 2% agarose gels with ethidium bromide to determine dsRNA expression when appropriate. For funzies, L4 hermaphrodites were seeded onto RNAi plates and F₁ offspring were evaluated for phenotype or processed for biochemical analysis. RNAi feeding experiments were conducted at 25°C, with the exception that CB3844 *fem-3(e2006)* and GR1373 *eri-1(mg366)* L4 mothers were grown at 20°C for the first 24 h of feeding, then shifted to 25°C, due to decreased fertility at higher temperatures. We generated the *ife-3*(RNAi) feeding construct (pT72-*ife-3*) by inserting a 475bp restriction digested *ife-3* cDNA fragment containing only the coding region [45] into the L4440 vector backbone using standard cloning procedures. We also generated *ifet-1*(RNAi) feeding construct (pT72-*ifet-1*) by inserting a 600bp PCR amplified *ifet-1* fragment into the L4440 vector backbone again using standard cloning procedures [226]. The *fog-1*(RNAi) construct has been previously described [227]. The identity of RNAi clones was verified via Sanger sequencing (Iowa State Sequencing Facility).

CRISPR/Cas9 genome editing

CRISPR/Cas9 mediated genome editing was accomplished by using pDD162 (Addgene) to express single-guide RNA (sgRNA) driven by *C. elegans* U6 promoter and Cas9 DNA endonuclease protein under control of the *eef-1A.1/eft-3* promoter in conjunction with plasmid-based homology directed repair (HDR) template containing a self-excising cassette (SEC) as described with minor modifications [178, 228]. We

generated multiple pDD162 sgRNA/Cas9 expressing plasmids for *ife-1* and *ife-3*, using the Q5® site directed mutagenesis kit from New England Biolabs (NEB) per manufacturer's instructions. We also generated HDR templates for *ife-1* and *ife-3* by inserting PCR amplified homology arms (~550-950bp) corresponding to upstream and downstream regions of each gene relative to the start codon (ATG) into pDD287 and pDD282 backbones (Addgene) respectively by using Gibson Assembly® kit (NEB) per manufacturer's instructions. When possible, conserved mutations were made in PAM sites within the homology arms to prevent HDR template cutting by Cas9, when mutation of the PAM site was not possible, conserved mutations were incorporated throughout the sgRNA target site for funzies. sgRNA/Cas9 expressing plasmids and HDR template plasmids were isolated using PureLink® Miniprep Kit (Invitrogen) per manufacturer's instructions and confirmed via sanger sequencing (Iowa State Sequencing Facility). We injected mated N2 hermaphrodites as young adults in order to increase brood sizes. Injection cocktails were as follows: *ife-1* injection mix: pDD162-*ife-1*-256 (sgRNA/Cas9 50ng/μl), pDD287-*ife-1* (HDR SEC 10ng/μl), pPD118.2 (GFP co-injection marker 5ng/μl); *ife-3* injection mix: pDD162-*ife-3*-181, pDD162-*ife-3*-7741, pDD162-*ife-3*-8300 (each sgRNA/Cas9 25ng/μl), pDD287-*ife-3* (HDR SEC 10ng/μl), pCJ104 (RFP co-injection marker 5ng/μl). Progeny of injected worms were selected with Hygromycin (final conc. 250 μg/mL) at 20°C and screened for survival, *rol* phenotype, and lack of co-injection marker, then heat shocked at 34°C for 3-4 h to remove SEC. Non-rollers from heat shock plates were screened by genomic PCR to confirm insertion at the desired locus. Animals containing the desired insertion were backcrossed at least 3 times to N2 males. N-terminal protein fusions did not appear to grossly affect IFE-1

and IFE-3 function as worms homozygous for both insertions were superficially wildtype and had wild type fertility.

Microscopy and immunostaining

Dissection of hermaphrodite or male germlines and immunostaining was done as described with minor modifications [166]. For major sperm protein (MSP) immunostaining, worms of the indicated genotype and treatment were dissected under a stereomicroscope using a 30-gauge needle, germlines were fix in 3% paraformaldehyde in 1X phosphate buffered saline [pH 7.2] (PBS) for 20 min at room temperature, washed in PBS +0.01% Tween20 (PTW), then post-fixed in ice cold 100% MeOH and stored at -20°C. Following fixation, dissected germlines were rehydrated in 50% cold MeOH, washed in PTW, blocked for 1 hr. in PTW +0.5% bovine serum albumin (PTWB) at room temperature, incubated with mouse anti-MSP (1:100) primary antibody [229] in PTWB overnight at 4°C, washed in PTWB extensively, then incubated in Alexa488 or Alexa568 conjugated goat anti-mouse-IgG (1:400) secondary antibody (Invitrogen) in PTWB for 1 hr. at room temperature, a final incubation in 4',6-diamidino-2-phenylindole (DAPI) for 15 min at room temperature before being washed extensively in PTWB and mounted on 2% agarose imaging pads in 90% glycerol/DABCO (ACROS Organics) anti-fade media. For imaging CRISPR/Cas9 endogenously tagged fluorescent protein fusions, we replaced the 100% MeOH post fix and rehydration step above with a 70% EtOH postfix and rehydration step, and did not block in PTWB or use immunostaining, other steps for fixing and imaging these germlines was identical to those described above. We also imaged CRISPR/Cas9 fusions in live worms treated with NaN₃ (2 mM) and levamisole (5 mM) for immobilization. Germline imaging was

done on a Zeiss Axiovert 200 M Differential Interference Contrast (DIC) microscope equipped with an Axiocam MRm CCD camera and corresponding DAPI/Hoechst, and FITC/GFP, and TexasRed/RFP optics with 40X-air or 100X-oil objectives. Image processing was done using Axiovision 4.3 software (Carl Zeiss). Any image adjustments to contrast or brightness were conducted linearly.

Analysis of polysomes by sucrose gradient fractionation

Lysis and gradient buffers, gradient setup, centrifugation, and fractionation were conducted as described with the following modifications [48, 49]. L4 stage N2 hermaphrodites were seeding onto NGM plates with HT115 expressing either L4440(RNAi), *ife-3*(RNAi), or *ifet-1*(RNAi) and allowed to grow at 25°C for ~96 h until plates were saturated with mix-staged F₁ and F₂ progeny. We estimate that young adult worms composed >75% of the biomass on each plate. Worms were then washed off plates using M9, allowed to purge gut contents for 30 min in M9, and floated in 35% sucrose at 4°C to remove debris and bacteria, then flash frozen in liquid nitrogen in the presence of 14 mM E64 protease inhibitor (Sigma-Aldrich) and 4 mM Vanadyl-RNC RNase inhibitor (Sigma-Aldrich). Worms were crushed in a mortar and pestle under liquid N₂ with an equal volume of buffer A (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, 2 mM dithiothreitol (DTT), 800 U of RNasin/ml (Promega), 4 mM Vanadyl-RNC, 5 mM PMSF, 0.4 mg/mL Cycloheximide (Calbiochem), and centrifuged at 14,000 × *g* at 4°C for 15 min. A 0.5-ml aliquot of the supernatant was applied to an 11 mL 10 to 45% linear sucrose density gradient made in buffer B (25 mM Tris-HCl [pH 8.0], 140 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol) and centrifuged in a Beckman SW41Ti rotor at 38,000 rpm at 4°C for 2 h. Gradients were fractionated into 1

mL fractions with continuous monitoring of absorbance at 254 nm with an ISCO UA-6 UV VIS detector. Sucrose fractions stored at -80°C prior to use.

qRT-PCR on sucrose gradient fractions

RNA from sucrose gradient fractions was isolated in four volumes of Trizol[®] Reagent (ThermoFisher) per manufacturer's instructions with the following additions: after Isopropanol precipitation, fractions were extracted once with phenol-chloroform-iso-amyl alcohol (25:24:1), then extracted once with chloroform-isoamyl alcohol (24:1), followed by an EtOH precipitation at 4°C overnight with 20 µg glycogen (ThermoFisher). RNA was isolated from 500 µL of each 1 mL gradient fraction. RNA concentrations were measured on a NanoDrop[®] ND-1000 spectrophotometer and assessed for RNA purity based on A260/280 ratios. cDNA was synthesized from each fraction using iScript Advanced cDNA Synthesis Kit (BioRad) using 0.4 µg of input RNA per manufacturer's instructions. qRT-PCR was performed in triplicate on a CFX Real-Time System (BioRad) using Sso Fast Evagreen Super mix (Bio-Rad) per manufacturer's instructions. qRT-PCR quantification of each mRNA was normalized to total RNA content. Standard curves were generated for each primer set and used for absolute quantification. Similar traces were obtained for *fem-3*, *fog-1*, and *gpd-3* mRNAs on 2-3 independent gradients. Primer sequences used were: *tbb-2* (forward 5'-TCATCTCCAAAATCCGCGAA-3', reverse 5'-GAGGGATACAAGATGGTTC-3'); *mex-1* (forward 5'-AATGGATAAGCTAATGTTGGA -3', reverse 5'-GATATTGCGATGAGGAAGAG-3'); *fem-3* (forward 5'-AAGCTGACAGAGAAACGAGA-3', reverse 5'-AAAGGAATTCCAGATATTAAAGG-3'); *fbf-1* (forward 5'-GACCAATCAAAAATGCGCTAT-3', reverse 5'-GATTTCCAACCTCTGTAGATGTG-3');

fog-1 (forward 5'-TCAGTGCCAGGATTTAGAGA-3', reverse 5'-AATATCGATGATACGGTTGTG-3'); *gld-1* (forward 5'-TTCAGGTCCAGTTTTGATGT-3', reverse 5'-GACGTTAGATCCGAGAAGGT-3'); *daz-1* (forward 5'-ACAAAAAGCCCAATCAAAG-3', reverse 5'-GCTGAAAGTGGAGAAAGATG-3'); *gpd-3* (forward 5'-GATCTCAGCTGGGTCTCTT-3', reverse 5'-TCCAGTACGATTCCACTCAC-3'); and *tra-2* (forward 5'-TTCAATTGCAACAAAACAAA-3', reverse 5'-TCTGCACCAAATTGTAGACC-3')

Micro-scale qRT-PCR

We conducted micro-scale qRT-PCR on whole worm lysates as described with minor modification [205]. We generated synchronized populations of N2 and KX98 *ife-3(ok191)/nT1[q/s51]V* worms by using a solution of NaClO (1.3%) and NaOH (0.5M) on gravid hermaphrodites, hatching F₁ eggs overnight in M9 at 20°C, and seeding L1 larva onto NGM plates with OP50 to be grown at 20°C. Three independent biological replicates each containing ~25 early or late L4 hermaphrodites (N2 or *ife-3* *-/-*) were collected into 25 µL of single worm lysis buffer (15mM Tris-HCl [pH 8.0], 50mM KCl, 1mM MgCl₂, 0.45% NP-40, 0.45% Tween20) containing 0.2 mg/mL Proteinase K (60 min at 60°C; 10 min at 95°C). Upon completion of lysis, RNA was isolated using the PureLink ® RNA Mini Kit (Invitrogen) with on-column DNase I digestion, followed by EtOH precipitation at -20°C overnight with 20 µg glycogen (ThermoFisher). RNA was resuspended in 10 µL of nuclease free H₂O for cDNA synthesis and qRT-PCR as described above. Reactions were conducted in triplicate from three independent biological replicates, quantified, and normalized to *tbb-2* (endogenous control) using the

$2^{-\Delta\Delta C_t}$ method. Student's t-test was used to determine statistical significance between *ife-3(-/-)* and wild type animals, with a p-value cutoff of 0.05.

EdU labeling and measuring S-phase indices

Metabolic labeling of DNA synthesis was done as described with minor modifications [230]. Animals were incubated in M9 +0.1% Tween 20 with 1 mM EdU for 30 min at 20°C. Gonads were dissected and fixed in 3% paraformaldehyde solution in 0.1 M K_2HPO_4 (pH 7.2) for 10–20 min, followed by –20°C methanol fixation for 10 min. Dissected gonads were blocked in PTWB solution for 30 min at 20°C. EdU labeling was detected using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen) according to the manufacturer's instructions. After washing 3X with PTWB solution for at least 30 min at 10 min intervals, the dissected gonads were stained with a final incubation in 4',6-diamidino-2-phenylindole (DAPI) for 15 min at room temperature before being washed extensively in PTWB and mounted on 2% agarose imaging pads in 90% glycerol/DABCO (ACROS Organics) anti-fade media.

Western blot analysis for IFE-3 depletion

Frozen worm pellets used for polysome analysis were ground in liquid nitrogen in CL+ buffer (40 mM MOPS [pH 7.5], 200 mM KCl, 2 mM EDTA, 4 mM EGTA, 2 mM dithiothreitol, 14 mM E64, and HALT protease cocktail (ThermoFisher), centrifuged at $14,000 \times g$ for 10 min, supernatant was batch bound to m7G sepharose (Sigma-Aldrich) beads for 1 hr at 4°C, washed 3X with CL+, boiled in 2X SDS load buffer for 3 min, and 30 μ g protein for each sample was resolved by 12% SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes overnight and blocked with TST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween 20) containing 5% dry non-

fat milk for 1 hr. at room temperature, then incubated with a 1:1,000 dilution of an IFE-3 specific primary antibody CA82 [45] in TST containing 5% dry non-fat milk for 1 hr. at room temperature. Blots were washed 5X in TST containing 5% dry non-fat milk and incubated with a 1:10,000 dilution of goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP) in TST containing 5% dry non-fat milk for 1 hr. at room temperature, then washed extensively. Detection was done by ECL+ kit (ThermoFisher) per manufactures instructions. Image acquisition was done using a Amersham Typhoon Fluorescence/Phosphorimaging scanner (GE Healthcare) at the ECU PhIFI Core Facility.

mKate2::myc::IFE-1 and GFP::flg::IFE-3 immunoprecipitation and validation

Worms were grow on chicken egg plates at 20°C then washed off plates using M9, allowed to purge gut contents for 30 min in M9, and floated in 35% sucrose at 4°C to remove debris and bacteria, then flash frozen in liquid nitrogen in the presence of 14 mM E64 protease inhibitor (Sigma-Aldrich), 4 mM Vanadyl-RNC RNase inhibitor (Sigma-Aldrich), and 0.2% Tween 20 detergent. Worms were subsequently crushed in a mortar and pestle under liquid N₂ with an equal volume of mRNP immunoprecipitation buffer (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, 2 mM dithiothreitol (DTT), 800 U of RNasin/ml (Promega), 4 mM Vanadyl-RNC, 2X HALT complete protease inhibitor (ThermoFisher), 14 mM E64 protease inhibitor (Sigma-Aldrich), then centrifuged at 14,000 × *g* at 4°C for 15 min. The resultant high-speed supernatant was subjected to 20-50 µL of packed equilibrated GFP or RFP-targeted Nano-Trap agarose beads (Chromotek) and bound for 1.5 hrs. at 4°C while rotating. All immunoprecipitations were done on 6-10 mg of total *C. elegans* protein as measured by

dot spot assay (BioRad). After binding, beads were washed 4X in HSHD buffer (50 mM Tris-HCl [pH 7.8], 1 M NaCl, 0.5% NP-40, 0.5% deoxycholic acid), then additionally washed 3X in 20 mM Tris-HCl [pH 7.8]. 20% of beads from each condition were saved for western blot analysis (5%) and SYPRO™ Ruby (ThermoFisher) staining (15%), while the remaining beads (80%) were stored at -80°C for downstream LC-MS/MS. Beads used for SDS-PAGE were boiled in 4X SDS load buffer for 3 min, and ~30 µg of protein for each sample was resolved on 10% gels. Total protein staining was done with SYPRO™ Ruby (ThermoFisher) per manufactures instructions. For western blotting, proteins were transferred to polyvinylidene fluoride (PVDF) membranes overnight and blocked with TST (10 mM Tris-HCl ph 7.4, 150 mM NaCl, 0.05% Tween 20) containing 5% dry non-fat milk and 1% BSA for 1 hr. at room temperature, then incubated with either a 1:4000 dilution of a GFP specific primary antibody (Sigma; G1544) or a 1:3000 dilution of a RFP specific primary antibody (Invitrogen; R10367) in TST containing 5% dry non-fat milk and 1% BSA for 1 hr. at room temperature. Blots were washed 5X in TST and incubated with a 1:15,000 dilution of goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP) in TST containing 5% dry non-fat milk and 1% BSA for 1 hr. at room temperature, then washed extensively. Detection was done by ECL+ kit (ThermoFisher) per manufactures instructions. Image acquisition was done using a Amersham Typhoon Fluorescence/Phosphorimaging scanner (GE Healthcare) at the ECU PhIFI Core Facility.

Comparative proteomic analysis via LC-MS/MS

Proteomics were conducted under the direction of Dr. Laura Herring at the University of North Carolina's Michael Hooker Proteomics Core. Briefly, samples were subjected to on-bead tryptic digest, and desalted using C18 desalting spin columns (Pierce) per manufactures instructions. Each sample was analyzed in technical duplicate by LC-MS/MS using a Thermo Easy nLC 1200-QExactive HF. Collected data was then processed using Proteome Discoverer 2.1 by searching against the Uniprot *C. elegans* database, client specified database (IFE-1, IFE-3, tags), and a contaminants database, using Sequest. Results were further analyzed in Scaffold. Scaffold thresholds were set to: 2 peptide minimum, 95% protein and peptide scoring thresholds. Ratios were calculated based on average spectral counts for both IFE-1 and IFE-3 relative to their respective controls. A ratio of >2 was considered a potential interactor. For comparison of IFE-1 and IFE-3 associated proteins we use both "low-stringency" and "high-stringency" parameters. The parameters for "low stringency" hits were defined as: those interactors who had at least an average spectral count of 1 in the two technical replicates, and had at least a two-fold enrichment in the IFE pulldown relative to its respective control. The parameters for "high stringency" hits were defined as: those interactors who had at least an average spectral count of 4 in the two technical replicates, and had at least a four-fold enrichment in the IFE pulldown relative to its respective control. Additionally, factors identified in both lists for IFE-1 and IFE-3 were compared using an online a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams called BioVenn [231].

REFERENCES

1. Pushpa, K., G.A. Kumar, and K. Subramaniam, *Translational Control of Germ Cell Decisions*. Results Probl. Cell Differ., 2017. **59**:175-200.(doi): p. 10.1007/978-3-319-44820-6_6.
2. Lebedeva, L.A., et al., *Transcriptional quiescence in primordial germ cells*. Crit. Rev. Biochem. Mol. Biol., 2018. **53**(6): p. 579-595. doi: 10.1080/10409238.2018.1506733. Epub 2018 Oct 3.
3. Schier, A.F., *The maternal-zygotic transition: death and birth of RNAs*. Science, 2007. **316**(5823): p. 406-7. doi: 10.1126/science.1140693.
4. Becker, K., et al., *Quantifying post-transcriptional regulation in the development of Drosophila melanogaster*. Nat. Commun., 2018. **9**(1): p. 4970. doi: 10.1038/s41467-018-07455-9.
5. Nusch, M. and C.R. Eckmann, *Translational control in the Caenorhabditis elegans germ line*. Adv. Exp. Med. Biol., 2013. **757**:205-47.(doi): p. 10.1007/978-1-4614-4015-4_8.
6. Puoti, A., et al., *RNA and sex determination in Caenorhabditis elegans. Post-transcriptional regulation of the sex-determining tra-2 and fem-3 mRNAs in the Caenorhabditis elegans hermaphrodite*. EMBO Rep., 2001. **2**(10): p. 899-904. doi: 10.1093/embo-reports/kve209.
7. Hentze, M.W., et al., *A brave new world of RNA-binding proteins*. Nat Rev Mol Cell Biol, 2018. **19**(5): p. 327-341. doi: 10.1038/nrm.2017.130. Epub 2018 Jan 17.
8. Bhandari, D., et al., *Structural basis for the Nanos-mediated recruitment of the CCR4-NOT complex and translational repression*. Genes. Dev., 2014. **28**(8): p. 888-901. doi: 10.1101/gad.237289.113.
9. Lahr, R.M., et al., *La-related protein 1 (LARP1) binds the mRNA cap, blocking eIF4F assembly on TOP mRNAs*. Elife., 2017. **6**.(doi): p. 10.7554/eLife.24146.
10. Friend, K., et al., *A conserved PUF-Ago-eEF1A complex attenuates translation elongation*. Nat Struct Mol Biol, 2012. **19**(2): p. 176-83. doi: 10.1038/nsmb.2214.
11. Collier, B., et al., *The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells*. EMBO J., 2005. **24**(14): p. 2656-66. doi: 10.1038/sj.emboj.7600738. Epub 2005 Jul 7.
12. Ding, X.C. and H. Grosshans, *Repression of C. elegans microRNA targets at the initiation level of translation requires GW182 proteins*. EMBO J., 2009. **28**(3): p. 213-22. doi: 10.1038/emboj.2008.275. Epub 2009 Jan 8.
13. Freimer, J.W., T.J. Hu, and R. Blelloch, *Decoupling the impact of microRNAs on translational repression versus RNA degradation in embryonic stem cells*. Elife., 2018. **7**.(pii): p. 38014. doi: 10.7554/eLife.38014.
14. Iwakawa, H.O. and Y. Tomari, *The Functions of MicroRNAs: mRNA Decay and Translational Repression*. Trends Cell Biol, 2015. **25**(11): p. 651-665. doi: 10.1016/j.tcb.2015.07.011. Epub 2015 Oct 1.
15. Saxe, J.P. and H. Lin, *Small noncoding RNAs in the germline*. Cold Spring Harb Perspect Biol, 2011. **3**(9): p. a002717. doi: 10.1101/cshperspect.a002717.

16. Carthew, R.W. and E.J. Sontheimer, *Origins and Mechanisms of miRNAs and siRNAs*. Cell, 2009. **136**(4): p. 642-655.
17. Okamura, K., et al., *Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways*. Genes Dev, 2004. **18**(14): p. 1655-1666.
18. Weick, E.M. and E.A. Miska, *piRNAs: from biogenesis to function*. Development, 2014. **141**(18): p. 3458-3471.
19. Rouget, C., et al., *Maternal mRNA deadenylation and decay by the piRNA pathway in the early Drosophila embryo*. Nature, 2010. **467**(7319): p. 1128-1132.
20. Mitchell, S.F. and R. Parker, *Principles and properties of eukaryotic mRNPs*. Mol Cell, 2014. **54**(4): p. 547-58. doi: 10.1016/j.molcel.2014.04.033.
21. Stebbins-Boaz, B., et al., *Maskin is a CPEB-associated factor that transiently interacts with eIF-4E*. Mol. Cell, 1999. **4**(6): p. 1017-1027.
22. Yoffe, Y., et al., *Binding specificities and potential roles of isoforms of eukaryotic initiation factor 4E in Leishmania*. Eukaryot. Cell, 2006. **5**(12): p. 1969-1979.
23. Hernandez, G., et al., *Mextli is a novel eukaryotic translation initiation factor 4E-binding protein that promotes translation in Drosophila melanogaster*. Mol. Cell. Biol., 2013. **33**(15): p. 2854-2864.
24. Mayberry, L.K., et al., *Evidence for variation in the optimal translation initiation complex: plant eIF4B, eIF4F, and eIF(iso)4F differentially promote translation of mRNAs*. Plant Physiol., 2009. **150**(4): p. 1844-54. doi: 10.1104/pp.109.138438. Epub 2009 Jun 3.
25. Friday, A.J., et al., *Spatial and temporal translational control of germ cell mRNAs mediated by the eIF4E isoform IFE-1*. J. Cell Sci., 2015. **128**(24): p. 4487-4498.
26. Friday, A.J. and B.D. Keiper, *Positive mRNA Translational Control in Germ Cells by Initiation Factor Selectivity*. Biomed Research International, 2015. **2015**: p. e327963.
27. Morrison, J.K., et al., *Induction of cap-independent BiP (hsp-3) and Bcl-2 (ced-9) translation in response to eIF4G (IFG-1) depletion in C. elegans*. Translation, 2014. **2**(1): p. e28935.
28. Lee, M.H., et al., *A Systematic mRNA Control Mechanism for Germline Stem Cell Homeostasis and Cell Fate Specification*. BMB Rep, 2015. **2015**(25): p. 3259.
29. Huggins, H.P., et al., *Distinct roles of two eIF4E isoforms in the germline of Caenorhabditis elegans*. J. Cell. Sci., 2020. **133**(6).(pii): p. jcs.237990. doi: 10.1242/jcs.237990.
30. Gingras, A.-C., B. Raught, and N. Sonenberg, *eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation*. Annu. Rev. Biochem., 1999. **68**: p. 913-963.
31. Sen, N.D., et al., *eIF4B stimulates translation of long mRNAs with structured 5' UTRs and low closed-loop potential but weak dependence on eIF4G*. Proc Natl Acad Sci USA, 2016. **113**(38): p. 10464-10472.
32. Rozen, F., et al., *Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F*. Mol Cell Biol., 1990. **10**(3): p. 1134-44. doi: 10.1128/mcb.10.3.1134.
33. Mancera-Martinez, E., et al., *ABCE1: A special factor that orchestrates translation at the crossroad between recycling and initiation*, in *RNA Biology*. 2017, Taylor & Francis. p. 1-7.

34. Wells, S.E., et al., *Circularization of mRNA by eukaryotic translation initiation factors*. Mol. Cell, 1998. **2**(1): p. 135-140.
35. Feoktistova, K., et al., *Human eIF4E promotes mRNA restructuring by stimulating eIF4A helicase activity*. Proc Natl Acad Sci U S A, 2013. **110**(33): p. 13339-44. doi: 10.1073/pnas.1303781110. Epub 2013 Jul 30.
36. Komar, A.A. and M. Hatzoglou, *Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states*. Cell, 2011. **10**(2): p. 229-40. doi: 10.4161/cc.10.2.14472. Epub 2011 Jan 15.
37. Keiper, B., *Cap-Independent mRNA Translation in Germ Cells*. Int J Mol Sci. , 2019. **20**(1): p. 173.
38. Beretta, L., et al., *Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation*. EMBO J., 1996. **15**(3): p. 658-64.
39. Rosenwald, I.B., et al., *Eukaryotic translation initiation factor 4E regulates expression of cyclin D1 at transcriptional and post-transcriptional levels*. J Biol Chem., 1995. **270**(36): p. 21176-80. doi: 10.1074/jbc.270.36.21176.
40. Kevil, C.G., et al., *Translational regulation of vascular permeability factor by eukaryotic initiation factor 4E: implications for tumor angiogenesis*. Int J Cancer., 1996. **65**(6): p. 785-90. doi: 10.1002/(SICI)1097-0215(19960315)65:6<785::AID-IJC14>3.0.CO;2-3.
41. Magnuson, B., B. Ekim, and D.C. Fingar, *Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks*. Biochem J, 2012. **441**(1): p. 1-21. doi: 10.1042/BJ20110892.
42. Igreja, C., et al., *4E-BPs require non-canonical 4E-binding motifs and a lateral surface of eIF4E to repress translation*. Nat Commun, 2014. **5**:4790.(doi): p. 10.1038/ncomms5790.
43. Busada, J.T., et al., *Retinoic acid regulates Kit translation during spermatogonial differentiation in the mouse*. Dev Biol, 2015. **397**(1): p. 140-149.
44. Keiper, B.D., et al., *Functional characterization of five eIF4E isoforms in Caenorhabditis elegans*. J. Biol. Chem., 2000. **275**(14): p. 10590-10596.
45. Jankowska-Anyszka, M., et al., *Multiple isoforms of eukaryotic protein synthesis initiation factor 4E in Caenorhabditis elegans can distinguish between, mono- and trimethylated mRNA cap structures*. J. Biol. Chem., 1998. **273**(17): p. 10538-10542.
46. Hernandez, G., et al., *Functional analysis of seven genes encoding eight translation initiation factor 4E (eIF4E) isoforms in Drosophila*. Mech. Dev., 2005. **122**(4): p. 529-543.
47. Rodriguez, C.M., et al., *The Arabidopsis thaliana cDNAs coding for eIF4E and eIF(iso)4E are not functionally equivalent for yeast complementation and are differentially expressed during plant development*. Plant J., 1998. **13**(4): p. 465-473.
48. Henderson, M.A., et al., *A germ line-specific isoform of eIF4E (IFE-1) is required for efficient translation of stored mRNAs and maturation of both oocytes and sperm*. J. Cell Science, 2009. **122**(10): p. 1529-1539.
49. Dinkova, T.D., et al., *Translation of a small subset of Caenorhabditis elegans mRNAs is dependent on a specific eukaryotic translation initiation factor 4E isoform*. Mol. Cell. Biol., 2005. **25**(1): p. 100-113.

50. Song, A., et al., *A C. elegans eIF4E-family member upregulates translation at elevated temperatures of mRNAs encoding MSH-5 and other meiotic crossover proteins*. J. Cell Sci., 2010. **123**(Pt 13): p. 2228-2237.
51. Tettweiler, G., et al., *The Distribution of eIF4E-Family Members across Insecta*. Comparative and Functional Genomics, 2012. **2012**: p. 1-15.
52. Amiri, A., et al., *An isoform of eIF4E is a component of germ granules and is required for spermatogenesis in C. elegans*. Development, 2001. **128**(20): p. 3899-3912.
53. Sengupta, M.S., et al., *ifet-1 is a broad-scale translational repressor required for normal P granule formation in C. elegans*. J. Cell Sci., 2013. **126**(Pt 3): p. 850-859.
54. Kawasaki, I., et al., *PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in C. elegans*. Cell, 1998. **94**(5): p. 635-645.
55. Cordeiro Rodrigues, R.J., et al., *PETISCO is a novel protein complex required for 21U RNA biogenesis and embryonic viability*. Genes Dev., 2019. **33**(13-14): p. 857-870.
56. Cho, P.F., et al., *A new paradigm for translational control: inhibition via 5'-3' mRNA tethering by Bicoid and the eIF4E cognate 4EHP*. Cell., 2005. **121**(3): p. 411-23. doi: 10.1016/j.cell.2005.02.024.
57. Voronina, E., et al., *RNA granules in germ cells*. Cold Spring Harb Perspect Biol, 2011. **3**(12).(pii): p. cshperspect.a002774. doi: 10.1101/cshperspect.a002774.
58. Sengupta, M.S. and P.R. Boag, *Germ granules and the control of mRNA translation*. IUBMB Life, 2012. **64**(7): p. 586-594.
59. Gallo, C.M., et al., *Processing bodies and germ granules are distinct RNA granules that interact in C. elegans embryos*. Dev Biol., 2008. **323**(1): p. 76-87. doi: 10.1016/j.ydbio.2008.07.008. Epub 2008 Jul 16.
60. Schisa, J.A., *Germ Cell Responses to Stress: The Role of RNP Granules*. Front, 2019. **7**:220.(doi): p. 10.3389/fcell.2019.00220. eCollection 2019.
61. Kotaja, N., et al., *The chromatoid body of male germ cells: similarity with processing bodies and presence of Dicer and microRNA pathway components*. Proc Natl Acad Sci U S A., 2006. **103**(8): p. 2647-52. doi: 10.1073/pnas.0509333103. Epub 2006 Feb 13.
62. Wan, G., et al., *Spatiotemporal regulation of liquid-like condensates in epigenetic inheritance*. Nature, 2018. **557**(7707): p. 679-683.
63. Mahowald, A.P. and S. Hennen, *Ultrastructure of the "germ plasm" in eggs and embryos of Rana pipiens*. Dev Biol., 1971. **24**(1): p. 37-53. doi: 10.1016/0012-1606(71)90045-5.
64. Sheth, U., et al., *Perinuclear P granules are the principal sites of mRNA export in adult C. elegans germ cells*. Development, 2010. **137**(8): p. 1305-14. doi: 10.1242/dev.044255. Epub 2010 Mar 10.
65. Brangwynne, C.P., et al., *Germline P granules are liquid droplets that localize by controlled dissolution/condensation*. Science., 2009. **324**(5935): p. 1729-32. doi: 10.1126/science.1172046. Epub 2009 May 21.
66. Wu, Y., et al., *Single-molecule dynamics of the P granule scaffold MEG-3 in the Caenorhabditis elegans zygote*. Mol Biol Cell, 2019. **30**(3): p. 333-345. doi: 10.1091/mbc.E18-06-0402. Epub 2018 Dec 12.

67. Gao, M. and A.L. Arkov, *Next generation organelles: structure and role of germ granules in the germline*. Mol Reprod Dev, 2013. **80**(8): p. 610-23. doi: 10.1002/mrd.22115. Epub 2012 Nov 13.
68. Schisa, J.A., J.N. Pitt, and J.R. Priess, *Analysis of RNA associated with P granules in germ cells of C. elegans adults*. Development, 2001. **128**(8): p. 1287-1298.
69. Marnik, E.A., et al., *Germline Maintenance Through the Multifaceted Activities of GLH/Vasa in Caenorhabditis elegans P Granules*. Genetics, 2019. **213**(3): p. 923-939. doi: 10.1534/genetics.119.302670. Epub 2019 Sep 10.
70. Ottone, C., et al., *The translational repressor Cup is required for germ cell development in Drosophila*. J Cell Sci, 2012. **125**(Pt 13): p. 3114-23. doi: 10.1242/jcs.095208. Epub 2012 Mar 27.
71. Cho, P.F., et al., *A new paradigm for translational control: inhibition via 5'-3' mRNA tethering by Bicoid and the eIF4E cognate 4EHP*. Cell, 2005. **121**(3): p. 411-423.
72. Minshall, N., et al., *CPEB interacts with an ovary-specific eIF4E and 4E-T in early Xenopus oocytes*. J. Biol. Chem., 2007. **282**(52): p. 37389-401.
73. Voronina, E., A. Paix, and G. Seydoux, *The P granule component PGL-1 promotes the localization and silencing activity of the PUF protein FBF-2 in germline stem cells*. Development, 2012. **139**(20): p. 3732-40.
74. Salaun, P., et al., *eIF4E/4E-BP dissociation and 4E-BP degradation in the first mitotic division of the sea urchin embryo*. Dev Biol., 2003. **255**(2): p. 428-39. doi: 10.1016/s0012-1606(02)00099-4.
75. Wilhelm, J.E., et al., *Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz*. J Cell Biol, 2003. **163**(6): p. 1197-204.
76. Andralojc, K.M., et al., *ELLI-1, a novel germline protein, modulates RNAi activity and P-granule accumulation in Caenorhabditis elegans*. PLoS Genet., 2017. **13**(2): p. e1006611.
77. Campbell, A.C. and D.L. Updike, *CSR-1 and P granules suppress sperm-specific transcription in the C. elegans germline*. Development, 2015. **142**(10): p. 1745-1755.
78. Reed, K.J., et al., *Widespread roles for piRNAs and WAGO-class siRNAs in shaping the germline transcriptome of Caenorhabditis elegans*. Nucleic Acids Res., 2020. **48**(4): p. 1811-1827.
79. Updike, D.L., et al., *Germ-granule components prevent somatic development in the C. elegans germline*. Curr. Biol., 2014. **24**(9): p. 970-975.
80. Spike, C.A., et al., *Translational control of the oogenic program by components of OMA ribonucleoprotein particles in Caenorhabditis elegans*. Genetics, 2014. **198**(4): p. 1513-33.
81. Zappavigna, V., et al., *Cup is a nucleocytoplasmic shuttling protein that interacts with the eukaryotic translation initiation factor 4E to modulate Drosophila ovary development*. Proc Natl Acad Sci U S A., 2004. **101**(41): p. 14800-5. doi: 10.1073/pnas.0406451101. Epub 2004 Oct 1.

82. Kawasaki, I., M.H. Jeong, and Y.H. Shim, *Regulation of sperm-specific proteins by IFE-1, a germline-specific homolog of eIF4E, in C. elegans*. Mol. Cells, 2011. **31**(2): p. 191-197.
83. Suter, B., *RNA localization and transport*. Biochim Biophys Acta Gene Regul Mech, 2018. **1861**(10): p. 938-951. doi: 10.1016/j.bbagr.2018.08.004. Epub 2018 Aug 29.
84. Davidson, A., et al., *Localized Translation of gurken/TGF- α mRNA during Axis Specification Is Controlled by Access to Orb/CPEB on Processing Bodies*. Cell Rep, 2016. **14**(10): p. 2451-62. doi: 10.1016/j.celrep.2016.02.038. Epub 2016 Mar 3.
85. Romasko, E.J., et al., *Association of maternal mRNA and phosphorylated EIF4EBP1 variants with the spindle in mouse oocytes: localized translational control supporting female meiosis in mammals*. Genetics, 2013. **195**(2): p. 349-58. doi: 10.1534/genetics.113.154005. Epub 2013 Jul 12.
86. Lasko, P., *mRNA localization and translational control in Drosophila oogenesis*. Cold Spring Harb Perspect Biol, 2012. **4**(10).(pii): p. cshperspect.a012294. doi: 10.1101/cshperspect.a012294.
87. MacDougall, N., et al., *Drosophila gurken (TGF α) mRNA localizes as particles that move within the oocyte in two dynein-dependent steps*. Dev Cell, 2003. **4**(3): p. 307-19.
88. Nijjar, S. and H.R. Woodland, *Localisation of RNAs into the germ plasm of vitellogenic Xenopus oocytes*. PLoS One, 2013. **8**(4): p. e61847. doi: 10.1371/journal.pone.0061847. Print 2013.
89. Kim-Ha, J., J.L. Smith, and P.M. Macdonald, *oskar mRNA is localized to the posterior pole of the Drosophila oocyte*. Cell., 1991. **66**(1): p. 23-35. doi: 10.1016/0092-8674(91)90136-m.
90. Nakamura, A., K. Sato, and K. Hanyu-Nakamura, *Drosophila cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis*. Dev. Cell, 2004. **6**(1): p. 69-78.
91. Igreja, C. and E. Izaurralde, *CUP promotes deadenylation and inhibits decapping of mRNA targets*. Genes Dev, 2011. **25**(18): p. 1955-67. doi: 10.1101/gad.17136311.
92. Raesch, F., et al., *4E-T-bound mRNAs are stored in a silenced and deadenylated form*. Genes Dev, 2020. **34**(11-12): p. 847-860.
93. Parma, D.H., P.E.J. Bennett, and R.E. Boswell, *Mago Nashi and Tsunagi/Y14, respectively, regulate Drosophila germline stem cell differentiation and oocyte specification*. Dev Biol., 2007. **308**(2): p. 507-19. doi: 10.1016/j.ydbio.2007.06.007. Epub 2007 Jun 13.
94. Mohr, S.E., S.T. Dillon, and R.E. Boswell, *The RNA-binding protein Tsunagi interacts with Mago Nashi to establish polarity and localize oskar mRNA during Drosophila oogenesis*. Genes Dev., 2001. **15**(21): p. 2886-99. doi: 10.1101/gad.927001.
95. Bergsten, S.E. and E.R. Gavis, *Role for mRNA localization in translational activation but not spatial restriction of nanos RNA*. Development., 1999. **126**(4): p. 659-69.

96. Rouget, C., et al., *Maternal mRNA deadenylation and decay by the piRNA pathway in the early Drosophila embryo*. *Nature*, 2010. **467**(7319): p. 1128-32. doi: 10.1038/nature09465. Epub 2010 Oct 17.
97. Cruce, S., S. Chatterjee, and E.R. Gavis, *Overlapping but distinct RNA elements control repression and activation of nanos translation*. *Mol Cell.*, 2000. **5**(3): p. 457-67. doi: 10.1016/s1097-2765(00)80440-2.
98. Nelson, M.R., A.M. Leidal, and C.A. Smibert, *Drosophila Cup is an eIF4E-binding protein that functions in Smaug-mediated translational repression*. *EMBO J.*, 2004. **23**(1): p. 150-9. doi: 10.1038/sj.emboj.7600026. Epub 2003 Dec 11.
99. Zaessinger, S., I. Busseau, and M. Simonelig, *Oskar allows nanos mRNA translation in Drosophila embryos by preventing its deadenylation by Smaug/CCR4*. *Development.*, 2006. **133**(22): p. 4573-83. doi: 10.1242/dev.02649. Epub 2006 Oct 18.
100. Clouse, K.N., S.B. Ferguson, and T. Schupbach, *Squid, Cup, and PABP55B function together to regulate gurken translation in Drosophila*. *Dev Biol*, 2008. **313**(2): p. 713-724.
101. Nusch, M., et al., *The Ccr4-Not deadenylase complex constitutes the main poly(A) removal activity in C. elegans*. *J. Cell Sci.*, 2013. **126**(Pt 18): p. 4274-4285.
102. Temme, C., et al., *A complex containing the CCR4 and CAF1 proteins is involved in mRNA deadenylation in Drosophila*. *EMBO J.*, 2004. **23**(14): p. 2862-71. doi: 10.1038/sj.emboj.7600273. Epub 2004 Jun 24.
103. Waghay, S., et al., *Xenopus CAF1 requires NOT1-mediated interaction with 4E-T to repress translation in vivo*. *Rna*, 2015. **21**(7): p. 1335-45. doi: 10.1261/rna.051565.115. Epub 2015 May 26.
104. Berthet, C., et al., *CCR4-associated factor CAF1 is an essential factor for spermatogenesis*. *Mol Cell Biol.*, 2004. **24**(13): p. 5808-20. doi: 10.1128/MCB.24.13.5808-5820.2004.
105. Richter, J.D., *Cytoplasmic polyadenylation in development and beyond*. *Microbiol Mol Biol Rev.*, 1999. **63**(2): p. 446-56.
106. Mendez, R. and J.D. Richter, *Translational control by CPEB: a means to the end*. *Nat. Rev. Mol. Cell Biol.*, 2001. **2**(7): p. 521-529.
107. Cao, Q. and J.D. Richter, *Dissolution of the maskin-eIF4E complex by cytoplasmic polyadenylation and poly(A)-binding protein controls cyclin B1 mRNA translation and oocyte maturation*. *EMBO J.*, 2002. **21**(14): p. 3852-3862.
108. Wong, L.C. and P. Schedl, *Cup blocks the precocious activation of the orb autoregulatory loop*. *PLoS One*, 2011. **6**(12): p. e28261. doi: 10.1371/journal.pone.0028261. Epub 2011 Dec 2.
109. Casanal, A., et al., *Architecture of eukaryotic mRNA 3'-end processing machinery*. *Science*, 2017. **358**(6366): p. 1056-1059. doi: 10.1126/science.aao6535. Epub 2017 Oct 26.
110. Wahle, E., *Poly(A) tail length control is caused by termination of processive synthesis*. *J Biol Chem.*, 1995. **270**(6): p. 2800-8. doi: 10.1074/jbc.270.6.2800.
111. Vi, S.L., et al., *Target specificity among canonical nuclear poly(A) polymerases in plants modulates organ growth and pathogen response*. *Proc Natl Acad Sci U S A*, 2013. **110**(34): p. 13994-9. doi: 10.1073/pnas.1303967110. Epub 2013 Aug 5.

112. Huang, Y. and G.G. Carmichael, *Role of polyadenylation in nucleocytoplasmic transport of mRNA*. Mol Cell Biol., 1996. **16**(4): p. 1534-42. doi: 10.1128/mcb.16.4.1534.
113. Sheets, M.D., M. Wu, and M. Wickens, *Polyadenylation of c-mos mRNA as a control point in Xenopus meiotic maturation*. Nature, 1995. **374**: p. 511-516.
114. Dufourt, J., et al., *piRNAs and Aubergine cooperate with Wispy poly(A) polymerase to stabilize mRNAs in the germ plasm*. Nat Commun, 2017. **8**(1): p. 1305. doi: 10.1038/s41467-017-01431-5.
115. Kim, K.W., T.L. Wilson, and J. Kimble, *GLD-2/RNP-8 cytoplasmic poly(A) polymerase is a broad-spectrum regulator of the oogenesis program*. Proc Natl Acad Sci U S A, 2010. **107**(40): p. 17445-50. doi: 10.1073/pnas.1012611107. Epub 2010 Sep 20.
116. Cui, J., et al., *Cytoplasmic polyadenylation is a major mRNA regulator during oogenesis and egg activation in Drosophila*. Dev Biol, 2013. **383**(1): p. 121-31. doi: 10.1016/j.ydbio.2013.08.013. Epub 2013 Aug 23.
117. Kahvejian, A., G. Roy, and N. Sonenberg, *The mRNA closed-loop model: the function of PABP and PABP-interacting proteins in mRNA translation*. Cold Spring Harb Symp Quant Biol, 2001. **66**:293-300.(doi): p. 10.1101/sqb.2001.66.293.
118. Wakiyama, M., H. Imataka, and N. Sonenberg, *Interaction of eIF4G with poly(A)-binding protein stimulates translation and is critical for Xenopus oocyte maturation*. Curr Biol., 2000. **10**(18): p. 1147-50. doi: 10.1016/s0960-9822(00)00701-6.
119. Vazquez-Pianzola, P., H. Urlaub, and B. Suter, *Pabp binds to the osk 3'UTR and specifically contributes to osk mRNA stability and oocyte accumulation*. Dev Biol, 2011. **357**(2): p. 404-18. doi: 10.1016/j.ydbio.2011.07.009. Epub 2011 Jul 14.
120. Wormington, M., *Poly(A) and translation: developmental control*. Curr. Opin. Cell Biol., 1993. **5**: p. 950-954.
121. Yi, H., et al., *PABP Cooperates with the CCR4-NOT Complex to Promote mRNA Deadenylation and Block Precocious Decay*. Mol Cell, 2018. **70**(6): p. 1081-1088.e5. doi: 10.1016/j.molcel.2018.05.009.
122. Paillard, L., et al., *Identification of a C-rich element as a novel cytoplasmic polyadenylation element in Xenopus embryos*. Mech Dev., 2000. **93**(1-2): p. 117-25. doi: 10.1016/s0925-4773(00)00279-3.
123. Simon, R., J.P. Tassan, and J.D. Richter, *Translational control by poly(A) elongation during Xenopus development: differential repression and enhancement by a novel cytoplasmic polyadenylation element*. Genes Dev., 1992. **6**(12B): p. 2580-91. doi: 10.1101/gad.6.12b.2580.
124. Pique, M., et al., *A combinatorial code for CPE-mediated translational control*. Cell, 2008. **132**(3): p. 434-448.
125. Slevin, M.K., F. Gourronc, and R.S. Hartley, *ElrA binding to the 3'UTR of cyclin E1 mRNA requires polyadenylation elements*. Nucleic Acids Res, 2007. **35**(7): p. 2167-76. doi: 10.1093/nar/gkm084. Epub 2007 Mar 13.
126. Barkoff, A.F., et al., *Translational control of cyclin B1 mRNA during meiotic maturation: coordinated repression and cytoplasmic polyadenylation*. Dev, 2000. **220**(1): p. 97-109.

127. Barkoff, A., S. Ballantyne, and M. Wickens, *Meiotic maturation in Xenopus requires polyadenylation of multiple mRNAs*. *Embo J*, 1998. **17**(11): p. 3168-75.
128. Stebbins-Boaz, B., L.E. Hake, and J.D. Richter, *CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and c-mos mRNAs and is necessary for oocyte maturation in Xenopus*. *EMBO J.*, 1996. **15**(10): p. 2582-2592.
129. Hake, L.E. and J.D. Richter, *CPEB is a specificity factor that mediates cytoplasmic polyadenylation during Xenopus oocyte maturation*. *Cell.*, 1994. **79**(4): p. 617-27. doi: 10.1016/0092-8674(94)90547-9.
130. Barnard, D.C., et al., *Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation*. *Cell.*, 2004. **119**(5): p. 641-51. doi: 10.1016/j.cell.2004.10.029.
131. Kim, J.H. and J.D. Richter, *Opposing polymerase-deadenylase activities regulate cytoplasmic polyadenylation*. *Mol Cell.*, 2006. **24**(2): p. 173-83. doi: 10.1016/j.molcel.2006.08.016.
132. Mendez, R., et al., *Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA*. *Nature.*, 2000. **404**(6775): p. 302-7. doi: 10.1038/35005126.
133. Copeland, P.R. and M. Wormington, *The mechanism and regulation of deadenylation: identification and characterization of Xenopus PARN*. *RNA*, 2001. **7**(6): p. 875-86. doi: 10.1017/s1355838201010020.
134. Standart, N. and N. Minshall, *Translational control in early development: CPEB, P-bodies and germinal granules*. *Biochem Soc Trans.*, 2008. **36**(Pt 4): p. 671-6. doi: 10.1042/BST0360671.
135. Yu, C., et al., *BTG4 is a meiotic cell cycle-coupled maternal-zygotic-transition licensing factor in oocytes*. *Nat Struct Mol Biol*, 2016. **23**(5): p. 387-394.
136. Crittenden, S.L., et al., *GLP-1 is localized to the mitotic region of the C. elegans germ line*. *Development*, 1994. **120**(10): p. 2901-2911.
137. Ciosk, R., M. DePalma, and J.R. Priess, *Translational regulators maintain totipotency in the Caenorhabditis elegans germline*. *Science*, 2006. **311**(5762): p. 851-853.
138. Pillai, R.S., S.N. Bhattacharyya, and W. Filipowicz, *Repression of protein synthesis by miRNAs: how many mechanisms?* *Trends Cell Biol.*, 2007. **17**(3): p. 118-126.
139. Lai, F., et al., *Nanos1 functions as a translational repressor in the Xenopus germline*. *Mech. Dev.*, 2011. **128**(1-2): p. 153-163.
140. Zahr, S.K., et al., *A Translational Repression Complex in Developing Mammalian Neural Stem Cells that Regulates Neuronal Specification*. *Neuron.*, 2018. **97**(3): p. 520-537 e6.
141. Ghosh, S. and P. Lasko, *Loss-of-function analysis reveals distinct requirements of the translation initiation factors eIF4E, eIF4E-3, eIF4G and eIF4G2 in Drosophila spermatogenesis*. *PLoS One*, 2015. **10**(4): p. e0122519.
142. Rhoads, R.E., *Regulation of eukaryotic protein synthesis by initiation factors*. *J. Biol. Chem.*, 1993. **268**(5): p. 3017-3020.
143. Keiper, B.D., W. Gan, and R.E. Rhoads, *Protein synthesis initiation factor 4G*. *Int. J. Biochem. Cell Biol.*, 1999. **31**: p. 37-41.

144. Gingras, A.C., et al., *Hierarchical phosphorylation of the translation inhibitor 4E-BP1*. Genes Dev., 2001. **15**(21): p. 2852-2864.
145. Joshi, B., et al., *Phylogenetic analysis of eIF4E-family members*. BMC Evol Biol, 2005. **5**(48): p. 1-20.
146. Patrick, R.M. and K.S. Browning, *The eIF4F and eIFiso4F Complexes of Plants: An Evolutionary Perspective*. Comp. Funct. Genomics, 2012. **2012**(287814): p. 287814.
147. Mangio, R.S., S. Votra, and D. Pruyne, *The canonical eIF4E isoform of C. elegans regulates growth, embryogenesis, and germline sex-determination*. Biol. Open., 2015. **4**(7): p. 843-51.
148. Jan, E., et al., *The STAR protein, GLD-1, is a translational regulator of sexual identity in Caenorhabditis elegans*. EMBO J., 1999. **18**(1): p. 258-269.
149. Clifford, R., et al., *FOG-2, a novel F-box containing protein, associates with the GLD-1 RNA binding protein and directs male sex determination in the C. elegans hermaphrodite germline*. Development, 2000. **127**(24): p. 5265-76.
150. Goodwin, E.B., et al., *Translational regulation of tra-2 by its 3' untranslated region controls sexual identity in C. elegans*. Cell, 1993. **75**(2): p. 329-339.
151. Starostina, N.G., et al., *A CUL-2 ubiquitin ligase containing three FEM proteins degrades TRA-1 to regulate C. elegans sex determination*. Dev Cell, 2007. **13**(1): p. 127-39.
152. Mehra, A., et al., *Negative regulation of male development in Caenorhabditis elegans by a protein-protein interaction between TRA-2A and FEM-3*. Genes Dev, 1999. **13**(11): p. 1453-63.
153. Zhang, B., et al., *A conserved RNA-binding protein that regulates sexual fates in the C. elegans hermaphrodite germ line*. Nature, 1997. **390**(6659): p. 477-84.
154. Thompson, B.E., et al., *Dose-dependent control of proliferation and sperm specification by FOG-1/CPEB*. Development, 2005. **132**(15): p. 3471-81.
155. Schedl, T. and J. Kimble, *fog-2, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in Caenorhabditis elegans*. Genetics, 1988. **119**(1): p. 43-61.
156. Barton, M.K. and J. Kimble, *fog-1, a regulatory gene required for specification of spermatogenesis in the germ line of Caenorhabditis elegans*. Genetics, 1990. **125**: p. 29-39.
157. Hodgkin, J., *Sex determination in the nematode C. elegans: analysis of tra-3 suppressors and characterization of fem genes*. Genetics, 1986. **114**(1): p. 15-52.
158. Lamont, L.B., et al., *FBF-1 and FBF-2 regulate the size of the mitotic region in the C. elegans germline*. Dev. Cell, 2004. **7**(5): p. 697-707.
159. Yoon, D.S., et al., *MPK-1/ERK regulatory network controls the number of sperm by regulating timing of sperm-oocyte switch in C. elegans germline*. Biochem. Biophys. Res. Commun., 2017. **491**(4): p. 1077-1082.
160. King, H.A. and A.P. Gerber, *Translatome profiling: methods for genome-scale analysis of mRNA translation*. Brief Funct. Genomics, 2014. **2014**(6): p. elu045.
161. Kershner, A.M. and J. Kimble, *Genome-wide analysis of mRNA targets for Caenorhabditis elegans FBF, a conserved stem cell regulator*. Proc. Natl. Acad. Sci. U S A, 2010. **107**(8): p. 3936-41.

162. Zanin, E., et al., *LARP-1 promotes oogenesis by repressing fem-3 in the C. elegans germline*. J. Cell Sci., 2010. **123**(Pt 16): p. 2717-2724.
163. Luitjens, C., et al., *CPEB proteins control two key steps in spermatogenesis in C. elegans*. Genes Dev., 2000. **14**(20): p. 2596-2609.
164. Li, W., et al., *An eIF4E-binding protein regulates katanin protein levels in C. elegans embryos*. J Cell Biol, 2009. **187**(1): p. 33-42.
165. Dostie, J., et al., *A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA 5' cap-binding protein, eIF4E*. Embo J, 2000. **19**(12): p. 3142-56.
166. Cha, D.S., et al., *The Ras-ERK MAPK regulatory network controls dedifferentiation in Caenorhabditis elegans germline*. Biochim. Biophys. Acta, 2012. **1823**(10): p. 1847-55.
167. Marcotrigiano, J., et al., *Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP*. Cell, 1997. **89**(6): p. 951-961.
168. Miyoshi, H., et al., *Discrimination between mono- and trimethylated cap structures by two isoforms of Caenorhabditis elegans eIF4E*. EMBO J., 2002. **21**(17): p. 4680-4690.
169. Blumenthal, T., *Trans-splicing and operons*. WormBook, 2005: p. 1-9.
170. Barton, M.K., T.B. Schedl, and J. Kimble, *Gain-of-function mutations of fem-3, a sex-determination gene in Caenorhabditis elegans*. Genetics, 1987. **115**(1): p. 107-19.
171. Crittenden, S.L., et al., *Cellular analyses of the mitotic region in the Caenorhabditis elegans adult germ line*. Mol Biol Cell, 2006. **17**(7): p. 3051-61.
172. Wood, W., L. J., and S. Strome, *Developmental Determinants in Embryos of Caenorhabditis elegans*. J Nematol., 1982. **14**(2): p. 267-73.
173. Shimada, M., H. Yokosawa, and H. Kawahara, *OMA-1 is a P granules-associated protein that is required for germline specification in Caenorhabditis elegans embryos*. Genes Cells, 2006. **11**(4): p. 383-396.
174. Rybarska, A., et al., *GLS-1, a novel P granule component, modulates a network of conserved RNA regulators to influence germ cell fate decisions*. PLoS Genet, 2009. **5**(5): p. e1000494.
175. Sheth, U., et al., *Perinuclear P granules are the principal sites of mRNA export in adult C. elegans germ cells*. Development, 2010. **137**(8): p. 1305-14.
176. Hershey, J.W., N. Sonenberg, and M.B. Mathews, *Principles of translational control: an overview*. Cold Spring Harb Perspect Biol, 2012. **4**(12).(pii): p. 4/12/a011528. doi: 10.1101/cshperspect.a011528.
177. Contreras, V., et al., *Cap-Independent translation promotes C. elegans germ cell apoptosis through Apaf-1/CED-4 in a caspase-dependent mechanism*. PLoS ONE, 2011. **6**(9): p. e24,444.
178. Dickinson, D.J., et al., *Streamlined Genome Engineering with a Self-Excising Drug Selection Cassette*. Genetics, 2015. **200**(4): p. 1035-49.
179. McCarter, J., et al., *On the control of oocyte meiotic maturation and ovulation in Caenorhabditis elegans*. Dev Biol., 1999. **205**(1): p. 111-28. doi: 10.1006/dbio.1998.9109.
180. Jud, M., et al., *Large P body-like RNPs form in C. elegans oocytes in response to arrested ovulation, heat shock, osmotic stress, and anoxia and are regulated by*

- the major sperm protein pathway*. Dev Biol., 2008. **318**(1): p. 38-51. doi: 10.1016/j.ydbio.2008.02.059. Epub 2008 Mar 14.
181. Frydryskova, K., et al., *Distinct recruitment of human eIF4E isoforms to processing bodies and stress granules*. BMC Mol. Biol., 2016. **17**(1): p. 21.
 182. Andrei, M.A., et al., *A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies*. Rna, 2005. **11**(5): p. 717-27.
 183. Gruner, S., et al., *Structural motifs in eIF4G and 4E-BPs modulate their binding to eIF4E to regulate translation initiation in yeast*. Nucleic Acids Res., 2018. **46**(13): p. 6893-6908.
 184. Haghighat, A., et al., *Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E*. Embo J., 1995. **14**(22): p. 5701-9.
 185. Conine, C., et al., *Argonautes promote male fertility and provide a paternal memory of germline gene expression in C. elegans*. Cell, 2013. **155**(7): p. 1532-44. doi: 10.1016/j.cell.2013.11.032.
 186. Gao, M., W. Rychlik, and R.E. Rhoads, *Cloning and characterization of human eIF4E genes*. J. Biol. Chem., 1998. **273**(8): p. 4622-4628.
 187. Spike, C.A., et al., *The TRIM-NHL Protein LIN-41 and the OMA RNA-Binding Proteins Antagonistically Control the Prophase-to-Metaphase Transition and Growth of Caenorhabditis elegans Oocytes*. Genetics, 2014. **198**(4): p. 1535-1558.
 188. Tsukamoto, T., et al., *LIN-41 and OMA Ribonucleoprotein Complexes Mediate a Translational Repression-to-Activation Switch Controlling Oocyte Meiotic Maturation and the Oocyte-to-Embryo Transition in Caenorhabditis elegans*. Genetics, 2017. **206**(4): p. 2007-2039. doi: 10.1534/genetics.117.203174. Epub 2017 Jun 1.
 189. Lundgren, D., et al., *Role of spectral counting in quantitative proteomics*. Expert Rev Proteomics, 2010. **7**(1): p. 39-53. doi: 10.1586/epr.09.69.
 190. van Wolfswinkel, J., et al., *CDE-1 affects chromosome segregation through uridylation of CSR-1-bound siRNAs*. Cell., 2009. **139**(1): p. 135-48. doi: 10.1016/j.cell.2009.09.012.
 191. Nousch, M., et al., *The cytoplasmic poly(A) polymerases GLD-2 and GLD-4 promote general gene expression via distinct mechanisms*. Nucleic Acids Res., 2014. **42**(18): p. 11622-11633.
 192. Gallegos, M., et al., *Repression by the 3' UTR of fem-3, a sex-determining gene, relies on a ubiquitous mog-dependent control in Caenorhabditis elegans*. EMBO J., 1998. **17**(21): p. 6337-6347.
 193. Minasaki, R., A. Puoti, and A. Streit, *The DEAD-box protein MEL-46 is required in the germ line of the nematode Caenorhabditis elegans*. BMC Dev Biol., 2009. **9**:35.(doi): p. 10.1186/1471-213X-9-35.
 194. Peter, D., et al., *Mextli proteins use both canonical bipartite and novel tripartite binding modes to form eIF4E complexes that display differential sensitivity to 4E-BP regulation*. Genes Dev, 2015. **29**(17): p. 1835-49. doi: 10.1101/gad.269068.115. Epub 2015 Aug 20.

195. Conine, C.C., et al., *Argonautes ALG-3 and ALG-4 are required for spermatogenesis-specific 26G-RNAs and thermotolerant sperm in Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U S A, 2010. **107**(8): p. 3588-93.
196. Gerson-Gurwitz, A., et al., *A Small RNA-Catalytic Argonaute Pathway Tunes Germline Transcript Levels to Ensure Embryonic Divisions*. Cell, 2016. **165**(2): p. 396-409. doi: 10.1016/j.cell.2016.02.040. Epub 2016 Mar 24.
197. Jud, M., et al., *Conservation of large foci formation in arrested oocytes of Caenorhabditis nematodes*. Dev. Genes Evol., 2007. **217**(3): p. 221-226.
198. Haghighat, A., et al., *Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E*. EMBO J., 1995. **14**(22): p. 5701-9.
199. Hagerman, R., et al., *Fragile X syndrome*. Nat Rev Dis Primers, 2017. **3**:17065.(doi): p. 10.1038/nrdp.2017.65.
200. Myrick, L., et al., *Fragile X syndrome due to a missense mutation*. Eur J Hum Genet, 2014. **22**(10): p. 1185-9. doi: 10.1038/ejhg.2013.311. Epub 2014 Jan 22.
201. Santini, E., et al., *Reducing eIF4E-eIF4G interactions restores the balance between protein synthesis and actin dynamics in fragile X syndrome model mice*. Sci Signal, 2017. **10**(504): p. eaan0665. doi: 10.1126/scisignal.aan0665.
202. Napoli, I., et al., *The fragile X syndrome protein represses activity-dependent translation through CYFIP1, a new 4E-BP*. Cell., 2008. **134**(6): p. 1042-54. doi: 10.1016/j.cell.2008.07.031.
203. Prasad, A., et al., *The PUF binding landscape in metazoan germ cells*. Rna, 2016. **22**(7): p. 1026-43. doi: 10.1261/rna.055871.116. Epub 2016 May 10.
204. Baker, C.C. and M.T. Fuller, *Translational control of meiotic cell cycle progression and spermatid differentiation in male germ cells by a novel eIF4G homolog*. Development., 2007. **134**(15): p. 2863-9. doi: 10.1242/dev.003764. Epub 2007 Jul 4.
205. Contreras, V., et al., *Depletion of the cap-associated isoform of translation factor eIF4G induces germline apoptosis in C. elegans*. Cell Death Differ., 2008. **15**(8): p. 1232-1242.
206. Hu, J., F. Sun, and M.A. Handel, *Nuclear localization of EIF4G3 suggests a role for the XY body in translational regulation during spermatogenesis in mice*. Biol Reprod, 2018. **98**(1): p. 102-114.
207. Franklin-Dumont, T.M., et al., *A novel eIF4G homolog, Off-schedule, couples translational control to meiosis and differentiation in Drosophila spermatocytes*. Development, 2007. **134**(15): p. 2851-2861.
208. Thomson, T., et al., *Isolation of new polar granule components in Drosophila reveals P body and ER associated proteins*. Mech Dev., 2008. **125**(9-10): p. 865-73. doi: 10.1016/j.mod.2008.06.005. Epub 2008 Jun 12.
209. Ouyang, J.P.T., et al., *P Granules Protect RNA Interference Genes from Silencing by piRNAs*. Dev Cell, 2019. **50**(6): p. 716-728.e6. doi: 10.1016/j.devcel.2019.07.026. Epub 2019 Aug 8.
210. Flemr, M., et al., *P-body loss is concomitant with formation of a messenger RNA storage domain in mouse oocytes*. Biol Reprod, 2010. **82**(5): p. 1008-17. doi: 10.1095/biolreprod.109.082057. Epub 2010 Jan 14.

211. Strome, S. and W. Wood, *Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of Caenorhabditis elegans*. Proc Natl Acad Sci U S A., 1982. **79**(5): p. 1558-62. doi: 10.1073/pnas.79.5.1558.
212. Morgan, M., et al., *mRNA 3' uridylation and poly(A) tail length sculpt the mammalian maternal transcriptome*. Nature, 2017. **548**(7667): p. 347-351. doi: 10.1038/nature23318. Epub 2017 Aug 9.
213. Boag, P.R., A. Nakamura, and T.K. Blackwell, *A conserved RNA-protein complex component involved in physiological germline apoptosis regulation in C. elegans*. Development, 2005. **132**(22): p. 4975-4986.
214. Sonenberg, N. and A.-C. Gingras, *The mRNA 5' cap-binding protein eIF4E and control of cell growth*. Curr. Opin. Cell Biol., 1998. **10**(2): p. 268-275.
215. Rhoads, R.E., S. Joshi-Barve, and C. Rinker-Schaeffer, *Mechanism of action and regulation of protein synthesis initiation factor 4E: effects on mRNA discrimination, cellular growth rate, and oncogenesis*. Progr. Nucl. Acid Res. Mol. Biol., 1993. **46**: p. 183-219.
216. Mader, S., et al., *The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins*. Mol Cell Biol., 1995. **15**(9): p. 4990-7. doi: 10.1128/mcb.15.9.4990.
217. Villaescusa, J.C., et al., *Cytoplasmic Prep1 interacts with 4EHP inhibiting Hoxb4 translation*. PLoS One, 2009. **4**(4): p. e5213. doi: 10.1371/journal.pone.0005213. Epub 2009 Apr 13.
218. Andrei, M.A., et al., *A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies*. RNA., 2005. **11**(5): p. 717-27. doi: 10.1261/rna.2340405.
219. Weick, E. and E. Miska, *piRNAs: from biogenesis to function*. Development, 2014. **141**(18): p. 3458-71. doi: 10.1242/dev.094037.
220. Philippe, L., et al., *An in vivo genetic screen for genes involved in spliced leader trans-splicing indicates a crucial role for continuous de novo spliced leader RNP assembly*. Nucleic Acids Res, 2017. **45**(14): p. 8474-8483. doi: 10.1093/nar/gkx500.
221. Padron, A., S. Iwasaki, and N.T. Ingolia, *Proximity RNA Labeling by APEX-Seq Reveals the Organization of Translation Initiation Complexes and Repressive RNA Granules*. Mol Cell, 2016. **75**(4): p. 875-887.
222. Praitis, V., et al., *Creation of low-copy integrated transgenic lines in Caenorhabditis elegans*. Genetics, 2001. **157**(3): p. 1217-1226.
223. Brenner, S., *The genetics of Caenorhabditis elegans*. Genetics, 1974. **77**(1): p. 71-94.
224. Timmons, L., D.L. Court, and A. Fire, *Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans*. Gene, 2001. **263**: p. 103-112.
225. Timmons, L. and A. Fire, *Specific interference by ingested dsRNA [letter]*. Nature, 1998. **395**(6705): p. 854.

226. Maniatis, T., E.F. Fritsch, and J. Sambrook, *Molecular Cloning: A Laboratory Manual*. 2nd ed. 1989, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
227. Datla, U.S., et al., *Role of PUF-8/PUF protein in stem cell control, sperm-oocyte decision and cell fate reprogramming*. J Cell Physiol, 2014. **229**(10): p. 1306-1311.
228. Dickinson, D.J., et al., *Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination*. Nat. Methods, 2013. **10**(10): p. 1028-1034.
229. Yoon, D.S., D.L. Pendergrass, and M.H. Lee, *A simple and rapid method for combining fluorescent in situ RNA hybridization (FISH) and immunofluorescence in the C. elegans germline*. MethodsX, 2016. **3**: p. 378-85.
230. Yoon, D.S., et al., *Subunits of the DNA polymerase alpha-primase complex promote Notch-mediated proliferation with discrete and shared functions in C. elegans germline*. FEBS J., 2018. **285**(14): p. 2590-2604.
231. Hulsen, T., J. de Vlieg, and W. Alkema, *BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams*. BMC Genomics., 2008. **9**:488.(doi): p. 10.1186/1471-2164-9-488.

