**ifet-1** is a broad-scale translational repressor required for normal P granule formation in *C. elegans*

Madhu S. Sengupta¹, Wai Yee Low¹, Joseph R. Patterson², Hyun-Min Kim³, Ana Traven¹, Traude H. Beilharz¹, Monica P. Colaiácovo², Jennifer A. Schisa² and Peter R. Boag¹,∗

¹Department of Biochemistry and Molecular Biology, Monash University, Victoria 3800, Australia  
²Department of Biology, Central Michigan University, Mount Pleasant, MI 48859, USA  
³Department of Genetics, Harvard Medical School, MA 02115, USA

*Author for correspondence (peter.boag@monash.edu)

Accepted 21 November 2012  
Journal of Cell Science 126, 850–859  
© 2013. Published by The Company of Biologists Ltd  
doi: 10.1242/jcs.119834

**Summary**

Large cytoplasmic ribonucleoprotein germ granule complexes are a common feature in germ cells. In *C. elegans* these are called P granules and for much of the life-cycle they associate with nuclear pore complexes in germ cells. P granules are rich in proteins that function in diverse RNA pathways. Here we report that the *C. elegans* homolog of the eIF4E-transporter IFET-1 is required for oogenesis but not spermatogenesis. We show that IFET-1 is required for translational repression of several maternal mRNAs in the distal gonad and functions in conjunction with the broad-scale translational regulators CGH-1, CAR-1 and PATR-1 to regulate germ cell sex determination. Furthermore we have found that IFET-1 localizes to P granules throughout the gonad and in the germ cell lineage in the embryo. Interestingly, IFET-1 is required for the normal ultrastructure of P granules and for the localization of CGH-1 and CAR-1 to P granules. Our findings suggest that IFET-1 is a key translational regulator and is required for normal P granule formation.

**Key words:** eIF4E transporter, P granule, Germ granules, Translational regulation, *Caenorhabditis elegans*

**Introduction**

Germ cells are unique as they are immortal and pluripotent. In all species examined, germ cells contain a distinctive electron dense, non-membranous, cytoplasmic structure called germ granules. Germ granules are highly enriched in RNAs and RNA-binding proteins and are key regulators of RNA metabolism in germ cells (Voronina et al., 2011). The presence of germ granules within germ cells can vary between species, for example in *C. elegans* germ granules are continually present and are required for normal proliferation and differentiation (Kawasaki et al., 1998; Spike et al., 2008). In mammals, germ granules form de novo and are present mainly in the later stages of germ cell development (Pepling et al., 2007). Despite this difference, it is clear that germ granules in both invertebrates and vertebrates share many of the same proteins and likely play similar functions in regulating multiple RNA pathways critical for gametogenesis and early embryonic development.

In *C. elegans*, germ granules are known as P granules. For most of the gonad, P granules are located in perinuclear foci tightly associated with the nuclear pore complex (NPC) (Fig. 1A) (Pitt et al., 2000; Schisa et al., 2001) and appear to extend the nuclear pore environment into the cytoplasm (Updike et al., 2011). Recent studies have found that nascent mRNAs are transported through the NPC to perinuclear P granules and that transcription is required for the localization of two P granule-specific components, PGL-1, the P granule nucleating factor and eIF4E-binding protein, and GLH-2, the *C. elegans* homolog of the DEAD-box RNA helicase Vasa (Sheth et al., 2010). Although perinuclear P granules are continuously associated with transcriptionally active germ cells, components within these granules appear to be highly dynamic. Fluorescence recovery after photobleaching (FRAP) experiments demonstrated a very rapid recovery of PGL-1::GFP fluorescence in perinuclear P granules (Sheth et al., 2010). In late stage oocytes, where transcription appears to be off, P granules detach from the nuclear pore and become dispersed throughout the cytoplasm (Fig. 1A). Over 40 proteins have now been localized to P granules including proteins involved in Dicer-dependent and Dicer-independent small RNA pathways, and regulators of transcription and translation (Voronina et al., 2011). Interestingly, some P granule components also function in somatic processing (P) bodies where they are important factors in the decapping mediated mRNA turnover pathway (Sheth and Parker, 2003). These include the DEAD box RNA helicase CGH-1 and the Lsm- and RGG-domain containing protein CAR-1, both of which are required for normal levels of germ cell apoptosis and gonad function (Navarro et al., 2001; Audhya et al., 2005; Boag et al., 2005). This has led to the speculation that germ granules and P bodies are evolutionarily related hubs of mRNA regulation in germ and somatic cells, respectively (Strome and Lehmann, 2007).

In P bodies, CGH-1 and CAR-1 homologs function as ‘general’ translational repressors and activators of mRNA decapping (Coller and Parker, 2005; Nissan et al., 2010). In mammalian cell culture, the CGH-1 homolog RCK directly interacts with the metazoan-specific eIF4E-transporter (4E-T), which is a nuclear/cytoplasmic shuttling protein required for P body formation. Knockdown of 4E-T results in loss of RCK from P bodies, while overexpression of 4E-T leads to inhibition of cap-dependent translation (Andrei et al., 2005; Ferraiuolo et al., 2007).
Interestingly, 4E-T homologs are also important regulatory factors during oogenesis in many species. In *Drosophila*, the 4E-T homolog CUP is important for oogenesis where it is required for translational repression and localization of *nanos* and *oskar* mRNAs (Wilhelm et al., 2003; Nakamura et al., 2004; Nelson et al., 2004; Zappavigna et al., 2004), as well as deadenylation of mRNAs (Igreja and Izaurralde, 2011). *Xenopus* 4E-T is expressed in early stage oocytes, interacts with an ovary-specific eIF4E and regulates translation in in vitro assays (Minshall et al., 2007). Mouse 4E-T homolog, Clast4, is also highly expressed during oogenesis, however, its role is less well defined (Villaescusa et al., 2006). The *C. elegans* homolog of 4E-T, IFET-1 (previously known as SPN-2/PQN-45), was recently shown to be required for translational regulation of *zif-1* and *mei-1* mRNAs during oocyte maturation and in one-cell stage embryos, respectively (Li et al., 2009; Guven-Ozkan et al., 2010). The function of IFET-1 in the earlier stages of oogenesis in *C. elegans* is unknown.

Here we report that IFET-1 is a broad scale translational repressor of germ cell mRNAs in the distal region of the gonad and is required for normal gonad development and P granule formation. Ultrastructural studies indicate that IFET-1 is required for formation of the electron dense crest and base of P granules that are thought to be sites of mRNA concentration (Schisa et al., 2001; Sheth et al., 2010). Our data support a model in which IFET-1 is required for retention of mRNAs in P granules which allows translational repressor proteins to bind the mRNA prior to export into the core of the gonad.

**Results**

IFET-1 is required for normal gonad development

IFET-1 (F56F3.1) has ~47% similarity at the amino acid level to human 4E-T and shares the gross overall protein structure, including predicted nuclear import and export signals, a glutamine-rich region in the C-terminus and an eIF4E-binding motif at the N-terminus (Fig. 1B). Microarray analyses indicate *ifet-1* is oogenesis-enriched (Reinke et al., 2004), and it is required for oocyte maturation and early embryonic development (Li et al., 2009; Guven-Ozkan et al., 2010). In mammalian somatic cells, 4E-T is required for P body formation and localization of the CGH-1 ortholog RCK/p54 (Andrei et al., 2005; Ferratuolo et al., 2005). To examine if 4E-T is important...
for perinuclear P granule formation we obtained the previously uncharacterized deletion mutant ifet-1(tm2944) (Fig. 1C), which appears to be a strong loss-of-function. Male ifet-1(tm2944) animals are fertile (data not shown), however, ifet-1(tm2944) hermaphrodites are sterile, indicating that IFET-1 has a major role in oogenesis. Gonads lacking IFET-1 appeared grossly normal in size, however, the distal region had a bulbous shape, similar to what has been described for animals lacking CGH-1 (Table 1; supplementary material Fig. S1) (Navarro et al., 2001). When grown at 20°C, ifet-1(tm2944) gonads displayed a mildly disorganized meiotic progression and contained a mixture of oocytes in diakinesis and pachytene stages in the proximal gonad (supplementary material Figs S2, S3). When grown at 25°C an unusual phenotype observed in ~14% of animals was a bifurcation of the gonad shortly after the transition zone (Fig. 1D; Table 1). The bifurcated arm lacked a distal tip cell, but immunostained positively for the pachytene stage marker SYP-1, a component of the central region of the synaptonemal complex, indicating these cells were at the pachytene stage of meiosis (Fig. 1D). A large number of bright small DAPI staining foci in the proximal region of the gonad (Fig. 1D) stained positively with the sperm-specific antibody SP56 (Table 1; supplementary material Fig. S1B), indicating that these animals had a strong masculinization of the germline (MOG) phenotype: 48% of ifet-1(tm2944) hermaphrodites had a mixture of sperm and oocytes in the proximal gonad, while 25% contained only sperm. The switch from sperm to oocyte production in the gonad is highly dependent on post-transcriptional gene regulation (Ellis, 2008). Therefore we investigated if the general translational regulators CGH-1, CAR-1 and PATR-1 synthetically enhanced the MOG phenotype. Knockdown of cgh-1/RNAi background resulted in 68% and 52% of gonads, respectively, ifet-1(tm2944); cgh-1(RNAi) background it resulted in a dramatic masculinization of the gonad and early embryo. We generated an antibody that specifically recognizes IFET-1 and immuno-stained 1-day-old adult hermaphrodite gonads (supplementary material Fig. S1A). IFET-1 levels were low in the distal gonad, dramatically increased as germ cells entered meiosis, and remained high throughout the remainder of the gonad, a staining pattern very similar to CGH-1 and CAR-1 (Navarro et al., 2001; Boag et al., 2005). IFET-1 colocalized with the majority of CGH-1/CAR-1 perinuclear P granules throughout the gonad (Fig. 2, data not shown). In the gonad, core IFET-1 was also abundant, with the majority of IFET-1 colocalized with CGH-1 in small foci (Fig. 2). During embryonic development IFET-1 again showed a remarkably similar staining pattern as CGH-1 and CAR-1. In one-cell embryos, IFET-1 was highly expressed and diffusely distributed throughout the cytoplasm and associated with P granules (Fig. 3A). IFET-1 was asymmetrically distributed in the embryo and was significantly enriched in the germ cell lineage (P cells) where it localized to P granules throughout embryogenesis. In the somatic cells of the embryo, IFET-1 abundance was dramatically reduced after the four-cell stage; however, small foci that colocalized with CGH-1 and CAR-1 were evident (Fig. 3A,B) and correspond to P bodies associated with decapping mediated mRNA turnover (Boag et al., 2008).

To test if IFET-1 was required for CGH-1 and CAR-1 localization we immuno-stained extruded gonads from 1-day-old ifet-1(tm2944) animals. Strikingly, both CGH-1 and CAR-1 failed to localize to perinuclear P granules in the absence of IFET-1 and were instead diffusely distributed throughout the gonad (Fig. 4A). Interestingly, in gonads lacking CGH-1, IFET-1 typically failed to localize to perinuclear P granules and was diffusely distributed throughout the gonad, while CAR-1 localized to sheet-like structures in the gonad core (Audhya et al., 2005; Boag et al., 2005). IFET-1 localized to P granules in less than 5% of germ cells (Fig. 4B). To examine if IFET-1 was required for the localization of other perinuclear P granule components, we investigated the localization of two constitutive components, the germ cell-specific proteins PGL-1 and GLH-1. The absence of IFET-1 had little effect on localization of GLH-1, but interestingly PGL-1 localization was severely affected (Fig. 4C). In 60% of ifet-1(tm2944) gonads PGL-1 failed to localize to perinuclear P granules, and was instead detected in small foci unassociated with the germ cells (Fig. 4C). The level of IFET-1 was required for CGH-1 and CAR-1 to localize to P granules

To further investigate the interactions between IFET-1 and CGH-1/CAR-1 it was important to determine their distributions in the gonad and early embryo. We generated an antibody that specifically recognizes IFET-1 and immuno-stained 1-day-old adult hermaphrodite gonads (supplementary material Fig. S1A). IFET-1 levels were low in the distal gonad, dramatically increased as germ cells entered meiosis, and remained high throughout the remainder of the gonad, a staining pattern very similar to CGH-1 and CAR-1 (Navarro et al., 2001; Boag et al., 2005). IFET-1 colocalized with the majority of CGH-1/CAR-1 perinuclear P granules throughout the gonad (Fig. 2, data not shown). In the gonad, core IFET-1 was also abundant, with the majority of IFET-1 colocalized with CGH-1 in small foci (Fig. 2). During embryonic development IFET-1 again showed a remarkably similar staining pattern as CGH-1 and CAR-1. In one-cell embryos, IFET-1 was highly expressed and diffusely distributed throughout the cytoplasm and associated with P granules (Fig. 3A). IFET-1 was asymmetrically distributed in the embryo and was significantly enriched in the germ cell lineage (P cells) where it localized to P granules throughout embryogenesis. In the somatic cells of the embryo, IFET-1 abundance was dramatically reduced after the four-cell stage; however, small foci that colocalized with CGH-1 and CAR-1 were evident (Fig. 3A,B) and correspond to P bodies associated with decapping mediated mRNA turnover (Boag et al., 2008).

To test if IFET-1 was required for CGH-1 and CAR-1 localization we immuno-stained extruded gonads from 1-day-old ifet-1(tm2944) animals. Strikingly, both CGH-1 and CAR-1 failed to localize to perinuclear P granules in the absence of IFET-1 and were instead diffusely distributed throughout the gonad (Fig. 4A). Interestingly, in gonads lacking CGH-1, IFET-1 typically failed to localize to perinuclear P granules and was diffusely distributed throughout the gonad, while CAR-1 localized to sheet-like structures in the gonad core (Audhya et al., 2005; Boag et al., 2005). IFET-1 localized to P granules in less than 5% of germ cells (Fig. 4B). To examine if IFET-1 was required for the localization of other perinuclear P granule components, we investigated the localization of two constitutive components, the germ cell-specific proteins PGL-1 and GLH-1. The absence of IFET-1 had little effect on localization of GLH-1, but interestingly PGL-1 localization was severely affected (Fig. 4C). In 60% of ifet-1(tm2944) gonads PGL-1 failed to localize to perinuclear P granules, and was instead detected in small foci unassociated with the germ cells (Fig. 4C). The level

---

**Table 1. Defects in ifet-1(tm2944) grown at 25°C**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sp (%)</th>
<th>Sp + Oo (%)</th>
<th>Oo (%)</th>
<th>Bulbous (%)</th>
<th>Bifurcation (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>ifet-1(tm2944)</td>
<td>25</td>
<td>48</td>
<td>27</td>
<td>97</td>
<td>14</td>
<td>77</td>
</tr>
<tr>
<td>cgh-1(RNAi)</td>
<td>0</td>
<td>3</td>
<td>97</td>
<td>99</td>
<td>0</td>
<td>69</td>
</tr>
<tr>
<td>ifet-1(tm2944); cgh-1(RNAi)</td>
<td>97</td>
<td>2</td>
<td>1</td>
<td>90</td>
<td>4</td>
<td>116</td>
</tr>
<tr>
<td>car-1(RNAi)</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>44</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>ifet-1(tm2944); car-1(RNAi)</td>
<td>68</td>
<td>27</td>
<td>5</td>
<td>34</td>
<td>5</td>
<td>44</td>
</tr>
<tr>
<td>patr-1(RNAi)</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>ifet-1(tm2944); patr-1(RNAi)</td>
<td>52</td>
<td>48</td>
<td>0</td>
<td>57</td>
<td>9</td>
<td>21</td>
</tr>
</tbody>
</table>

Sp, sperm; Oo, oocytes.
of PGL-1 was the same as in wild-type and ifet-1(tm2944) adult hermaphrodites (supplementary material Fig. S5A).

With the clear genetic and cell biological interaction uncovered between IFET-1 and CGH-1/CAR-1 in germ cell differentiation, we next asked if the proteins physically interact. We immunoprecipitated endogenous CGH-1 from adult hermaphrodite protein extracts and determined by western blot that IFET-1 co-precipitated. IFET-1 co-immunoprecipitated with CGH-1, but not non-specific IgG control antibody (supplementary material Fig. S5B). Levels of CGH-1 and CAR-1 were not altered in the absence of IFET-1 (supplementary material Fig. S5A), indicating that the lack of perinuclear P granule localization was not simply due to degradation of these proteins. Together these data suggest that IFET-1 may play a major role in the localization or retention of CGH-1/CAR-1 to perinuclear P granules. Interestingly, IFET-1 has a less dramatic effect on PGL-1 and GLH-1 localization compared to CGH-1 and CAR-1. Previous studies have indicated that the absence of CGH-1 results in defects in PGL-1 localization (Updike and Strome, 2009); therefore, it is possible that the absence of CGH-1 or CAR-1 from perinuclear P granules in ifet-1(tm2944) may contribute to the mislocalization of PGL-1.

**Ultrastructural analysis of perinuclear P granules**

The formation of perinuclear P granules in pachytene germ cells appears to be defective in ifet-1(tm2944) worms based on immunocytochemistry data with CAR-1, CGH-1 and PGL-1 (Fig. 4). Therefore we compared the ultrastructure of perinuclear P granules in wild-type and ifet-1(tm2944) pachytene stage germ cells by transmission electron microscopy (TEM). P granules were detected as electron dense structures along the nuclear envelope of wild-type and ifet-1(tm2944) nuclei. No significant difference was found in the number of perinuclear P granules per nucleus in ifet-1(tm2944) compared to wild type (n = 128 and 153 P granules, respectively). Although variation in the size and shape of P granules was noted, no significant differences in the gross length or height of P granules were detected (Fig. 5). The pachytene nuclei are normally transcriptionally active, and clusters of nuclear pores are organized beneath the P granules.

![Fig. 2. IFET-1 colocalizes with CGH-1 in perinuclear P granules and in the gonad core.](image)

![Fig. 3. IFET-1 colocalizes with CGH-1 in the embryo.](image)
leading to the speculation that newly synthesized and translationally repressed maternal mRNAs may preferentially interact with P granules as they are exported through the clusters of nuclear pores (Pitt et al., 2000; Schisa et al., 2001; Sheth et al., 2010). Since IFET-1 is required to repress the translation of several 3' UTR reporter transgenes (Table 2), we were especially interested in any differences in the substructure of P granules that might be consistent with differential levels or trafficking of RNA within ifet-1(tm2944) P granules. In the meiotic pachytene nuclei, perinuclear P granules often have an electron-dense 'crest' and 'base' that are ~70–100 nm from the nuclear envelope (Sheth et al., 2010). Strikingly we found significantly fewer perinuclear P granules with electron dense crests or bases in ifet-1(tm2944) (37%, n=48 P granules) compared to wild type (70%, n=108 P granules, P<0.05)
Table 2. IFET-1 is required for translational regulation of multiple mRNAs

<table>
<thead>
<tr>
<th>3'UTR</th>
<th>Treatment</th>
<th>Gonad zones</th>
</tr>
</thead>
<tbody>
<tr>
<td>puf-5 (n=100)</td>
<td>control (RNAi)</td>
<td>A B C D E</td>
</tr>
<tr>
<td>rme-2 (n=82)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>lip-1 (n=60)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>pal-1 (n=80)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>spn-4 (n=90)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>mex-3 (n=105)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>daz-1 (n=58)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>pie-1 (n=100)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>gld-2 (n=150)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>daz-1 (n=58)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>pup-2 (n=150)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>oma-2 (n=150)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
<tr>
<td>pie-1 (n=100)</td>
<td>ifet-1(RNAi)</td>
<td></td>
</tr>
</tbody>
</table>

Black, high level of GFP expression; dark grey, medium level of GFP expression; light grey, low level of GFP expression. See Fig. 1A for illustration of gonad zones.

IFET-1 is required for the translational regulation of multiple mRNAs

In mammalian somatic cells 4E-Ts are thought to act as broad translational inhibitors by disruption of the binding of eIF4G to eIF4E, thereby inhibiting the formation of the pre-initiation complex (Ferraiuolo et al., 2005). To examine if IFET-1 is required for the normal spatial translational regulation of germ cell mRNAs, we used RNAi to knockdown ifet-1 in eight strains that express a green fluorescent protein (GFP) reporter under the control of different 3' UTRs (Merritt et al., 2008). RNAi knockdown of ifet-1 produces gonads with a less severe phenotype compared to the mutant, with the formation of oocytes and the occasional viable progeny produced. Compared to wild-type gonads expressing the reporter genes, ifet-1(RNAi) gonads displayed a consistent alteration in GFP localization for each of the 4F-3'UTR strains. In the wild-type background, GFP expressed under the control of the puf-5, rme-2, lip-1 or pal-1 3' UTRs were first detected in the late stages of pachytene (zones D/E, see Fig. 1A) and became more abundant as germ cells entered diplotene and diakinesis (supplementary material Fig. S4) (Merritt et al., 2008). In all four cases, ifet-1(RNAi) resulted in GFP expression being detected earlier/more distally in the transition zone (zone B, see Fig. 1A) and extended to the proximal end of the gonad (Table 2; supplementary material Fig. S4). GFP under the control of the spn-4 or mex-3 3' UTRs had a different expression pattern in wild-type gonads, with expression first detected in the mitotic cells (zones A and B), not seen in the transition zone, pachytene, or diplotene cells (zones B–E), and then detectable in diakinesis stage oocytes. In ifet-1(RNAi) gonads, GFP was detectable throughout the gonad (zones A–E). GFP under the control of either the daz-1 or pie-1 3' UTRs was expressed throughout the gonad in wild-type animals, while in ifet-1(RNAi) gonads increased expression levels were detected in the mitotic region (zones A and B, and zones C–D for daz-1; Table 2). Interestingly, puf-5, rme-2, lip-1, pal-1 spn-4 and mex-3 3' UTRs expressed GFP at consistently lower levels in ifet-1(RNAi) animals compared to the wild type, suggesting that these mRNAs were either not fully de-repressed, not as efficiently translated, or degraded more rapidly. We were unable to test if knockdown of both ifet-1 and cgl-1 in the gonads further released the translational repression of the selected mRNAs as these gonads were highly masculinized (Table 1).

Regulation of poly(A) tail length is an important mechanism to regulate translation, mRNA storage and stability. The Drosophila elf4E-binding protein CUP has recently been shown to play a role in promoting deadenylation of mRNAs (Igreja and Izaurralde, 2011). To test if IFET-1 also plays a role in regulating poly(A) tail length, we selected several predicted cytoplasmic poly(A) polymerase GLD-2 target mRNAs (egg-1, pup-2, oma-2 and pie-1) (Kim et al., 2010) and assayed their poly(A) tail length in wild-type, gld-2 and ifet-1(tm2944) mutants (Fig. 6). As expected, in gld-2 animals the poly(A) tails were shorter compared to wild-type, reflecting their translational inhibition prior to activation by cytoplasmic adenylation (Fig. 6) (Kim et al., 2010). The distribution of poly(A)-tail lengths was distinctly shorter for each of the GLD-2 target

Fig. 6. Analysis of poly(A) tail length in ifet-1(tm2944) animals. The poly(A) tail length of four mRNAs in wild-type, ifet-1(tm2944) and gld-2 animals was examined using the ePAT assay (see Materials and Methods). In the wild type, the distribution can extend to ~150 bases. The A~150 and A~12 annotations on the left hand side show the position of the ampiclons having poly(A) tails of 12 and 150 residues, respectively. In the absence of the cytoplasmic poly(A)-polymerase GLD-2, all the mRNAs examined had a short poly(A) tail. In the absence of IFET-1, the poly(A) tail length distribution was enriched for shorter poly(A) tails compared with wild type.
mRNAs in \textit{ifet-1(tm2944)} compared to wild-type. The slightly longer size distribution of the poly(A)-tails in \textit{ifet-1(tm2944)} animals is consistent with the interpretation of premature translation and associated deadenylation in transcript aging (Goldstrohm and Wickens, 2008). The size difference in poly(A) tail length between \textit{gld-2} and \textit{ifet-1(tm2944)} animals is consistent with the requirement of CUP for deadenylation and repression of specific mRNAs in \textit{Drosophila} (Igreja and Izaurralde, 2011), suggesting that IFET-1/CUP may function as conserved factors for regulation of poly(A) tail length.

**Discussion**

In this study we have uncovered a role for the eIF4E-binding protein IFET-1 in gonad development and fertility. In the absence of IFET-1 multiple germ cell reporter mRNAs are prematurely translated, indicating for the first time that IFET-1 functions in early stage germ cells (pachytene). The inhibition of translation is likely mediated by IFET-1 competing with eIF4G to bind eIF4E, thereby inhibiting the formation of the pre-initiation complex. This inhibition appears to use a similar strategy employed by the decapping mediated mRNA turnover in somatic P bodies, where orthologs of CGH-1, CAR-1 and PATR-1 are additional factors required for efficient translational inhibition and mRNA decay. Importantly, we demonstrate that IFET-1 is required for localization of CGH-1 and CAR-1 to perinuclear P granules, and IFET-1 impacts the localization of the constitutive P granule component PGL-1. Interestingly, the substructure of perinuclear P granules is abnormal in the absence of IFET-1 suggesting that RNA transit through the granules may be defective.

**IFET-1 is a component of an evolutionarily conserved RNA regulation pathway**

In oocytes and neurons an evolutionarily conserved RNA-protein (RNP) complex plays an important role in regulating timing of protein production (Garneau et al., 2007). In \textit{C. elegans}, \textit{D. melanogaster}, \textit{Xenopus} and mouse, this complex regulates translation of specific mRNAs during oogenesis (Paynton, 1998; Nakamura et al., 2001; Navarro et al., 2001; Wilhelm et al., 2003; Nakamura et al., 2004; Boag et al., 2005; Matsumoto et al., 2005; Minshull et al., 2007). Components of the complex include orthologs of the DEAD box RNA helicase CGH-1, Y-box proteins, LSM domain containing proteins, eIF4E and eIF4E-binding proteins. Homology searches indicate that IFET-1 is related to the eIF4E-binding proteins 4E-T and CUP, and it is predicted to perform a similar role in inhibiting cap-dependent translation by competing with eIF4G to bind eIF4E, thereby inhibiting the formation of the pre-initiation complex (Sonenberg and Hinnebusch, 2009). Supporting this model, our data shows the spatial control of translational repression is defective in gonads in which IFET-1 is knocked down, with premature expression of eight mRNAs normally translated only in the proximal region of the gonad (Table 2). Additionally, the poly(A) tails of four out of five mRNAs were intermediate in length compared to wild-type and \textit{gld-2} animals, suggesting that these tails represent the regular shorting of mRNAs during active translation (Goldstrohm and Wickens, 2008).

The pleiotropic phenotypes observed in \textit{ifet-1(tm2944)} hermaphrodites (Fig. 1; supplementary material Fig. S1; Table 1) may be the result of premature translation of multiple mRNAs. The MOG phenotype we identified in \textit{ifet-1(tm2944)} hermaphrodites may reflect defects in post-transcriptional gene regulatory networks upon which the germ cell sex determination pathway is highly dependent (Ellis, 2008). We identified a striking enhancement of the MOG phenotype when the decapping activators \textit{cgh-1}, \textit{car-1} and \textit{patr-1} were knocked down in the \textit{ifet-1(tm2944)} background (Table 1). Interestingly, we were unable to find MOG animals when \textit{cgh-1}, \textit{car-1} or \textit{patr-1} were knocked down individually or in combination. These data place IFET-1 at the heart of the translational repressive complex that functions early in germ cell development, and are consistent with the predicted function of inhibiting the rate-limiting step of eIF4E-eIF4G binding in cap-dependent translation.

Consistent with previous reports that IFET-1 orthologs are important components of P bodies, we showed that IFET-1 co-localizes with CGH-1 in small foci in somatic cells of the embryo which we have previously identified as P bodies (Fig. 3) (Boag et al., 2008). This suggests that IFET-1 is involved in decapping-mediated mRNA decay in these cells. In \textit{S. cerevisiae}, it has been recently proposed that the first step in mRNA decapping is the inhibition of translation initiation via the combined action of Dhh1, Scd6 and Pat1 (CGH-1, CAR-1 and PATR-1 in \textit{C. elegans}) (Nissan et al., 2010). Our data suggests that a similar mechanism is used to inhibit translational initiation during repression of germ cell mRNAs and for entry of somatic mRNAs into the decapping mediated mRNA turnover pathway.

**IFET-1 is required for normal perinuclear P granule formation**

An important finding of our studies was the requirement of IFET-1 for CGH-1 and CAR-1 localization to perinuclear P granules. This observation mirrors the requirement of 4E-T for localization of CGH-1/RCK to P bodies in somatic cells (Andrej et al., 2005; Ferraiuolo et al., 2005). An important difference between the perinuclear P granules and P bodies is the origin of the mRNA substrate upon which the protein complex forms. In the case of P bodies, the mRNAs are predominantly derived from a pool of mRNAs that have been translated and were translationally repressed prior to their localization to P bodies (Parker and Sheth, 2007). In the case of perinuclear P granules, the mRNAs are delivered directly to the RNA-granules via the nuclear export machinery. Interestingly, we were unable to detect any significant alteration in GLH-1 localization in the absence of IFET-1, and only incomplete mislocalization of PGL-1, which may also be due to secondary effects related to the lack of CGH-1/CAR-1 (Updike and Strome, 2009).

The failure of CGH-1 and CAR-1 to localize to perinuclear P granules in the absence of IFET-1 suggests that IFET-1 is required either for their transport to perinuclear P granules, or for their retention. IFET-1 homologs 4E-T and CUP are both nucleocytoplasmic shuttling proteins that are important for eIF4E localization. 4E-T is required for the importation of eIF4E into the nucleus (Dostie et al., 2000), while CUP is required for the posterior localization of eIF4E within the cytoplasm of developing oocytes (Zappavigna et al., 2004). Interestingly, in addition to binding the mRNA cap, eIF4E is able to bind to specific motifs in the 3’ UTR of some mRNAs (Culjkovic et al., 2005; Rong et al., 2008). IFET-1 contains predicted nuclear localization (NLS) and export signals (NES) and may also be required for eIF4E localization. It will be important to test if the predicted NLS/NES are functional in IFET-1 and to identify the domains that determine P granule localization of each of these proteins.
Our ultrastructure studies reveal an important defect in perinuclear P granule substructure. In the absence of IFET-1, perinuclear P granules are generally normal in size and distribution, suggesting that the hydrophobic size exclusion mesh (Updike et al., 2011) of the P granules is not affected, consistent with our GLH-1 immunostaining data. Although the size of P granules is not affected, the substructure is significantly altered, with many perinuclear P granules lacking the electron dense crest and/or base substructures which are thought to correspond to areas of localized RNA-enrichments (Sheth et al., 2010). The absence of these substructures suggests that RNAs are either entering P granules at a decreased rate, or are moving through the perinuclear P granules more rapidly. Interestingly the number of NPCs per perinuclear P granule is significantly reduced in the absence of IFET-1 (1.35 NPCs/P granule compared to 2.34 NPCs/P granule in wild type) and may indicate that the rate of mRNA nuclear export is reduced in the absence of IFET-1.

Proposed model of IFET-1 function
We propose a model in which IFET-1 is required for the retention of a subset of germ cell mRNAs in perinuclear P granules and for establishment of their translational repression. In the absence of IFET-1 and perinuclear P granule localization of CGH-1/CAR-1, many germ cell mRNAs are precociously translated and the electron dense crests and bases of perinuclear P granules are not observed. These data are consistent with mRNAs not being retained in the perinuclear P granules and instead rapidly transiting into the cytoplasm where they are available for translation. IFET-1, CGH-1 and CAR-1 all share similar expression patterns in the gonad, in which expression is low in the mitotic cells and dramatically increases concurrent with the increase in transcription as germ cells enter the transition zone (Navarro et al., 2001; Boag et al., 2005). We believe that IFET-1/CGH-1/CAR-1 functions in these transcriptionally active germ cells to generate a robust translational repression complex on germ cell mRNAs shortly after their export to perinuclear P granules and prior to the mRNP complex being exported into the gonad core (Fig. 7). In this model the IFET-1/CGH-1/CAR-1/PATR-1 repression complex acts as a general inhibitor of translation through the binding of IFET-1 to eIF4E, thereby repressing cap-dependent translation initiation. CGH-1/CAR-1/PATR-1 likely act as ‘activators’ of mRNA-protein remodeling; however, the mechanism remains unknown.

An important question that remains to be addressed is how is mRNA specificity achieved? One mechanism is to have sequence-specific mRNA binding proteins bind to or act cooperatively with the general inhibitor complex. In Drosophila oocytes, CUP physically binds the RNA-binding protein Bruno and represses translation of oskar mRNA via the binding of Bruno to a specific element in the 3′ UTR of the mRNA (Nakamura et al., 2004). In C. elegans, it has recently been shown that IFET-1 binds to the sequence-specific RNA-binding proteins OMA-1/-2 and is required for translational repression of zif-1 and mei-1 mRNAs in the proximal gonad and one cell-embryo (Li et al., 2009; Guven-Ozkan et al., 2010). OMA-1/-2 are strongly and exclusively detected in the cellularizing oocytes (Detwiler et al., 2001), a period when transcription is already inhibited. This suggests that the OMA proteins bind mRNAs that are already translationally repressed prior to their loading into the cellularizing oocytes. In Xenopus oocytes, two distinct translational repressive complexes have been identified. In the early oocyte a large RNP complex containing Xp54 (CGH-1), RAP55B (CAR-1), Pat1 (PATR-1), 4E-T and an ovary-specific eIF4E, repress translation of specific mRNAs (Minshull et al., 2007). In late stage oocytes a second complex is present containing the eIF4E-binding protein Maskin, which increases in abundance at this stage and is important for translational repression and subsequent re-adenylation and translation of specific mRNAs (Cao and Richter, 2002). This suggests that a dynamic two-step mechanism to regulate translation of some germ cell mRNAs by inhibiting the rate-limiting step in formation of the pre-initiation complex is a conserved feature of oogenesis. Given the importance of IFET-1/CGH-1/CAR-1 in mRNA regulation in the gonad, it will be critical to determine how these proteins are trafficked to the perinuclear P granules and the mechanism by which individual mRNAs are identified for translational repression.

Materials and Methods
Strains
Wild-type (N2 Bristol) and mutant strains were maintained using standard methods (Brenner, 1974). The ifet-1(tm2944)III deletion mutant was generated by the National BioResource Project for the Nematode and was outcrossed 8 times and balanced using qC1 [dpy-19(e1259) glp-1(q339) qIs26]. Additional strains used were: cgh-1(ok492)III and the GFP expressing strains: JH2379 (pie-lp:gfpg:histone H2B:pie-1 3′ utr), JH2313 (pie-lp:gfpg:histone H2B:rme-2 3′ utr), JH2223 (pie-lp:gfpg:histone H2B,diz-1 3′ utr), JH2156 (pie-lp:gfpg:orf-lp-1 ORF-lp-1 3′ utr), JH2311 (pie-lp:gfpg:histone H2B:pm-4 3′ utr), JH2013 (pie-lp:gfpg:pal-1 3′ utr), JH2319 (pie-lp:gfpg:pie-1 ORF-lp-1 3′ utr), JH2223 (pie-lp:gfpg:orflp-1 ORF-lp-1 3′ utr).
Antibodies and immunolocalization

Rat affinity-purified polyclonal antibodies were raised against the C-terminal 17 amino acids of IFET-1 (GL Biochem (Shanghai)), Immunostaining with the purified IFET-1 antibody was reduced to background levels in the ifet-1(tm2944). Additional antibodies used were: rabbit anti-CHG-I (1:300), chicken anti-CAR-I (1:300) (Boag et al., 2005), monoclonal K76 (1:10) and SP56 (1:300) (Strome and Wood, 1983), chicken anti-GLH-I (1:200) (Grudel et al., 1996), guinea pig anti-SYP-I (1:200), rabbit anti-AIR-2 (1:100), rabbit anti-LAB-1 (1:50), rabbit anti-SMC-3 (1:200), and rabbit anti-HTP-3 (1:200). Species-specific Alexa A488 and A555 (Invitrogen) were used as secondary antibodies, DNA was stained using DAPI and samples were mounted in Dako fluorescent mounting medium. Immunofluorescence images in Figs 2 and 4 were collected using a Zeiss LSM META 510 Confocal microscope. Immunofluorescence images in Figs 1 and 3 and supplementary material Figs S2 and S3 were collected with an IX-71 microscope (Olympus) controlled by the DeltaVision system (Applied Precision). Images were subjected to deconvolution analysis using the SoftWorx 3.0 program (Applied Precision) as in Nabeshima et al. (Nabeshima et al., 2005).

Immunoprecipitation and western blot analysis

Immunoprecipitation of CGH-I was conducted as described previously (Boag et al., 2005). Western blot analysis of IFET-1 expression was carried out in wild-type and ifet-1(tm2944) adult hermaphrodites. The worms were treated with 1× reducing sample buffer and denatured at 95°C for 5 minutes. Samples were run on 10% SDS-PAGE gels and transferred using standard protocols. The following primary antibodies were used: rat anti-IFET-1 (1:500), chicken anti-CAR-I (1:500), rabbit anti-CHG-I (1:500) and mouse monoclonal anti-mouse c-myc (1:1000; Sigma-Aldrich). Proteins were detected with species-specific HisP conjugated secondary antibodies goat anti-rabbit (1:1000; Dako), goat anti-rabbit (1:1000; Dako), rabbit anti-chicken (1:1000; Invitrogen) and goat anti-mouse (1:1000; Invitrogen). Blots were visualized using ECL detection system (GE Healthcare).

RNA

To analyse the GFP reporter strains, young adult hermaphrodites were injected with double-stranded RNA (1 µg/µl) and allowed to lay eggs for 8 hours before being transferred to new plates. F1 adult hermaphrodites were examined for GFP expression by live imaging. For analysing the MOG phenotype, RNAi was performed by feeding wild-type or ifet-1(tm2944) animals from L1 larvae at 25°C as described previously (Kamath and Ahringer, 2003) and gonads examined 55 hours post L1. pcb19 was used as a control which contains a fragment of Arabidopsis thaliana gene with no homology to any known C. elegans gene.

Transmission electron microscopy

Adult wild-type and ifet-1(tm2944) worms grown at 24°C were prepared according to the methods of Pitt et al. (Pitt et al., 2000). Worms were embedded using Spurr’s resin, and the worms were oriented for longitudinal sections (Spurr, 1969). Samples were sectioned using a PowerTom ultramicrotome. Sections were stained (uranyl acetate (sa) and Reynolds’ lead citrate) for 2 minutes, rinsed and dried with ddH2O (Reynolds, 1963). Grids were imaged after they dried using a Phillips CM10 transmission electron microscope. Images were captured using Kodak 4489 Electron Microscope Film; negatives were developed with Kodak D19 Developer, fixed with Reynold’s lead citrate for 2 minutes, and rinsed with ddH2O (Reynolds, 1963). Grids were imaged at 160,000x magnification; negatives were reprocessed with Kodak P3 film and scanned using a Metatek digitizing table. The final images were processed using Adobe Photoshop® CS2. Nuclear membranes were measured by drawing dots on the membrane to sub-divide it into a series of straight lines. Guidelines were placed on each dot, and the measure tool was used to measure from point to point on the nuclear membrane in µm. Measurements in µm were converted to mm (total enlargement x magnification/1000)), where total enlargement was the measurement between two points on the scanned film negative divided by the measurement between the same points on the final image. All statistics were performed using Minitab® 15.

Poly(A) tail length analysis

We used the ePAT approach to examine poly(A) tail length as described in Jänick et al. (Jänick et al., 2012). The ePAT method introduces a sequence tag at the 3’ end of adenylated RNA and uses this as an anchor to prime reverse transcription. The combination of gene specific primer and a primer complementary to the tag of adenylated RNA and uses this as an anchor to prime reverse transcription. The et al. (Jänick et al., 2012). The ePAT method introduces a sequence tag at the 3' end of adenylated RNA to prime reverse transcription. The poly(A) tail length is visualized as a smear of amplicons of slower migration that extend up (~150 bases) from the size control. The following gene specific primers were used: pup-2 5’-CCCCACTCTGAACGGATCC-3’; pie-1 5’-CTCTAGACCATACAA-3’; egg-1 5’-CCCTAAATTGACCTGGAAATCT-3’. PCR amplification was by 28 cycles using AmpliTaq Gold 360 master. The products were resolved on a 2% high-resolution agarose gel and imaged using as previously described using an LAS 3000 imager and Multi Gauge software (Fujifilm).

Acknowledgements

We thank the International C. elegans Gene Knockout Consortium and the Caenorhabditis Genetics Center for generating deletions and sending strains. The K76 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa. The GLH-1 and SP56 antibodies were gifts from Karen Bennett (Columbia, MO) and Susan Strome (Santa Cruz, CA) respectively. The pCB19 RNAi control was generously provided by Carolyn Behm (Acton, ACT, Australia). Fluorescent imaging was conducted at the Monash Micro Imaging facility. We thank Amy Walker for comments on the manuscript.

Author contributions

M.S.S. and P.R.B. conceived and designed the experiments. M.S.S., W.A.L., J.R.P., H.M.K. and T.H.B. performed the experiments. M.S.S., P.R.B., H.M.K., M.P.C., J.R.P., J.A.S., T.H.B. and A.T. analyzed the data. M.S.S. and P.R.B. wrote the paper.

Funding

This work was supported by the National Health and Medical Research Council of Australia (NHMRC) [grant number 066575 to P.R.B.]; and the National Institutes of Health (NIH) [grant numbers R15 GM09313-01 to J.A.S. and R01 GM072551 to M.P.C.]. Deposited in PMC release after 12 months.

Supplementary material available online at http://jcs.biologists.org/lookup/suppl?doi=10.1242/jcs.119834/DC1

References


Fig. S1. Immunostaining analysis of ifet-1(tm2944) gonads. (A) The anti-IFET-1 antibody specifically recognizes IFET-1. Immunostaining of the distal end of extruded gonads from one-day old adult wild-type and ifet-1(tm2944) animals. IFET-1 levels are low at the mitotic region of the distal gonad and increase as germ cells enter the transition zone. No signal above background was detected in the ifet-1(tm2944) gonads. The distal region of ifet-1(tm2944) gonads displayed a bulbous appearance. Bar represents 20 μm. (B) One-day old ifet-1(tm2944) adult hermaphrodites contain sperm in the pachytenne region of the gonad. Extruded gonads were immunostained with the sperm-specific antibody SP56 and DNA visualized with DAPI. No SP56 staining is evident in wild-type gonads, while ifet-1(tm2944) gonads contain a mixture of SP56 positive and negative cells. Bar represents 10 μm.
Fig. S2. Disorganization of meiotic progression in *ifet-1(tm2944)* grown at 20°C. Low magnification images of whole-mount gonads from age-matched wild-type (N2) and *ifet-1(tm2944)* adult hermaphrodites. Analysis of chromosome morphogenesis by examining DAPI-stained chromosomes reveals that *ifet-1(tm2944)* mutants exhibit a disorganized meiotic progression, especially at diakinesis, compared to wild-type. SYP-1 signal is observed associated with chromosomes from transition zone to diakinesis in wild-type gonads. SYP-1 localization upon entry into meiosis is normal in *ifet-1(tm2944)* mutants. However, loss of SYP-1 signal in *ifet-1(tm2944)* gonads varies throughout nuclei located in what corresponds to the diakinesis region in wild-type, suggesting an apparent disorganization of meiotic progression in this region. Bar represents 10 μm.
Fig. S3. Analysis of markers of meiotic progression in ifet-1(tm2944) grown at 20°C. (A) SYP-1 immunolocalization reveals the presence of mixed-stage meiotic nuclei throughout late prophase in ifet-1 germlines (27.5%, n=11/40; n= number of gonads examined). Yellow arrows indicate a diakinesis oocyte (i) and a pachytene nucleus (ii) closely positioned at the end of prophase. (B) SYP-1 and LAB-1 co-immunostaining reveals that in 62.5% (n=25/40) of germlines, nuclei transition normally through late prophase, although they fail to organize into a single row. 10% (n=4/40) of germlines show normal meiotic progression with oocytes acquiring the single file organization characteristic of wild-type upon exit from pachytene (data not shown). (C) AIR-2 immunostaining highlights the presence of late diakinesis oocytes in ifet-1(tm2944) gonads. Yellow arrows indicate mixed stages. (D) Immunolocalization of SMC-3 and HTP-3 reveal normal axis morphogenesis throughout meiotic progression in ifet-1(tm2944) compared to control.
**Fig. S4. Derepression of GFP reporter genes in the absence of IFET-1.** Representative images of GFP reporter stains that express GFP under the control of specific 3' UTRs. When *ifet-1* was knocked down by RNAi, GFP expression was detected further distally in the gonad.
Fig. S5. Analysis of IFET-1 interactions with CGH-1 and CAR-1. (A) Western blot analysis of CGH-1, CAR-1 and PGL-1 levels in one-day old adult hermaphrodite animals. Compared to wild-type animals, no change in CGH-1, CAR-1 or PGL-1 levels was detected in the absence of IFET-1. The multiple species of CGH-1 and CAR-1 likely represent alternative phosphorylation states of these proteins. Tubulin was used as a loading control. (B) IFET-1 co-immunoprecipitates with CGH-1 in one-day-old adult hermaphrodites extracts.