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An isoform of eIF4E is a component of germ granules and is required for spermatogenesis in *C. elegans*

Anahita Amiri[1], Brett D. Keiper[2], Ichiro Kawasaki[1,3], Yuan Fan[1], Yuji Kohara[3], Robert E. Rhoads[2] and Susan Strome[1,*]

[1]Department of Biology, Jordan Hall, 1001 E. Third St., Indiana University, Bloomington, IN 47405-3700, USA
[2]Louisiana State University Health Sciences Center, Department of Biochemistry and Molecular Biology, 1501 Kings Highway, Shreveport, LA 71130-3932, USA
[3]Genome Biology Laboratory, Center for Genetic Resource Information, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan
*Author for correspondence (e-mail: sstrome@bio.indiana.edu)

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SUMMARY

Control of gene expression at the translational level is crucial for many developmental processes. The mRNA cap-binding protein, eIF4E, is a key player in regulation of translation initiation; appropriate levels of eIF4E are essential for normal cell-cycle regulation and tissue differentiation. The observation that eIF4E levels are elevated during gametogenesis in several organisms suggests that eIF4E might have a specific role in gamete formation as well. We show that one of the five isoforms of *C. elegans* eIF4E, IFE-1, is enriched in the germline and is a component of germ granules (P granules). The association of IFE-1 with P granules requires the P-granule protein PGL-1. In vitro PGL-1 interacts directly with IFE-1, but not with the other four isoforms of eIF4E. Analysis of animals depleted of IFE-1 by RNAi shows that IFE-1 is required for spermatogenesis, specifically for efficient progression through the meiotic divisions and for the production of functional sperm, in both hermaphrodites and males. The requirement for IFE-1 is highly sensitive to temperature. IFE-1 is not required for oogenesis, as *ife-1(RNAi)* hermaphrodites produce viable progeny when normal sperm are supplied. Consistent with a primary role in spermatogenesis, *ife-1* mRNA levels are highest in regions of the gonad undergoing spermatogenesis. Our results suggest that *C. elegans* spermatogenesis requires either this specific isoform of eIF4E or an elevated level of eIF4E.

Key words: eIF4E, Translation initiation, *Caenorhabditis elegans*, Spermatogenesis, P granules

INTRODUCTION

One of the levels at which gene expression is controlled is translation of mRNA. Translational control is crucial for normal embryonic development, proper regulation of the cell cycle, tissue induction and differentiation, and germline development (Morris, 1995; Pain, 1996; Hake and Richter, 1997; Clemens and Bommer, 1999; Saffman and Lasko, 1999). Translational regulation can be achieved via *cis*-acting elements in the 5′ and 3′ untranslated regions (UTRs) of mRNAs, *trans*-acting factors that interact with these UTRs, and the binding of core translational components to mRNAs (Jackson and Wickens, 1997).

Recruitment of mRNAs to ribosomes to initiate translation is mediated by initiation factors of the eIF4 group and the poly(A)-binding protein (PABP). The eIF4 group includes eIF4A, an RNA helicase; eIF4B, an RNA-binding protein that stimulates eIF4A; eIF4E, a cap-binding protein; and eIF4G, the central organizing protein that colocalizes eIF4E, eIF4A, eIF3, PABP, the eIF4E kinase Mnk and RNA in the 48S initiation complex.

Intracellular levels of eIF4E strongly affect the rate of translation, specifically of mRNAs that are more strongly dependent on the cap (Altmann et al., 1989; De Benedetti et al., 1991), and thus eIF4E is an attractive target for regulation of translation. In fact, the level, availability, and activity of eIF4E are regulated by several processes. First, the level of eIF4E is transcriptionally regulated; e.g. transcription of the eIF4E gene is increased in fibroblasts in response to growth factor treatment (Rosenwald et al., 1993; Jones et al., 1996). Second, the availability of eIF4E is regulated by its association with eIF4E-binding proteins (4E-BPs), which, when bound to eIF4E, prevent its binding to eIF4G, thereby inhibiting translation initiation (Sonenberg, 1996). Third, the activity of eIF4E is regulated by phosphorylation. Phosphorylation of eIF4E by Mnk in response to extracellular stimuli, such as hormones, growth factors and mitogens, is generally correlated with an increase in translation rate (Rhoads, 1993; Gingras et al., 1999).

The importance of regulating the level of eIF4E is underscored by the findings that moderate overexpression of eIF4E can cause deregulated cell proliferation and malignant

transformation (Lazaris-Karatzas et al., 1990; De Benedetti and Rhoads, 1990; Lazaris-Karatazas and Sonenberg, 1992). Conversely, depletion of eIF4E by antisense RNA slows growth rate (De Benedetti et al., 1991) and partially reverses oncogenic transformation in cancer cells (Rinker-Schaeffer et al., 1993). Furthermore, a direct correlation between the amount of eIF4E and malignant transformation has been reported in many cell lines and tumors (De Benedetti and Harris, 1999). The recurrence of head and neck carcinomas after surgery is strongly correlated with eIF4E levels in the tumor margins (Nathan et al., 1997). Normal development also depends on proper levels or availability of eIF4E. In immature oocytes of *Xenopus*, the availability of eIF4E is regulated by its association with the CPEB-Maskin complex (Stebbins-Boaz et al., 1999). Maskin contains an eIF4E-binding motif similar to that found in eIF4G and 4E-BPs. The binding of eIF4E by the CPEB-Maskin complex precludes its interaction with eIF4G. During progesterone-induced oocyte maturation the Maskin-eIF4E interaction is weakened, allowing eIF4E and PABP to form a complex with eIF4G; complex formation is required for the recruitment of polyadenylated maternal mRNAs to ribosomes (Stebbins-Boaz et al., 1999; Keiper and Rhoads, 1999).

In *Caenorhabditis elegans*, there are five isoforms of eIF4E, called IFE-1, IFE-2, IFE-3, IFE-4 and IFE-5 (Jankowska-Anyszka et al., 1998; Keiper et al., 2000). Based on their primary sequences, cap-binding specificity and requirement for viability, IFE proteins have been grouped into three classes (Keiper et al., 2000). Class A contains IFE-3, which is most similar to human eIF4E-1 and is essential for viability. Class B includes IFE-1, IFE-2 and IFE-5. Depletion of any individual class B member by RNA-mediated interference (RNAi) did not affect viability. However, blocking the expression of all three proteins caused 99% embryonic lethality, suggesting that they function redundantly in embryonic development. Class C contains IFE-4, which is the most divergent isoform of eIF4E in *C. elegans* and is completely dispensable. Class A and C members bind m^7GTP-containing caps exclusively, while class B members bind both m^7GTP- and $m_3^{2,2,7}$GTP-containing caps.

A potential role for IFE-1 in germline development was brought to our attention by its interaction with the germ-granule component, PGL-1, in a yeast two-hybrid screen. Germ granules (also called P granules in *C. elegans* and polar granules in *Drosophila*) are non-membrane-bound organelles that contain RNAs and proteins and are candidate 'determinants' of the germline. Several proteins have been found to be associated with P granules either constitutively or transiently. These include PGL-1 (Kawasaki et al., 1998), GLH-1, GLH-2 (Gruidl et al., 1996), GLH-3, GLH-4 (Kuznicki et al., 2000), GLD-1 (Jones and Schedl, 1995; Jan et al., 1999), PIE-1 (Mello et al., 1996; Tenenhaus et al., 1998), MEX-1 (Guedes and Priess, 1997), MEX-3 (Draper et al., 1996) and POS-1 (Tabara et al., 1999b). Interestingly, all of these proteins have one or more RNA-binding motif(s) and therefore might have RNA-related functions. The presence of these proteins as well as of RNAs (Subramaniam and Seydoux, 1999; Schisa et al., 2001) in these granules suggests that P granules are involved in some aspect of RNA metabolism or translation.

In this study, we show that *ife-1* is expressed primarily in the germline, that IFE-1 protein associates with P granules, and that this association is dependent on PGL-1 protein. IFE-1 appears to be distinct from the other IFEs in being required specifically for the normal execution of spermatogenesis. This requirement is especially pronounced at elevated temperature. Depletion of IFE-1 causes a delay in spermatogenesis and production of defective sperm.

MATERIALS AND METHODS

Nematode stains and maintenance

General methods for maintaining *C. elegans* are described in Brenner (Brenner, 1974). Strains used were wild-type variety Bristol, strain N2; LGI, *glp-4(bn2)*; LGIII, *unc-32(e189), fem-2(b245)*; LGIV, *pgl-1(ct131, bn102), him-3(e1147), fem-3(q20gf)*; LGV, *rde-1(ne219)*. Strains were maintained at 16°C, except for the temperature-sensitive mutants *glp-4*, *fem-3* and *pgl-1*, which were maintained at 16°C and analyzed at 25°C (for *glp-4* and *fem-3*) and 26°C (for *pgl-1*).

Isolation and sequencing of *ife-1* cDNA

ife-1 cDNA was isolated in a yeast two-hybrid screen using *pgl-1* cDNA as 'bait' and oligo(dT)-primed *C. elegans* cDNAs (Barstead and Waterston, 1989) as 'prey' (I. K., A. A., Y. F., T. Karashima, Y. K. and S. S., unpublished). *ife-1* cDNA was subcloned from the yeast vector into the *Xho*I site in Bluescript KS^+ vector to form pBS-*ife-1*. The cDNA sequence was verified using an ABI PRISM DNA sequencing kit and ABI PRISM 310 Genetic Analyzer (PE Applied Biosynthesis). pBS-*ife-1* contains the full-length *ife-1* ORF.

Northern and in situ hybridization analyses

Northern hybridization analysis was performed as in Holdeman et al. (Holdeman et al., 1998). An *ife-1*-specific RNA probe was made using Strip-EZ™ T7/T3 Kit (Ambion). The *ife-1* probe (160 bp) corresponds to nucleotides 620-780 of *ife-1* cDNA. The *rpp-1* transcript, which encodes a ribosomal protein (Evans et al., 1997), was used as a loading control. In situ hybridization was carried out as in Tabara et al. (Tabara et al., 1996) using the cDNA clone yk504h9 as a probe.

GST-PGL-1 construction, expression and pull-down experiments

Full-length *pgl-1* cDNA (2190 bp) was amplified by PCR from pBS-*pgl-1* (Kawasaki et al., 1998), using primers 5′-CTCGAGATGGAG-GCTAACAAGCGAGAA-3′ and 5′-GCGGCCGCTTAGAAACCT-CCGCGTCCAC-3′. The PCR product was digested with *Xho*I and *Not*I and ligated to a glutathione-S-transferase (GST) gene fusion vector, pGEX-5X-3 (Amersham Pharmacia Biotech), pre-linearized by digestion with *Sal*I and *Not*I, to form pGEX-*pgl-1*.

Escherichia coli strain BL21 (DE3) harboring pGEX-*pgl-1* plasmid or a derivative of pGEX-2T (Pharmacia) were grown at 37°C in Luria-Bertani medium plus ampicillin. Expression of GST-PGL-1 or GST were induced by growth in 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours. Cells were lysed by sonication in lysis buffer (20 mM Hepes, pH 7.4, 0.5 M NaCl, 0.5 mM dithiothreitol (DTT), and 1 Complete™ Protease Inhibitor tablet per 10 ml (Boehringer-Mannheim)). Lysates were incubated at 4°C in the presence of 1% Triton X-100 for 30 minutes and centrifuged at 12,000 ***g***. Supernatants were filtered through 0.45 μm filters. GST-PGL-1 and GST were bound to Glutathione-Sepharose (Pharmacia) and washed repeatedly with lysis buffer. Protein purity and concentration were assessed by SDS-PAGE and staining with Coomassie Blue. Before use in GST pull-down assays, the protein concentrations were equalized by dilution with unbound beads. Protein-bound beads were washed three times in binding buffer (100

mM NaCl, 5 mM DTT, 20 mM Hepes, pH 7.4 and 0.5% non-fat dry milk) and used directly in pull-down assays (Kraemer et al., 1999).

Radiolabeled IFE proteins were synthesized by in vitro transcription-translation with the TNT-coupled reticulocyte lysate system (Promega), pBS-*ife-1*, pBS-*ife-2*, pBS-*ife-3*, pBS-*ife-4*, pBS-*ife-5* and [^{35}S]Met. For binding assays, 20 μl of in vitro-translated reaction product were incubated with 20 μl of bead-bound GST-PGL-1 or GST in 1 ml of binding buffer (see above) for 2 hours at 4°C on a rotator. After five washes with 1 ml of binding buffer, bead-bound radioactive proteins were resuspended in SDS sample buffer and separated by SDS-PAGE. Radiolabeled proteins were detected by autoradiography or by PhosphorImager. For RNase A treatment, [^{35}S]IFE-1 was diluted twofold in binding buffer containing RNase A (5 mg/ml) or mock-treated with water for 1 hour at 30°C. RNA was extracted from an aliquot of the RNase A-treated or mock-treated samples, electrophoresed and stained with Ethidium Bromide (Luitjens et al., 2000). Binding assays were performed as described above.

Cap-binding assay of ^{35}S-PGL-1 and ^{35}S-IFE-1

Radiolabeled proteins were synthesized in reticulocyte lysate as described above from the plasmids pBS-*ife-1* and pBS-*pgl-1*. Reticulocyte lysate (20 μl) was diluted into 0.3 ml buffer B (20 mM Hepes, pH 7.6, 50 mM KCl, 1 mM EDTA, 0.5 mM DTT, 5% (v/v) glycerol, 1 mM PMSF and 10 μg/ml leupeptin) and passed three times over a 0.2-ml column of m^{7}GTP-Sepharose. After a 0.5-ml wash, cap-bound proteins were eluted with 0.3 ml of 200 μM m^{7}GTP. Lysate, flow-through, and eluted protein were resolved by SDS-PAGE and radiolabeled proteins detected by autoradiography or by PhosphorImager.

Preparation of *C. elegans* cap-binding proteins

C. elegans wild-type N2 and *glp-4* mutant worms were cultured at 25°C on agar medium supplemented with chicken egg yolk and *E. coli* strain OP50 (Brenner, 1974; Sulston and Hodgkin, 1988; McDermott et al., 1996). Animals were harvested, cleaned by sucrose flotation (Sulston and Hodgkin, 1988), pelleted, resuspended in an equal volume of water and drop-frozen in liquid N_2. Frozen worms were crushed with a mortar and pestle under liquid N_2 and thawed in the presence of buffer components to yield the following final concentrations: 20 mM MOPS, pH 7.5, 1 mM EDTA, 2 mM EGTA, 100 mM KCl, 0.5 mM DTT, 80 μg/ml each of leupeptin and pepstatin, 10 μg/ml E-64 (Boehringer Mannheim), 1 mg/ml TAME (Sigma Chemical), 50 mM NaF and 10 mM β-glycerophosphate. Homogenates were centrifuged at 20,000 ***g*** for 15 minutes at 4°C, and the supernatants were applied immediately to affinity chromatography columns as described previously (Jankowska-Anyszka et al., 1998; Keiper et al., 2000). Briefly, cleared *C. elegans* extracts (1-3 ml) were applied to 0.2-ml columns of m^{7}GTP-Sepharose equilibrated in buffer A (20 mM MOPS, pH 7.5, 1 mM EDTA, 100 mM KCl, 10% (v/v) glycerol and 0.5 mM DTT). To assay the specific retention of PGL-1 on m^{7}GTP-Sepharose, GTP or m^{7}GTP were added to the cleared extract to a final concentration of 100 μM before chromatography to compete guanine nucleotide or cap-binding interactions, respectively. Columns were washed with 10 ml of buffer A. Proteins were eluted with 1 ml of buffer A containing 100 μM m^{7}GTP and quantitated by Bradford assay (Maniatis et al., 1989). Procedures for the preparation of cap-affinity purified protein from *ife-1(RNAi)* worms were identical, except that protein was eluted with 200 μM m^{7}GTP and precipitated with 10% (w/v) trichloroacetic acid in the presence of 120 μg linear acrylamide before electrophoresis.

Immunoblotting

Cleared worm lysates or cap-binding column elution fractions derived from the same amount of total protein were resolved on 6%, 10% or 12% gels by SDS-PAGE. After electrophoresis, proteins were transferred to PVDF membranes and immunostained using affinity-purified monospecific antibodies against each IFE (Jankowska-Anyszka et al., 1998; Keiper et al., 2000) or antiserum to PGL-1 (Kawasaki et al., 1998). Alternatively, gels were stained with silver nitrate to visualize proteins (Maniatis et al., 1989).

Construction of IFE-1::GFP

The histone cDNA fragment was excised from pJH4.52 (*pie-1*::GFP::histone H2B in pBS; Strome et al., 2001) by *Spe*I digestion and replaced with *ife-1* cDNA. *ife-1* cDNA was amplified by PCR from pBS-*ife-1* using primers 5′-TGAACTAGTGAAACGGAGCAA-ACGACG-3′ and 5′-TGAACTAGTGGATTTCTCGGCGACTGG-3′. The PCR product was digested with *Spe*I and subcloned into pJH4.52 (minus the histone insert) to form pSS4.52.1. Sequencing revealed a PCR-introduced amino acid change (A161→T) in IFE-1. Worms were transformed with pSS4.52.1, and GFP-expressing worms were maintained as described in Strome et al. (Strome et al., 2001).

Fluorescence microscopy

Whole worm staining was carried out as described in Kawasaki et al. (Kawasaki et al., 1998). Immunostaining of extruded germlines or embryos was carried out as in Strome and Wood (Strome and Wood, 1983). We used the following primary antibodies: rabbit anti-GFP (Clontech) diluted 1:250; rabbit anti-PGL-1 (Kawasaki et al., 1998) diluted 1:30,000; chicken anti-GLH-2 (Gruidl et al., 1996) diluted 1:100; mouse monoclonal anti-α-tubulin (DM1-α) (Amersham); and mouse monoclonal SP56 (Ward et al., 1986). Secondary antibodies (from Jackson ImmunoResearch) were rhodamine-conjugated goat anti-rabbit IgG, rhodamine-conjugated goat anti-mouse IgG, and FITC-conjugated donkey anti-chicken IgY (IgG), all diluted 1:100. Samples were examined by fluorescence microscopy as described in Kawasaki et al. (Kawasaki et al., 1998).

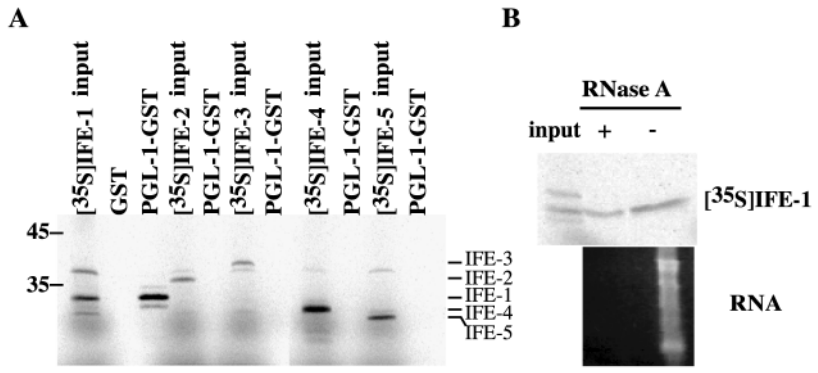

Fig. 1. IFE-1 and PGL-1 proteins interact directly in vitro. [^{35}S]IFE proteins were tested for binding to GST-PGL-1. Bound proteins were analyzed by SDS-PAGE on 12% gels. The input lanes contain 10% of the amount of radiolabeled protein used in the binding assays. The identity of the upper band in the IFE-1 input lane, which is also present in other IFE input lanes, is not known. (A) [^{35}S]IFE proteins were tested for binding to GST-PGL-1 or GST as indicated. (B) [^{35}S]IFE-1 was treated with RNase A or water, before incubating with GST-PGL-1. The slight variation in intensity of the [^{35}S]IFE-1 band in the '+' and '-' lanes was not observed in a duplicate experiment. The Ethidium Bromide-stained agarose gel of RNA extracted after RNase A treatment or mock treatment shows that endogenous RNAs were reduced to below detection by RNase A treatment.

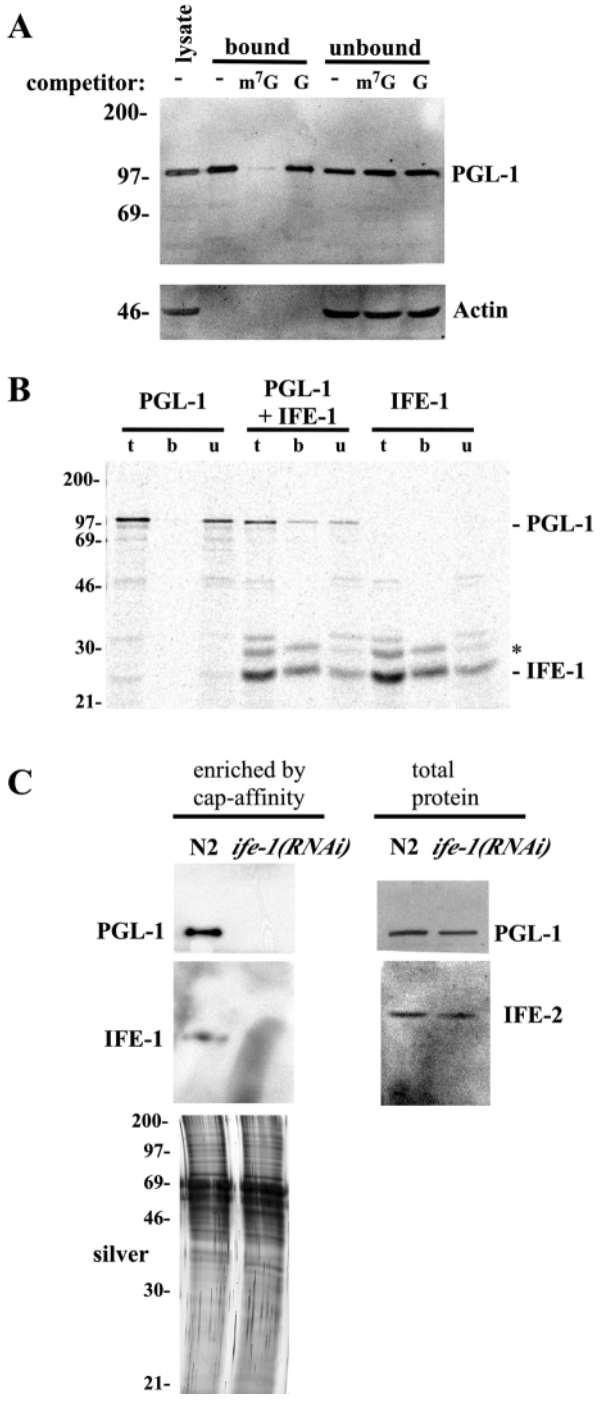

Fig. 2. PGL-1 is retained by m^{7}GTP-Sepharose through interaction with IFE-1. (A) Retention of native PGL-1 on m^{7}GTP-Sepharose. Cleared lysates were prepared from wild-type (N2) worms and incubated with m^{7}GTP-Sepharose. Bound proteins were eluted with m^{7}GTP as described in Materials and Methods. To verify the specificity of cap interaction, either 100 μM GTP (G), 100 μM m^{7}GTP (m^{7}G) or buffer A (-) were added to lysates as competitors before chromatography. Eluted fractions (bound; ~0.3 μg protein), column flow-through (unbound, 10% volume; ~24 μg protein) and original lysate (lysate, 10% volume; ~30 μg protein) were resolved by SDS-PAGE on a 6% gel and immunoblotted using anti-PGL-1 antiserum or anti-chicken actin antiserum (Sigma). (B) Retention of [^{35}S]PGL-1 on m^{7}GTP-Sepharose via [^{35}S]IFE-1. [^{35}S]PGL-1 synthesized in reticulocyte lysate (total, t) is not retained on m^{7}GTP-Sepharose (bound, b) unless IFE-1 is also synthesized in the lysate. Instead, PGL-1 alone is found entirely in the column flow-through (unbound, u). Equivalent volumes of total, bound and unbound fractions were resolved by SDS-PAGE on a 10% gel and ^{35}S-labeled proteins visualized by a phosphorimager. The asterisk indicates the migration of a protein of unknown identify (as seen in Fig. 1). (C) Depletion of IFE-1 in *ife-1(RNAi)* worms (generated by feeding; see Materials and Methods). m^{7}GTP-binding proteins were enriched from wild-type (N2) or *ife-1(RNAi)* worm extracts, and analyzed by western blot (1.4 μg protein per lane) in order to detect endogenous IFE-1 and cap-retained PGL-1. Silver staining of a similar gel verified equal loading of m^{7}GTP-binding proteins. Western blotting of total protein (30 μg per lane) from worm extracts verified the presence of PGL-1 and another eIF4E isoform, IFE-2, in *ife-1(RNAi)* worms.

RNA-mediated interference (RNAi)

To make double-stranded RNA (dsRNA), sense and antisense transcripts were generated from pBS-*ife-1* using the MEGAscript kit (Ambion). The two strands were denatured at 85°C for 5 minutes and annealed by cooling slowly to room temperature (Subramaniam and Seydoux, 1999). dsRNA (200 ng/μl) was injected into young adult hermaphrodites. Injected animals were allowed to purge their embryos for 8-16 hours at 16°C. Injected animals were then picked to individual plates every 24 hours at either 16°C, 20°C or 25°C. F1 progeny of the injected mothers were picked to individual plates and examined for sterility/fertility by counting their progeny. As a control, the F1 progeny of animals injected with water were analyzed. For mating experiments, injected mothers were mated with N2 males 8-12 hours after injection. The F1 males were then crossed with *unc-32* or *fem-2* mutant hermaphrodites.

To address whether IFE-1 functions during larval development, we used an *unc-32; rde-1* strain (Tabara et al., 1999a). Injected *unc-32; rde-1* mothers were mated to N2 males 12 hours after injection and transferred to fresh plates every 24 hours. *ife-1(RNAi); rde-1*/+ F1 progeny were analyzed for sterility/fertility as above.

To prepare *ife-1(RNAi)* worms for western analysis, we used the feeding method of delivering dsRNA to worms (Kamath et al., 2000). Briefly, full-length *ife-1* cDNA was subcloned into the *Xho*I site of the feeding vector L4440 (Timmons and Fire, 1998) to form L4440-*ife-1*. *E. coli* strain H115 (DE3) harboring L4440-*ife-1* plasmid or L4440 vector alone were grown at 37°C in LB medium plus ampicillin. Bacteria were grown on NGM plates containing 80 μg/ml IPTG and ampicillin at 25°C. Worms were transferred to fresh plates every day for 2 days, and the progeny from the second and third plates were analyzed for sterility/fertility as above.

RESULTS

IFE-1 and PGL-1 interact directly

The interaction of IFE-1 with a P-granule-specific protein, PGL-1, was originally observed in a yeast two-hybrid screen using full-length PGL-1 fused to the GAL4 DNA-binding domain as ‘bait’ and oligo-(dT)-primed *C. elegans* cDNAs fused to the GAL4 activation domain as ‘prey’ (I. K., A. A., Y. F., T. K., Y. K. and S. S., unpublished). To verify that this interaction was authentic, we performed in vitro binding assays. Full-length PGL-1 fused to GST was expressed and purified from *E. coli*, immobilized on glutathione-agarose beads, and tested for binding to radiolabeled full-length IFE-1 protein produced by in vitro translation in a rabbit reticulocyte lysate. ^{35}S-labeled IFE-1 was incubated with equal amounts of GST-PGL-1 or GST attached to beads. After extensive washes,

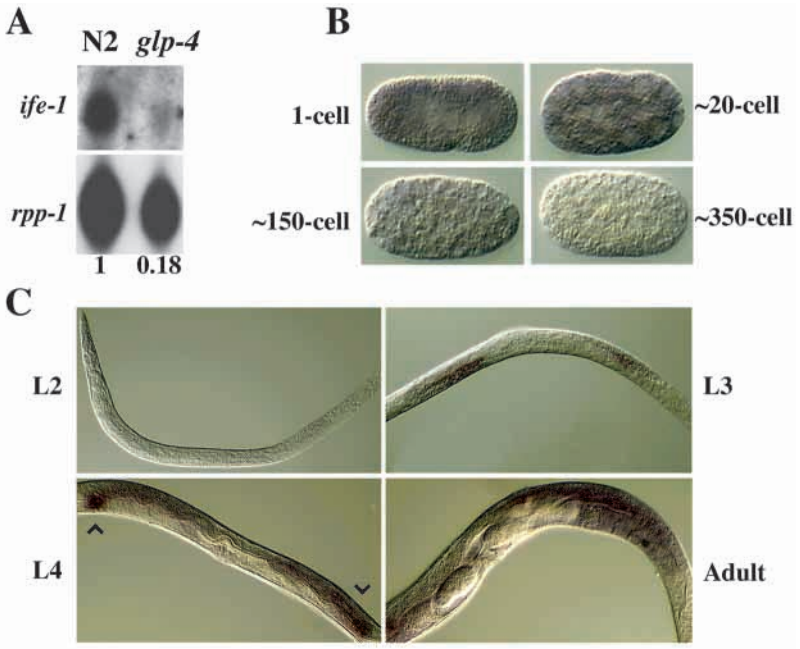

Fig. 3. *ife-1* mRNA is enriched in the germline. (A) Northern analysis of *ife-1* mRNA. Poly(A)$^+$ RNA was prepared from synchronous populations of wild-type (N2) and *glp-4(bn2ts)* hermaphrodites grown at 25°C, at which temperature *glp-4* mutants produce a severely underproliferated germline. The *rpp-1* mRNA, which encodes a ribosomal protein, was used as a loading control. Relative levels of *ife-1* mRNA are shown at the bottom. (B) Distribution of *ife-1* mRNA in embryos, as revealed by whole-mount in situ hybridization. The cDNA clone yk504h9 was used as a probe. *ife-1* mRNA is present in all cells until the 100-200-cell stage and gradually disappears by the 300-400-cell stage. (C) Distribution of *ife-1* mRNA in larvae and adult hermaphrodites. *ife-1* mRNA first becomes detectable in the germline of L3 stage larvae and is maintained in the germline throughout adulthood. The signal is more intense in the regions of the gonad undergoing spermatogenesis (carets).

binding was assessed by SDS-PAGE analysis of the bead samples. Radiolabeled IFE-1 (26 kDa) bound to GST-PGL-1 but not to GST alone (Fig. 1A), suggesting that IFE-1 and PGL-1 are able to bind to each other in vitro in the absence of other *C. elegans* proteins.

Because IFE-1 is a cap-binding protein and PGL-1 has an RNA-binding motif (RGG box; Kawasaki et al., 1998), there was a possibility that PGL-1 and IFE-1 interact indirectly via an RNA 'bridge' in both yeast two-hybrid and GST pull-down assays. To test this, reticulocyte lysate containing radiolabeled IFE-1 was treated with RNase A before incubation with GST-PGL-1. RNA extraction from the RNase A-treated lysate showed that all detectable RNAs were eliminated (Fig. 1B). RNase A treatment did not inhibit the binding of [^{35}S]IFE-1 and GST-PGL-1 (Fig. 1B), suggesting that the interaction between IFE-1 and PGL-1 is not dependent on RNA and therefore is direct.

We used GST pull-down assays to test whether PGL-1 also binds to the other four IFEs. Radiolabeled IFE-2, IFE-3, IFE-4 and IFE-5 were synthesized in vitro and incubated with GST or GST-PGL-1. None of the other IFEs bound to GST (not shown) or GST-PGL-1 (Fig. 1A), suggesting that PGL-1 interacts with a motif or region unique to IFE-1.

To investigate whether native IFE-1 and PGL-1 proteins interact in vivo, we determined whether PGL-1 is present in cap-binding fractions from adult hermaphrodites. Immunoblotting revealed that roughly 15% of PGL-1 was specifically retained on m^{7}GTP-Sepharose and eluted with m^{7}GTP (Fig. 2A). Since relatively few proteins remain bound to the affinity resin (<0.1% of soluble protein; see also Fig. 4A), PGL-1 was actually enriched approximately 180-fold in the bound fraction. Preincubation of worm extracts with 100 μM m^{7}GTP (m^{7}G), but not 100 μM GTP (G), greatly reduced the retention of PGL-1. As a control, we tested whether PGL-

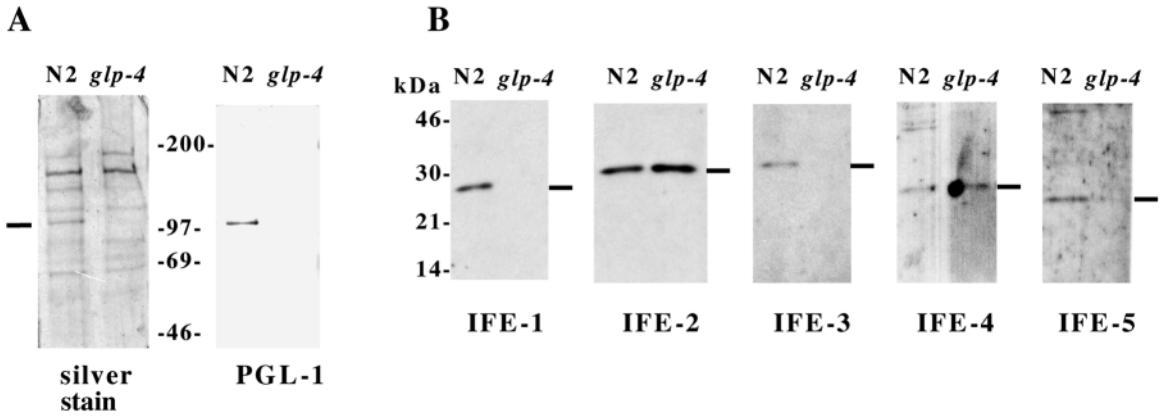

Fig. 4. IFE-1, IFE-3 and IFE-5 are enriched in the germline. m^{7}GTP affinity-purified proteins derived from 1.5 mg of total protein from wild-type (N2) or germline-deficient (*glp-4*) worms were resolved by SDS-PAGE on 6% (A) and 12% (B) gels. (A) The gel on the left was stained with silver nitrate, the gel on the right was immunoblotted using anti-PGL-1 antiserum. (B) The gels were immunoblotted using antibodies to isoform-specific C-terminal peptides for each of the IFE proteins. Both wild-type and *glp-4* worms were grown at 25°C, the restrictive temperature for germline development in the *glp-4* strain.

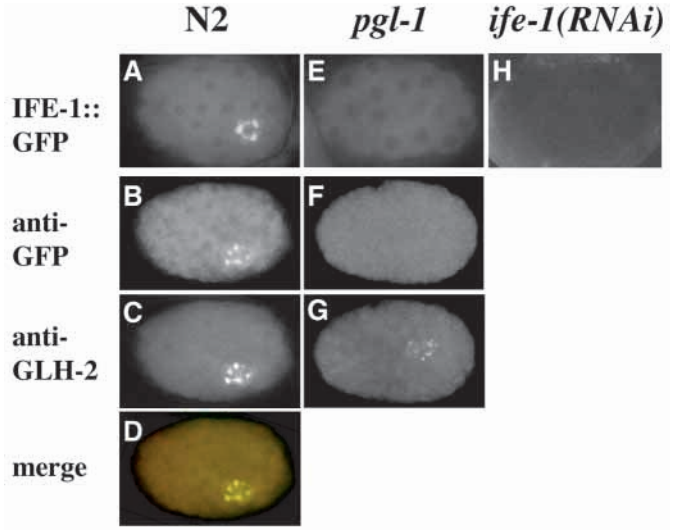

Fig. 5. IFE-1::GFP is associated with P granules in wild-type (N2) but not in *pgl-1* embryos. The genotypes of embryos are listed above the columns. Embryos measure ~30×50 μm. (A,E) Fluorescence micrographs of 16-20-cell stage embryos. IFE-1::GFP is localized in perinuclear granules in the germline cell (P_4) in N2 but not in *pgl-1*. (H) Fluorescence micrograph of a four-cell stage embryo. IFE-1::GFP signal is not detected in the cytoplasm or P granules of RNAi embryos. (B-D,F,G) Immunofluorescence micrographs of 70-90-cell stage embryos co-stained with anti-GFP (B,F) and anti-GLH-2 (C,G) antibodies. Anti-GFP staining is detectable in perinuclear granules in the germ cells (Z_2 and Z_3) of N2 but not in *pgl-1* germ cells. (D) The merged image of B,C demonstrates double staining of P granules by anti-GFP and anti-GLH-2 antibodies in N2.

1 has the ability to bind to the cap column by itself. In vitro synthesized [^{35}S]PGL-1 was not retained on m^{7}GTP-Sepharose, unless ^{35}S-IFE-1 was also present (Fig. 2B), suggesting that PGL-1 does not bind directly to m^{7}GTP. Furthermore, PGL-1 was absent in the cap-bound fraction from *ife-1(RNAi)* worm lysates (Fig. 2C). Thus, our cap-binding results suggest that PGL-1 in worm lysates is retained on m^{7}GTP-Sepharose via its association with an authentic cap-binding protein, IFE-1.

ife-1 mRNA is enriched in the germline

A role for IFE-1 in germline development was suggested by its interaction with the P-granule component PGL-1. To determine whether *ife-1* is expressed primarily in the germline, an RNA probe to the unique 3′ end of the *ife-1* cDNA was used to probe northern blots of RNA from adult wild-type and *glp-4(bn2)* mutant worms. *glp-4* is a conditional mutant that makes very few germ cells at the restrictive temperature (Beanan and Strome, 1992). *ife-1* RNA was 5.5-fold more abundant in wild-type than in *glp-4* mutant worms, suggesting that *ife-1* transcript is enriched in the germline (Fig. 3A).

To analyze the temporal and spatial distribution of *ife-1* mRNA, we performed in situ hybridization on wild-type worms at different developmental stages. In hermaphrodites, newly synthesized *ife-1* mRNA was first detected in the germline of L3 stage larvae (Fig. 3C). At this stage germ cells in the distal region of the gonad are mitotically proliferating and those in the proximal region are entering meiosis. As

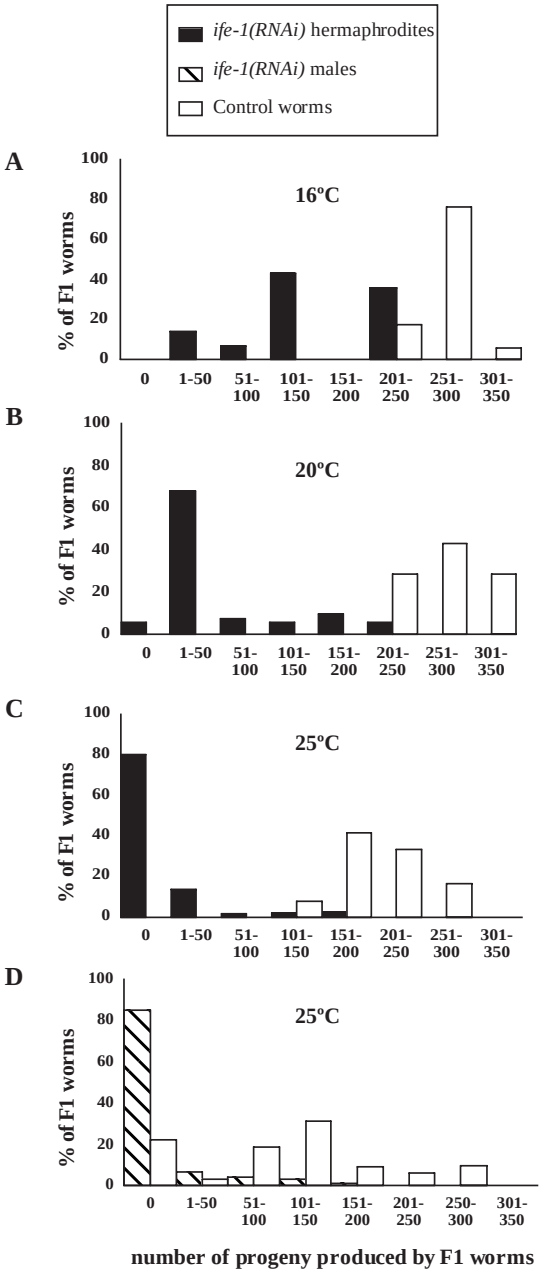

Fig. 6. *ife-1(RNAi)* worms display temperature-sensitive sterility and reduced brood size. The F1 hermaphrodite progeny of mothers injected with *ife-1* dsRNA or with water (control) were analyzed for sterility and brood size at (A) 16°C, (B) 20°C and (C) 25°C. The numbers of *ife-1(RNAi)* F1 worms analyzed were 14, 53 and 225 at 16°C, 20°C and 25°C, respectively. The numbers of control F1 progeny analyzed were 13, 13 and 14 at 16°C, 20°C and 25°C, respectively. At 25°C, 80% of RNAi worms did not produce any progeny. The remaining 20% of RNAi worms at 25°C and all RNAi worms at 16°C and 20°C produced a smaller number of progeny than control worms. (D) F1 males from matings between injected mothers and N2 males were grown at 25°C and tested for the ability to produce outcross progeny when mated with *unc-32* or *fem-2* animals. The number of *ife-1(RNAi)* and control males examined was 135 and 31, respectively. Of RNAi males, 85% did not produce any cross-progeny after mating. The remaining 15% of RNAi males produced a smaller number of cross-progeny than control males.

germline development progressed, the hybridization signal intensified in the regions of the gonad undergoing spermatogenesis (Fig. 3C, L4). The signal persisted after spermatogenesis was complete; a reduced level of *ife-1* mRNA was detectable in adult germlines undergoing oogenesis.

In embryos, the distribution of *ife-1* mRNA was similar to a pattern described previously as class I maternal mRNAs (Seydoux and Fire, 1994). These mRNAs show a uniform distribution in all cells during early cleavage stages and disappear at later stages. *ife-1* mRNA was detected in all cells in embryos until the 100-200-cell stage and gradually disappeared by the 300-400-cell stage (Fig. 3B). These in situ hybridization results suggest that *ife-1* mRNA in embryos is due to maternal transcription and that new *ife-1* expression does not start until the L3 stage.

IFE-1, IFE-3 and IFE-5 proteins are enriched in the germline

Northern and in situ hybridization results predicted that IFE-1 protein should be enriched in the germline. To assess the abundance of IFE-1 and other IFEs in the germline, we carried out a combination of affinity chromatography and western analysis. Proteins that bind to mRNA caps were enriched from extracts of wild-type and *glp-4* mutant worms by m^7GTP-Sepharose affinity chromatography. Interestingly, PGL-1 appears to be among the most abundant high molecular weight components among the cap-associated proteins (Fig. 4A, silver stained gel), supporting the notion that PGL-1 becomes enriched by association with IFE-1. The bound fractions were subjected to immunoblotting with anti-IFE antibodies (Fig. 4B). IFE-1, IFE-3 and IFE-5 signals were detectable in eluates from wild type but not in eluates from *glp-4* mutants, which are deficient in germline tissue, suggesting that these proteins are predominantly present in the germline. IFE-2 and IFE-4, on the other hand, are at least as abundant in *glp-4* worms as in wild type, suggesting that they are predominantly present in somatic cells.

IFE-1 associates with P granules in vivo and requires PGL-1 for this association

The isoform-specific anti-peptide antibodies to IFE-1, which specifically recognize IFE-1 in western blot analyses (Jankowska-Anyszka et al., 1998; Keiper et al., 2000; Fig. 4), did not yield a specific signal in immunostaining. Therefore, to examine the subcellular localization of IFE-1 in the germline, a construct expressing IFE-1 fused to green fluorescent protein (GFP) was expressed in worms. The construct uses the *pie-1* promoter to drive expression of IFE-1::GFP in the germline and in early embryos (Strome et al., 2001). IFE-1::GFP was distributed throughout the cytoplasm and also localized to distinct particles in the germline and in embryos (n>1000 embryos examined; Fig. 5A). The size and distribution of these particles were very similar to those of P granules (Strome and Wood, 1982). To verify this assignment, worms were stained simultaneously with an anti-GFP antibody (Fig. 5B) and an antibody to a known P-granule protein, GLH-2 (Gruidl et al., 1996; Fig. 5C). The particles stained with anti-GFP coincided with those stained by anti-GLH-2 (Fig. 5D), indicating that at least some IFE-1::GFP is associated with P granules.

The results shown in Figs 1, 2 suggested that IFE-1 may associate with P granules via a direct interaction with PGL-1. To test this possibility, we introduced the IFE-1::GFP-expressing construct into *pgl-1(ct131)* null mutants. In 99% of the *pgl-1* embryos examined (n>160), IFE-1::GFP signal was not detectably associated with P granules but was still present in the cytoplasm (compare Fig. 5E,5H). Co-staining embryos with anti-GFP and anti-GLH-2 antibodies confirmed that *pgl-1* mutants contain P granules but that those P granules lack detectable IFE-1::GFP (Fig. 5F,G). These results suggest that PGL-1 is required for IFE-1 localization to P granules in wild-type animals.

Two lines of evidence support the assumption that the localization of GFP-tagged IFE-1 reflects the pattern of native IFE-1. First, the same *pie-1*-based vector has been used to express several other GFP-tagged proteins (γ-tubulin, β-tubulin and histone); these other proteins showed the expected localization (to centrosomes, microtubules and chromosomes, respectively) (Strome et al., 2001) and did not associate with P granules. Thus, the association of IFE-1::GFP with P granules is not due to the GFP part of the fusion protein but appears to be specific to the IFE-1 portion. Second, the association of IFE-1::GFP with P granules is dependent upon PGL-1, which was shown to bind IFE-1 in independent assays (Figs 1, 2).

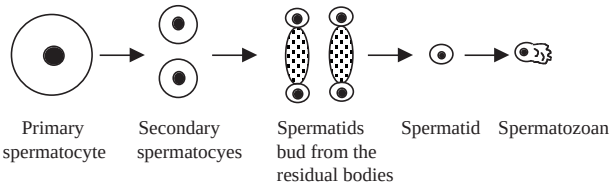

Fig. 7. Summary of the steps of spermatogenesis in wild-type *C. elegans*. Primary spermatocytes undergo two meiotic divisions to produce secondary spermatocytes and spermatids. Spermatids bud from residual bodies and undergo spermiogenesis to form spermatozoa.

Depletion of IFE-1 causes temperature-sensitive sterility and reduction in brood size

The role of IFE-1 in germline development was examined by RNA-mediated interference (RNAi). Injection of double-stranded RNA (dsRNA) into hermaphrodites has been shown to result in a gene-specific, loss-of-function phenotype in the injected mothers and their progeny (Rocheleau et al., 1997; Fire et al., 1998; Montgomery et al., 1998). RNAi can also be achieved by feeding worms *E. coli* that produce dsRNA (Timmons and Fire, 1998; Kamath et al., 2000). Disruption of *ife-1* expression has been shown previously to have no lethal effects on injected worms or their F1 progeny (Keiper et al., 2000). To verify that RNAi effectively depleted IFE-1 protein, we examined GFP fluorescence in *ife-1::gfp* worms injected with dsRNA prepared from full-length *ife-1* cDNA. IFE-1::GFP signal was no longer detected in the embryos of injected hermaphrodites (Fig. 5H). Furthermore, IFE-1 protein was not detectable in lysates prepared from worms fed *E. coli* that produce *ife-1* dsRNA but was present in control lysates prepared from worms fed *E. coli* harboring only the feeding vector (Fig. 2C). Wild-type hermaphrodites were then injected with *ife-1* dsRNA, allowed to develop and reproduce at 16°C, 20°C and 25°C, and the F1 worms were examined. *ife-1(RNAi)*

F1 hermaphrodites displayed temperature-sensitive sterility (Fig. 6A-C; Table 1). At 25°C, 80% of the F1 progeny of injected hermaphrodites were sterile (i.e. produced no progeny). A similar level of sterility was induced by the feeding method of RNAi (data not shown). The remaining fertile animals showed greatly reduced brood sizes (an average of 36 progeny from fertile *ife-1(RNAi)* worms compared with 221 progeny from control worms) (Fig. 6C). Unlike *pgl-1* mutants, which show 75-85% sterility at 25°C and 100% sterility at 26°C (Kawasaki et al., 1998), *ife-1(RNAi)* worms did not display higher sterility at 26°C (data not shown). At lower temperatures (16° and 20°C), most *ife-1(RNAi)* F1 hermaphrodites were fertile, but their brood sizes were considerably lower than wild type. Control hermaphrodites produced an average of 266 and 280 progeny at 16°C and 20°C, while *ife-1(RNAi)* F1 hermaphrodites produced an average of 139 and 45 at those temperatures, respectively (Fig. 6A,B). Thus, depletion of IFE-1 protein caused temperature-sensitive sterility and reduction of brood size in hermaphrodites.

The requirement for IFE-1 in male germline development was similarly investigated by blocking the expression of *ife-1* in males. *ife-1(RNAi)* males were generated by mating dsRNA-injected hermaphrodites with wild-type males. The ability of the resulting F1 males to produce outcross progeny when mated was then tested at 25°C. To enable scoring of outcross, the mating partners were homozygous *unc-32* or *fem-2*. *unc-32* hermaphrodites produce uncoordinated selfcross progeny, which can be easily distinguished from heterozygous progeny derived from mating. *fem-2* hermaphrodites produce only oocytes and thus no selfcross progeny at 25°C. *ife-1(RNAi*) males displayed higher sterility (85% did not produce outcross progeny, compared with 22% for control males) and when fertile produced fewer outcross progeny than control males (Fig. 6D). These results show that IFE-1 is required for normal germline development in both hermaphrodites and males.

To investigate whether RNAi-induced sterility is due to depletion of the maternal load of IFE-1 or of larvally synthesized IFE-1, we used an *rde-1* mutant (Tabara et al., 1999a). Homozygous *rde-1* hermaphrodites are resistant to RNAi, but heterozygous outcross progeny (i.e. *rde-1/+*) are not resistant to RNAi. *unc-32; rde-1* hermaphrodites were injected with *ife-1* dsRNA and later mated with wild-type males to generate *ife-1(RNAi); unc-32/+; rde-1/+* F1 hermaphrodites. The injected *rde-1/rde-1* mothers were resistant to RNAi and therefore provided a maternal load of IFE-1 to their progeny. The heterozygous *rde-1/+* offspring were no longer resistant to RNAi and therefore susceptible to inhibition of larval *ife-1* expression. We observed that 49% of the *ife-1(RNAi); unc-32/+; rde-1/+* F1 hermaphrodites were sterile at 25°C (n=38). Thus, inhibiting only larval synthesis of IFE-1 led to the same phenotype (sterility) as that caused by inhibiting both maternal and larval synthesis. This is consistent with IFE-1 functioning during larval development. The fact that inhibiting larval *ife-1* expression resulted in fewer sterile F1s (49%) than after inhibiting both maternal and larval expression (80% sterile F1s) suggests that maternally provided IFE-1 is also important for proper germline development in progeny worms.

An essential, germline-specific role is unique to IFE-1

We used RNAi to determine whether *ife-2*, *ife-4*, and *ife-5* also serve an essential role in germline development. *ife-3* was

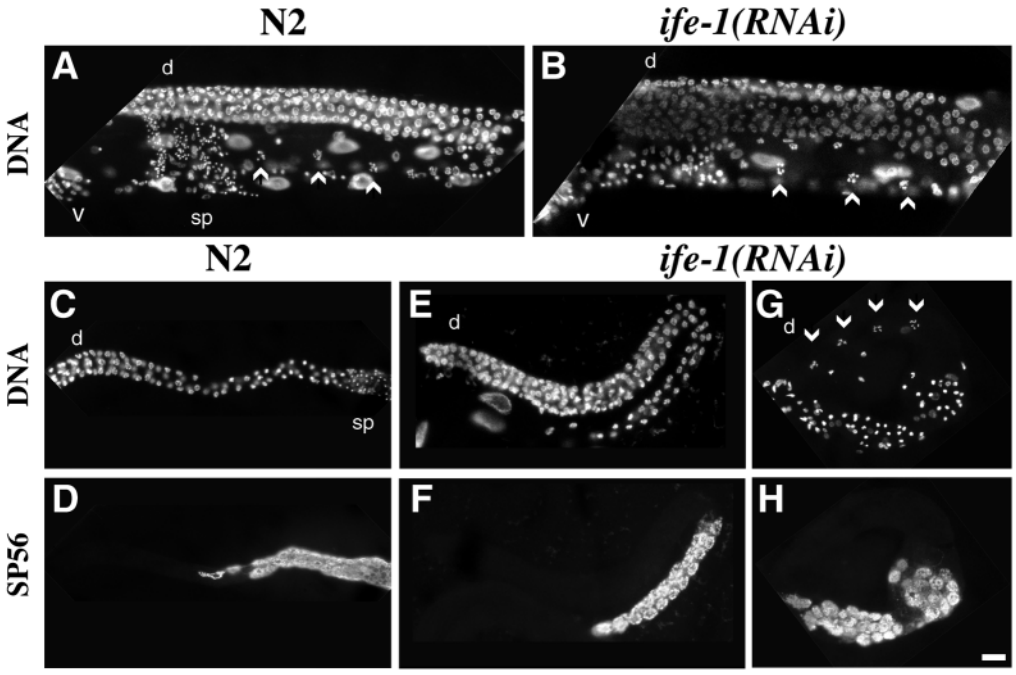

Fig. 8. *ife-1(RNAi)* hermaphrodites display defects in spermatogenesis. The genotypes of gonads are above the panels. d, distal portion of each arm; sp, sperm; carets, oocytes; v, vulva. (A,B) Fluorescence micrographs of intact hermaphrodites stained with Hoechst 33342. Only one gonad arm is shown. (A) Germline nuclei in a wild-type (N2) adult. Mitotic nuclei are in the distal region. As these nuclei progress toward the proximal region, they enter meiosis. Mature sperm (sp) are stored in the spermatheca. Oocyte nuclei, arrested at diakinesis, can be seen to the right of the spermatheca. (B) Germline nuclei in an *ife-1(RNAi)* adult. Mitotic, pachytene, and oocyte nuclei are observed as in wild type. However, no sperm are present in the spermatheca. (C-H) Immunofluorescence micrographs of gonad arms dissected from L4-young adult hermaphrodites. Gonads were fixed and stained with the DNA dye, DAPI (C,E,G) and with the monoclonal antibody SP56, which stains spermatocytes and sperm (D,F,H). (C) Germline nuclei in a wild-type L4. (D) SP56 stains primary spermatocytes, secondary spermatocytes, and sperm. (E) Germline nuclei in an *ife-1(RNAi)* L4. (F) SP56 stains spermatocytes, revealing that this germline entered spermatogenesis. (G) Oocytes and spermatocytes in an *ife-1(RNAi)* young adult that recently switched from spermatogenesis to oogenesis. Oocytes are located distal to the spermatocytes. Note the absence of mature sperm. (H) SP56 stains the spermatocytes. Scale bar: 10 μm.

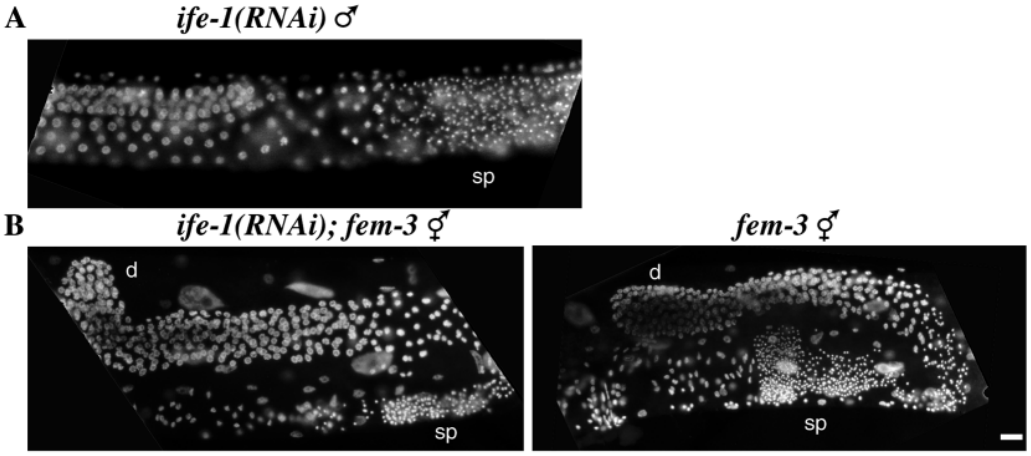

Fig. 9. *ife-1(RNAi)* worms are able to produce sperm if the period of spermatogenesis is not abbreviated by a switch to oogenesis. Fluorescence micrographs of intact adult gonad arms stained with Hoechst 33342. The genotypes of gonads are above the panels. d, distal portion of each arm; sp, sperm. Mature-looking sperm are seen in each gonad arm. Scale bar: 10 μm.

excluded from our analysis, as all hermaphrodites injected with *ife-3* dsRNA produce dead embryos (Keiper et al., 2000). *ife-4(RNAi)* and *ife-5(RNAi)* F1 worms were fertile at both 20°C and 25°C, and *ife-2(RNAi)* F1 worms showed negligible sterility (Table 1). These results suggest that when other IFEs are present, IFE-2, IFE-4 and IFE-5 are not required for germline development.

A previous study of worms depleted for multiple IFEs suggested that *ife-1*, *ife-2* and *ife-5* function redundantly during embryogenesis; one of the three proteins must be present to ensure embryo survival, and *ife-2* appears to be the most important of the three proteins (Keiper et al., 2000). To test whether these genes also function redundantly in germline development, *ife-1(RNAi);ife-2(RNAi)*, *ife-1(RNAi);ife-5(RNAi)* and *ife-1(RNAi);ife-2(RNAi);ife-5(RNAi)* F1 worms were analyzed for enhancement of sterility at 20°C and 25°C. These RNAi worms did not display higher sterility than *ife-1(RNAi)* worms at either temperature (Table 1), suggesting that neither IFE-2 nor IFE-5 functions redundantly with IFE-1 in the germline.

IFE-1 is required for proper progression through spermatogenesis

We observed that although *ife-1(RNAi)* F1 hermaphrodites failed to produce embryos, they laid oocytes. This phenotype could reflect production of defective oocytes, failure to make sperm, production of defective sperm, or some combination of these defects. To distinguish between these possibilities, sterile *ife-1(RNAi)* adult hermaphrodites were mated with wild-type males. When provided with wild-type sperm, *ife-1(RNAi)* animals became fertile (n=6), producing viable embryos that developed to adulthood, suggesting that IFE-1 is required for spermatogenesis but not for oogenesis.

The defect in spermatogenesis was characterized in the germline of *ife-1(RNAi)* F1 hermaphrodites. In wild-type worms, spermatogenesis initiates during the L4 larval stage (Fig. 7). Spermatogonial nuclei proliferate mitotically in the distal region of the gonad, entering meiosis as the cells move toward the loop region. Pachytene-stage primary spermatocytes separate from the central cytoplasmic core, the rachis, and divide to form secondary spermatocytes, and then haploid spermatids, which mature into motile spermatozoa. In males, spermatogenesis continues throughout adulthood. In hermaphrodites, the germline switches from spermatogenesis to oogenesis during young adulthood; oocytes arrested in prophase of meiosis I are fertilized by sperm stored in the spermatheca. The stages of spermatogenesis and oogenesis can be recognized by their characteristic chromatin morphology after staining with a DNA dye (Fig. 8A). Sterile *ife-1(RNAi)* adult hermaphrodites contained oocyte nuclei, but lacked nuclei typical of haploid sperm (n>100 worms examined) (Fig. 8B). The few *ife-1(RNAi)* adult hermaphrodites that were fertile contained some sperm nuclei (not shown). These results suggest that *ife-1(RNAi)* worms produce no or few progeny because they produce no or few normal sperm.

To test whether germ cells in *ife-1(RNAi)* hermaphrodites fail to enter spermatogenesis or else enter but fail to complete spermatogenesis, we stained L4 and young adult

Table 1. Temperature-sensitive sterility of *ife-1(RNAi)* compared with other *ife*s

	20°C		25°C	
Genotype*	% sterile F1 progeny‡	Number of F1 progeny scored	% sterile F1 progeny‡	Number of F1 progeny scored
ife-1(RNAi)	3	88	80	225
ife-2(RNAi)	3	32	2	41
ife-4(RNAi)	0	15	0	21
ife-5(RNAi)	0	29	0	65
ife-1(RNAi);ife-2(RNAi)	3	28	73	52
ife-1(RNAi);ife-5(RNAi)	0	45	54	80
ife-1(RNAi);ife-2(RNAi);ife-5(RNAi)	5	128	45	22

*Injection of *ife-3* dsRNA results in embryonic lethality.

‡The F1 progeny of dsRNA-injected mothers were scored for the production of F2 progeny. Animals that failed to produce progeny were scored as sterile. The reduced sterility observed with combinatorial RNAi might be due to the suppression effect that has been reported for certain dsRNAs in pools (Kuznicki et al., 2000).

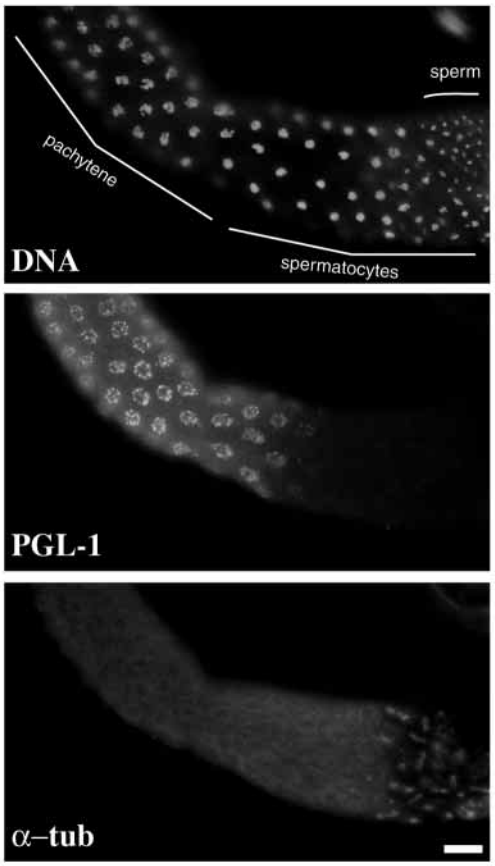

Fig. 10. PGL-1 disappears from P granules during spermatogenesis. Immunofluorescence micrograph of a gonad arm dissected from an adult male. The gonad was fixed and stained with the DNA dye, DAPI, anti-PGL-1 antiserum and anti-α-tubulin. The stages of spermatogenesis are indicated in the top panel. PGL-1 disappears from P granules after the pachytene stage and before the formation of meiotic spindles. Scale bar: 10 μm.

hermaphrodites with the spermatogenesis-specific antibody SP56 and a DNA dye (Fig. 8C-H). In wild type, SP56 binds to the plasma membrane, membranous organelles, and pseudopodial cytoplasm in primary spermatocytes and at later stages of spermatogenesis (Roberts et al., 1986). We observed SP56 staining in all *ife-1(RNAi)* gonad arms examined (n>100) (Fig. 8F,H), demonstrating that germ cells initiated spermatogenesis. The most mature spermatogenic cells observed in sterile RNAi hermaphrodites resembled late primary or secondary spermatocytes, based on the degree of DNA condensation (Fig. 8G) and on the occasional presence of meiotic spindles (not shown). Therefore, spermatogenesis and oogenesis appear to be initiated in proper sequence, but spermatogenesis is not completed in most *ife-1(RNAi)* hermaphrodites.

As described above, IFE-1 depletion also causes males to be sterile. To determine whether *ife-1(RNAi)* males also lack sperm, we stained RNAi males with a DNA dye. All *ife-1(RNAi)* males examined (n>20) contained highly condensed sperm nuclei, suggesting that at least some spermatocytes completed meiosis (Fig. 9A). Therefore, in contrast to *ife-1(RNAi)* hermaphrodites, spermatogenesis generally proceeds beyond the spermatocyte stage in *ife-1(RNAi)* males. Nevertheless, the sperm produced are apparently defective (Fig. 6D).

The presence of mature-looking sperm in *ife-1(RNAi)* males raised the question of whether the requirement for IFE-1 in spermatogenesis is different in males and hermaphrodites, or whether the requirement is similar and the absence of mature sperm in hermaphrodites is due to the onset of oogenesis. In wild-type hermaphrodites, by the time that mature oocytes are produced, most sperm are mature and motile. As oocytes pass through the narrow spermatheca, sperm are often swept into the uterus. However, these sperm are able to move back to the spermatheca (reviewed by Singson, 2001). Immotile spermatocytes, present in *ife-1(RNAi)* hermaphrodites, would be unable to migrate back to the spermatheca and would be lost. To test whether preventing the onset of oogenesis would enable *ife-1(RNAi)* hermaphrodites to produce mature-looking sperm, as in males, we used a *fem-3(gf)* mutant. *fem-3(gf)* hermaphrodites do not undergo the sperm/oocyte switch and as a result produce sperm throughout adulthood (Barton et al., 1987). As expected, after staining with a DNA dye, we observed that L4 and young adult *fem-3* mutants contained hundreds of sperm. As in *ife-1(RNAi)* hermaphrodites, 85% of L4 and young adult *ife-1(RNAi); fem-3* hermaphrodites contained only spermatocytes at 25°C (n=47). However, after allowing *ife-1(RNAi); fem-3* hermaphrodites to age (2 days beyond mid-L4 stage), 88% (n=26) contained mature-looking sperm, although the number of sperm was reduced compared to *fem-3* controls (Fig. 9B). Thus, progression past the spermatocyte stage can occur in RNAi hermaphrodites if oogenesis is prevented and the period of spermatogenesis is extended. This result suggests that depleting IFE-1 causes a delay but not an arrest in spermatogenesis in hermaphrodites.

To test whether males also are delayed in spermatogenesis, we compared similar stages of N2 and *ife-1(RNAi)* males at 25°C. At 32 hours past hatching, 83% of N2 males had sperm (n=6) and 0% of *ife-1(RNAi)* males had sperm (n=5). Six hours later, 71% of *ife-1(RNAi)* males contained a small number of sperm (n=7). Thus, similar to hermaphrodites, *ife-1(RNAi)* males are delayed in production of sperm. Sperm produced in *ife-1(RNAi)* males and in aged *ife-1(RNAi); fem-3* hermaphrodites have a normal appearance by Nomarski and DAPI, and can be activated to form a pseudopod (data not shown). Their failure to generate progeny may be due to defects in motility and/or fertilization.

PGL-1 disappears from P granules during spermatogenesis

P granules are present in the germ cells of both hermaphrodites and males throughout most of their development. We previously observed that P granules, as detected by anti-GLH-1 and anti-GLH-2, are partitioned to the residual body during spermatogenesis and thus are absent in mature sperm (Gruidl et al., 1996; A. A., unpublished). Interestingly, unlike GLH-1 and GLH-2, PGL-1 disappears from P granules after the pachytene stage of spermatogenesis (Fig. 10). Because the association of IFE-1 with P granules depends on PGL-1 (Fig. 5), it is likely that IFE-1 also is released from P granules after the pachytene stage. The stages of spermatogenesis known to be affected by IFE-1 depletion coincide with stages when IFE-1 is predicted to be dissociated from P granules. Based on these results, we speculate that P granules regulate the level and

perhaps the function(s) of IFE-1 during spermatogenesis; association with P granules may reduce the availability of IFE-1 during germline proliferation, and release from P granules may increase the level of IFE-1 to function during post-pachytene stages of spermatogenesis.

DISCUSSION

This study shows that one of the five *C. elegans* isoforms of eIF4E (IFE-1) plays a unique and essential role in germline development. IFE-1 is specifically required during spermatogenesis, and this requirement is more pronounced at elevated temperatures. Interestingly, IFE-1 is a component of P granules. The association of a translation initiation factor with P granules supports the view that these granules are involved in translational regulation.

ife-1 mRNA and protein are enriched in the germline.

Expression of *ife-1* in worms is significantly enriched in the germline, and mRNA accumulates in the region of the gonad undergoing spermatogenesis. Elevated expression of eIF4E in specific tissues and cell types has been noted in other systems. Specifically, eIF4E mRNA levels are elevated in the primordial germ cells of *Drosophila* (Hernandez et al., 1997), germ cells in rat testes (Miyagi et al., 1995) and oocytes in zebrafish (Fahrenkrug et al., 1999), suggesting that germ cells often, perhaps universally, require high levels of this translation initiation factor. eIF4E levels are also elevated in many solid tumors and malignant cell lines, with the highest levels being reported in breast and prostate cancers, and in head and neck squamous cell carcinomas (reviewed by De Benedetti and Harris, 1999). These observations have led to speculations that eIF4E is not simply a general translation factor, but also may act as a tissue-specific translational enhancer.

IFE-1 functions in spermatogenesis in *C. elegans*

Our RNAi studies suggest that IFE-1 is unique among the five IFEs in being essential for proper germline development. Blocking the expression of *ife-1* causes sterility in 80-85% of both hermaphrodites and males grown at high temperature (25°C); the remaining 15-20% of worms produce a greatly reduced number of progeny. Because RNAi causes defects specifically in spermatogenesis, we infer that 80-85% of RNAi animals produce no functional sperm and the remaining worms produce only a small number of functional sperm. Worms depleted of IFE-1 and grown at lower temperature (16-20°C) also produce reduced numbers of progeny. Thus, IFE-1 appears to be required for production of a normal number of functional sperm, especially at elevated temperature.

Hermaphrodites and males display similar RNAi-induced defects in spermatogenesis. In both sexes, the initial defect is a delay in production of mature sperm. In hermaphrodites, this delay generally leads to a spermless phenotype, probably because immature sperm are not motile. In this scenario, the first oocytes produced encounter spermatocytes (instead of sperm); the oocytes are not fertilized and as they are ovulated and laid, they carry the spermatocytes out of the oviduct, spermatheca and uterus. Interestingly, preventing the onset of oogenesis and prolonging the window of spermatogenesis in RNAi hermaphrodites (in *fem-3(gf)* mutants) allows at least some mature sperm to be formed, as seen in males. From testing the fertility of *ife-1(RNAi)* males, it is apparent that the sperm formed in males at 25°C are defective. They may be defective in motility or in their ability to fertilize oocytes. In the rare cases in which a few mature sperm were observed in *ife-1(RNAi)* sterile hermaphrodites, the sperm also were defective, as evidenced by the presence of unfertilized oocytes in the uterus. Thus, in both hermaphrodites and males, IFE-1 appears to be required for normal entry into and/or progression through spermatogenesis and for formation of functional sperm. We do not know if IFE-1 function is required at multiple steps in spermatogenesis, or only at a single early step. If the latter, then defective or slow progression through that step must lead to formation of abnormal sperm.

Worms depleted of IFE-1 do not display obvious defects in other aspects of germline development, such as proliferation, the ability to switch from spermatogenesis to oogenesis, and oogenesis. In fact, RNAi hermaphrodites produce healthy embryos when normal sperm are provided by mating with wild-type males. Thus, it seems that blocking the expression of *ife-1* in *C. elegans* leads to a preferential decrease in translation of a limited set of mRNAs, which are specifically involved in spermatogenesis.

A substantial body of evidence indicates that alteration in the amount of active eIF4E exerts different effects on the translation of different mRNAs (reviewed by Clemens and Bommer, 1999; De Benedetti and Harris, 1999). Overexpression of eIF4E in tissue culture cells leads to oncogenic transformation, probably owing to elevated translation of growth-promoting mRNAs whose translation is normally limited by inhibitory features in their 5′ UTRs. Such features include long, highly structured, G/C-rich sequences and upstream open reading frames (uORFs). Many mRNAs with such inhibitory features encode proteins that operate to promote cell growth and proliferation (Kozak, 1991). It has been suggested that these mRNAs are translated poorly in resting cells, owing to their inability to compete with other mRNAs (without inhibitory structures in their 5′ UTRs) for the cap-dependent unwinding machinery, for which the availability of active eIF4E may be crucial (Rhoads, 1991; Koromilas et al., 1992). Increasing the level or availability of eIF4E (e.g. after growth stimulation) would ameliorate the competition between mRNAs for the translation initiation machinery, leading to deregulated expression of these growth-promoting proteins. Similarly, it is possible that some of the mRNAs involved in spermatogenesis in *C. elegans* have such inhibitory structures in their 5′ UTRs. In the absence of IFE-1, these mRNAs would not be able to compete with other mRNAs for the low level of eIF4E, leading to defects in spermatogenesis.

Why is the sterility seen after blocking the expression of *ife-1* sensitive to temperature? It is known that eIF4E activity is sensitive to some cellular stresses (reviewed by Sonenberg and Gingras, 1998). *C. elegans* spermatogenesis occurs more rapidly at high temperature. The stress associated with high-temperature growth might require higher levels of eIF4E. If IFE-1 is a prevalent isoform of eIF4E, then blocking the expression of IFE-1 would likely reduce the overall rate of translation or the rate of translation of certain mRNAs (see above). Either could lead to problems in spermatogenesis. Whatever the underlying molecular mechanism, at low temperatures other IFEs provide sufficient eIF4E activity to

form at least some functional sperm, but at high temperatures IFE-1 is required. It would be interesting to test whether overexpression of other IFEs eliminates the requirement for IFE-1 in spermatogenesis at high temperatures.

IFE-1 is a component of P granules

We have demonstrated that IFE-1 protein is associated with P granules via PGL-1. PGL-1 is a constitutive component of P granules and is required for several processes in germline development, including proliferation, meiosis and gametogenesis (Kawasaki et al., 1998). *pgl-1* mutants show a variety of defects, ranging from no germline proliferation to some germline proliferation and production of defective gametes.

Are the spermatogenesis defects seen in *pgl-1* mutants the result of dissociation of IFE-1 from P granules? About 19% of *pgl-1* null hermaphrodites grown at 26°C and containing a relatively well proliferated germline produce mature-looking sperm (n=54; A. A., unpublished). This value is similar to the percentage (~20%) of *ife-1(RNAi)* hermaphrodites that produce sperm and are fertile at 25°C. These data are consistent with, but do not prove, that the absence of sperm in most *pgl-1* mutants results from dissociation of IFE-1 from P granules.

P granules and translational control

P granules contain RNA. Originally it was shown that P-granule-associated RNA contains poly(A) and SL1, a *trans*-spliced leader sequence found on many *C. elegans* mRNAs (Seydoux and Fire, 1994). More recently, it was shown that *nos-2* RNA is associated transiently with P granules in embryos (Subramaniam and Seydoux, 1999) and that *pos-1*, *skn-1*, *par-3*, *mex-1*, *gld-1* and *nos-2* RNAs are associated with P granules in the maternal germline (Schisa et al., 2001). The presence of RNAs in P granules suggests that these granules are important for RNA delivery, stability or translation. Consistent with this view, all P-granule proteins identified to date are predicted to bind RNA. The recent discovery that P granules are associated with nuclear pores in the germline (Pitt et al., 2000) suggests that these granules have access to the majority of mRNAs as they transit from the nucleus to the cytoplasm. Certain mRNAs may be specifically retained in P granules by resident RNA-binding proteins. Or alternatively, as suggested by Schisa et al. (Schisa et al., 2001), granule-associated proteins may facilitate export of mRNAs from the nucleus out to the cytoplasm.

At present, it is not known whether there are any other translation factors in P granules. It was reported recently that VASA protein, which is the *Drosophila* homolog of the *C. elegans* GLH proteins (Gruidl et al., 1996, Kuznicki et al., 2000) and is a component of polar granules, interacts with translation initiation factor 2, dIF2 (Carrera et al., 2000). Although it has not been shown that dIF2 is itself a component of polar granules, this discovery suggests that there might be other translation factors in germ granules.

Are germ granules active sites of translation? Although, mitochondrial ribosomal RNAs are associated with germ granules in *Drosophila* and *Xenopus* embryos (Kobayashi et al., 1993, Kobayashi et al., 1998), neither mitochondrial nor nuclearly encoded ribosomal RNAs are enriched in P granules in the *C. elegans* maternal germline (Schisa et al., 2001). This observation argues against a scenario in which P granules are active sites of mRNA translation. One possibility is that P granules serve an inhibitory role in translational control. For example, P granules may sequester IFE-1 and thereby reduce the level of eIF4E available for initiating translation in the cytoplasm. Furthermore, the IFE-1 retained in P granules may be inactive. This prediction is based on the observation that PGL-1 contains the motif, YXXXXLϕ (where ϕ is a hydrophobic amino acid and X is any amino acid; Mader et al., 1995), through which eIF4G, 4E-BPs and Maskin are known to bind eIF4E. If PGL-1 interacts with IFE-1 via this motif, then PGL-1 interaction would probably prevent the association of IFE-1 with eIF4G and thereby inhibit translation initiation. Interestingly, in wild-type worms PGL-1 levels drop to below detectable after the pachytene stage of spermatogenesis. This may liberate IFE-1, perhaps to participate in promoting the completion of meiosis.

Alternatively, as was suggested for nuclearly localized eIF4E (Dostie et al, 2000), it is possible that IFE-1 in P granules serves a unique, non-translational role. For example, P-granule-associated IFE-1 may participate in nucleocytoplasmic transport of mRNA or function in regulating mRNA stability.

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