Regulation of Sperm-Specific Proteins by IFE-1, a Germline-Specific Homolog of eIF4E, in *C. elegans*

Ichiro Kawasaki*, Myung-Hwan Jeong, and Yhong-Hee Shim*

IFE-1 is one of the five *C. elegans* homologs of eIF4E, which is the mRNA 5' cap-binding component of the translation initiation complex eIF4F. Depletion of IFE-1 causes defects in sperm, suggesting that IFE-1 regulates a subset of genes required for sperm functions. To further understand the molecular function of IFE-1, proteomic analysis was performed to search for sperm proteins that are down-regulated in *ife-1(ok1978)*; *fem-3(q20)* mutants relative to the *fem-3(q20)* control. The *fem-3(q20)* mutant background was used because it only produces sperm at restrictive temperature. Total worm proteins were subjected to 2D-DIGE, and differentially expressed protein spots were further identified by MALDI-TOF mass spectrometry. Among the identified proteins, GSP-3 and Major Sperm Proteins (MSPs) were found to be significantly down-regulated in the *ife-1(ok1978)* mutant. Moreover, RNAi of *gsp-3* caused an *ife-1*-like phenotype. These results suggest that IFE-1 is required for efficient expression of some sperm-specific proteins, and the fertilization defect of *ife-1* mutant is caused mainly by a reduced level of GSP-3.

INTRODUCTION

Germ granules are distinctive organelles found in the germ cells of many species, including nematode *C. elegans*. They have been considered as instructors of germline development since their specific segregation to the germline was observed in fruit flies, frogs, and nematodes (reviewed in Saffman and Lasko, 1999). By studying a family of proteins, the PGL proteins, we previously demonstrated that the germ granules in *C. elegans* (also called as P granules) are indeed essential for the germline development (Kawasaki et al., 1998; 2004). The PGL proteins are constitutive protein components of P granules and function redundantly in *C. elegans* germline development. Among these proteins, PGL-1 is the most critical component. PGL-1 has been shown to specifically interact with IFE-1 *in vitro* and *in vivo* (Amiri et al., 2001). IFE-1 is one of the five *C. elegans* homologs of eIF4E, which is the mRNA 5' cap-binding component of the translation initiation complex eIF4F (Jankowska-Anyszka et al., 1998). Using RNAi analysis, we further demonstrated that IFE-1 is specifically required for sperm functions (Amiri et al., 2001). These findings suggest that IFE-1 and pos-

sibly PGL-1 are involved in translational control of a subset of genes that are required for proper sperm functions.

To further understand the molecular functions of IFE-1 on sperm activity, comparative proteomic analysis was performed between an *ife-1* mutant and wild-type control with the aim of identifying proteins that were down-regulated in the *ife-1* mutant. Through the analysis, 16 such proteins were identified. Among them, 4 proteins were encoded by genes that were previously annotated as "male enriched" or "male-germline enriched" through a genome-wide microarray gene expression analysis (Reinke et al., 2004). Among the 4 genes, RNAi of *gsp-3* caused an *ife-1*-like phenotype. That is, *gsp-3(RNAi)* hermaphrodites produced unfertilized oocytes or early-arrested embryos, and this defect was rescued by mating with wild-type males, as observed in *ife-1* mutants. These results suggest that IFE-1 is essential for the expression of some sperm-specific proteins, including the predicted phosphatase GSP-3, and the fertilization defect of the *ife-1* mutant may be mainly caused by a reduced level of GSP-3.

MATERIALS AND METHODS

Worm culture and strains

Caenorhabditis elegans strains were cultured and manipulated as described previously (Brenner, 1974). All strains were maintained at 16°C or 20°C on Nematode Growth Medium (NGM) agar plates containing *Escherichia coli* strain OP50, and up-shifted to 25°C when necessary. The following strains were used: wild-type strain N2, SS712: *ife-1(bn127) III*, RB1606: *ife-1(ok1978) III*, JK816: *fem-3(q20) IV*, YHS15: *ife-1(ok1978) III*; *fem-3(q20) IV*. RB1606 was outcrossed six times against N2 before phenotype analysis and construction of YHS15 to assure no additional mutations are accompanied.

Microscopic analysis

Images of live worms on agar plates were taken using a Nikon Digital Sight DS-L1 digital camera attached to a Nikon SMZ 1500 dissecting microscope (Nikon). To acquire images of DNA-stained extruded gonad arms, young adult animals were dissected in 10 μ l of M9 buffer on a poly-lysine coated glass slide, covered with a coverslip, freeze-cracked in liquid nitrogen, and fixed first in 100% cold methanol for 10 min, then in fresh 3% paraformaldehyde for 10 min. Specimens were then incu-

Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea

*Correspondence: ikawasak@mac.com (IK); yshim@konkuk.ac.kr (YHS)

Received October 25, 2010; revised November 1, 2010; accepted November 1, 2010; published online December 22, 2010

Keywords: *Caenorhabditis elegans*, eIF4E, IFE-1, proteomic analysis, sperm

Table 1. *ife-1* mutant phenotype. *bn127* and *ok1978* hermaphrodite worms were grown from the L1 larval stage on individual plates at either 20°C or 25°C (n = 12 for each).

<i>ife-1</i> allele (Temp.)	Mother's sterility	Brood size	Unfertilized oocytes	Embryonic lethality	Larval lethality	Male freq.
<i>bn127</i> (20°C)	33%	41 ± 27	49%	27%	3%	5%
<i>ok1978</i> (20°C)	8%	236 ± 41	22%	11%	8%	1%
<i>bn127</i> (25°C)	100%	0	100%			
<i>ok1978</i> (25°C)	100%	0	100%			

Their sterility, progeny's brood size, percent unfertilized oocytes among the total laid eggs, progeny's embryonic lethality, progeny's larval lethality, and progeny's male frequency were scored. The progeny's brood sizes are presented as means ± SEM. Brood size is the total number of fertilized embryos laid by a mother. Embryonic lethality is the fraction of non-hatched embryos among the fertilized embryos. Larval lethality is the fraction of arrested larvae among the hatched progeny. Male frequency is the fraction of males among the adult progeny.

Fig. 1. (A) Gene structure and mutations of *ife-1*. *ife-1* (F53A2.6 on chromosome III) encodes two isoforms (a and b) of a *C. elegans* homolog of the mRNA 5' cap-binding protein, eIF4E, in 4 types of transcripts. *bn127* is a 590 bp deletion, which removes more than half of the exon 2 and most of the exon 3 including the 3' UTR of *ife-1*. *ok1978* is a DNA rearrangement consisting of a 1047 bp deletion and an 8 bp insertion, which removes the stop codon and the 3' UTR of *ife-1*, intergenic sequence, and the 5' portion of the downstream gene, F53A2.7. White boxes, caretts, and black boxes indicate 5' or 3' UTRs, introns, and coding sequences, respectively. (B) *ife-1(ok1978)* hermaphrodites grown at 25°C are sterile. Wild-type N2 hermaphrodite worms grown at 25°C are fertile and produce fertilized eggs, which eventually hatch into larvae (arrowheads). *ife-1(ok1978)* hermaphrodite worms grown at 25°C are 100% sterile as observed for the *ife-1(bn127)* hermaphrodite worms, and produce unfertilized oocytes (arrows). Scale bar, 100 μm.

bated in PBS containing 0.5 μg/ml of Hoechst 33342 DNA dye and mounted. Mounted specimens were observed under a fluorescence microscope (Axioskop 2 MOT, ZEISS). Images were acquired using an Orca ERG digital camera (Hamamatsu) and processed with Openlab (Improvision) and Photoshop (Adobe) software.

Whole-worm protein preparation and Cy-Dye labeling

The worm strains JK816: *fem-3(q20)* and YHS15: *ife-1(ok1978)*; *fem-3(q20)* were propagated at 16°C on seeded NGM plates for a few generations until a high enough number of worms were obtained. The worms were then collected, lysed in an alkaline bleach solution (2% NaClO, 0.5 N NaOH) by pipetting, and washed repeatedly with M9 buffer to obtain a large number of live eggs. The obtained eggs were allowed to hatch in S-basal buffer in the absence of food overnight to synchronize them at the L1 larval stage. The obtained L1 larvae were seeded on large NGM plates (ca 1500 L1 larvae/plate × 20 plates), up-shifted to 25°C, and grown for 2 days until they

reached the adult stage. The adult worms, which were masculinized by temperature-sensitive *fem-3(q20)* gain-of-function mutation and thus produced sperm continuously, were collected, cleaned up by sucrose floatation, washed with M9 buffer and distilled water, centrifuged, and stored as a worm pellet at -70°C. The worm pellet volume obtained for each strain was approximately 200 μl. Whole-worm proteins were extracted as previously described (Ahn et al., 2006). 50 μg of worm protein was mixed with 400 pmol Cy-Dye (GE Healthcare), and incubated on ice in the dark for 30 min. Proteins from each strain were labeled with either Cy3 or Cy5, and mixed with a Cy2-labeled internal standard. The internal standard was prepared by pooling 25 μg of protein from each strain. After incubation, the samples were quenched by adding 1 μl of 10 mM lysine.

2-D DIGE and 2-DE preparative gel

2-D DIGE was carried out as previously described (Na et al., 2009). Aliquots in sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTE, 40 mM Tris, pH 8.8) were applied to

Table 2. List of 16 down-regulated proteins in *ife-1(ok1978)*; *fem-3(q20gf)* mutant compared with the *fem-3(q20gf)* control.

Protein or gene ID	Fold change control vs <i>ife-1</i>	T-test	Expression profile
GLutamiNe synthetase family member, <i>gln-2</i> (K03H1.1) (III)	2.68	0.0049	M.G.
T05A7.1 (II)	1.96	6.20E-07	
K07H8.1 (IV)	1.96	0.00045	F.G.
Nuclear Pore complex Protein family member, <i>npp-21</i> (R07G3.3) (II)	1.90	0.00029	F.G.
ACTin family member, <i>act-4</i> (M03F4.2) (X)	1.80	0.00023	
Major Sperm Proteins, Y59H11AM.1 (IV), <i>msp-32</i> (R05F9.3) (II), <i>msp-38</i> (K08F4.8) (IV), and others	1.64	0.0072	M.G.
Yeast Glc Seven-like Phosphatases family member, <i>gsp-3</i> (W09C3.6) (I)	1.63	0.00094	M.G.
Y24D9A.8a (IV)	1.59	0.0032	
R08A2.2 (V)	1.48	0.00071	
F29C4.7 (IV)	1.44	0.0054	
Lipid Binding Protein family member, <i>lbp-9</i> (Y40B10A.1) (V)	1.41	0.00074	M.
C34B2.7 (I)	1.38	1.20E-06	F.
PeRoxireDoXin family member, <i>prdx-2</i> (F09E5.15) (II)	1.37	0.001	
Guanine nucleotide exchange factor for RAS, <i>sos-1</i> (T28F12.3) (V)	1.37	0.0044	
Cytochrome C Heme-Lyase family member, <i>cchl-1</i> (T06D8.6) (II)	1.32	0.00098	F.
Heat Shock Protein family member, <i>hsp-6</i> (C37H5.8) (V)	1.31	0.0022	F.G.

Values for fold change are the average of the three independent 2D-DIGE analyses. Expression profiles are derived from the data obtained by Reinke et al. (2004). M.G., male-germline enriched; M., male enriched; F.G., female-germline enriched; F., female enriched

Fig. 2. *ife-1(ok1978)* mutant gonads developed at 25°C. Wild-type N2 and *ife-1(ok1978)* mutant gonads were dissected from adult-stage hermaphrodites (hermaph.) and males grown at 25°C, and stained with the DNA dye, Hoechst 33342. While N2 adult hermaphrodite gonads contained both oocytes and sperm, *ife-1(ok1978)* adult hermaphrodite gonads contained only oocytes including unfertilized endomitotic oocytes (arrows) at 25°C. Both N2 adult male gonads and *ife-1(ok1978)* adult male gonads contained enough spermatids at 25°C, suggesting that the *ife-1* mutant has a defect in sperm function rather than in spermatogenesis. Asterisks, distal end of gonad arms. Brackets, regions of mature sperm. Scale bar, 100 μ m.

IPG strips pH 3-10NL (GE Healthcare). IEF was performed at 110,000 Vhr (24 cm). The second dimension was analyzed on 9-17% linear gradient polyacrylamide gels at a constant current

of 40 mA per gel for approximately 5 h. Three independent gels were prepared and analyzed. The CyDye-labeled gels were visualized using a Typhoon 9400 imager (GE Healthcare). Excitation/emission wavelengths used for each DIGE fluor were; Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm). Images were preprocessed to remove areas that were extraneous to those of interest using ImageQuant V2005 (GE Healthcare). Intra-gel analysis was performed using DeCyder DIA (Difference In-gel Analysis) version 6.5.11 (GE Healthcare). Inter-gel matching and statistical analysis were performed using DeCyder BVA (Biological Variance Analysis) version 6.5.11 (GE Healthcare). For a preparative gel, IEF/SDS-PAGE was performed using 1 mg of non-labeled internal standard proteins, and the proteins were fixed in 40% methanol and 5% phosphoric acid for 1 h. Gels were then stained with Coomassie Brilliant Blue G250 (GE Healthcare) for 12 h, and destained using ultrapure distilled water.

Protein identification by MALDI-TOF-MS and MS/MS

A spot that was more than 1.3-fold down-regulated in the *ife-1* mutant and was statistically significant ($p < 0.05$) in three independent gels was manually excised from the preparative gel and analyzed by MALDI-TOF-MS (Applied Biosystems) after trypsin digestion as previously described (Na et al., 2009). Protein identification was performed by searching the Swiss-Prot and NCBI nr databases using the Matrix Science (<http://www.matrixscience.com>) search engine.

RNAi analysis

RNAi analysis was performed using the “RNAi-by-soaking” method as described previously (Maeda et al., 2001), with some modifications. N2 worms were synchronized at the L1 larval stage in the absence of food, and soaked for 24 h in each of the double-strand RNA solutions transcribed *in vitro* from the respective cDNA clones (yk clones) listed in Table 3. Worms

Table 3. RNAi phenotype of male or male-germline enriched genes that are down-regulated in the *ife-1(ok1978)* mutant. RNAi analysis was performed as described in "Materials and Methods".

Sequence	Gene	yk clone	20°C	25°C
-	-	-	Fertile (60/60)	Fertile (60/60)
K03H1.1 (III)	<i>gln-2</i>	203d8	Fertile (239/241)	Fertile (264/266)
F58A6.8 (II)	<i>msp-45</i>	410f8	Fertile (191/199)	Fertile (250/253)
C34F11.6 (II)	<i>msp-49</i>	409f8	Fertile (238/245)	Fertile (274/279)
ZK1251.6 (IV)	<i>msp-76</i>	437g6	Fertile (173/174)	Fertile (216/224)
K07F5.1 (IV)	<i>msp-81</i>	438b11	Fertile (231/237)	Fertile (280/285)
W09C3.6 (I)	<i>gsp-3</i>	385e11	Many worms produced only unfertilized oocytes (134/238)	Many worms produced only unfertilized oocytes (171/272)
Y40B10A.1 (V)	<i>lbp-9</i>	516e12	Fertile (180/186)	Fertile (235/241)

were then retrieved and transferred to individual seeded plates where they resumed development either at 20°C or 25°C. After a few days, the plates were examined for the production of fertilized embryos.

RESULTS

ok1978 is a reduction-of-function mutation of *ife-1*

In previous studies, both the RNAi of *ife-1*, *ife-1(RNAi)*, and a molecular-null mutation of *ife-1*, *ife-1(bn127)* were shown to cause temperature-sensitive sterility (Amiri et al., 2001; Henderson et al., 2009). The sterility was mainly caused by defects in the sperm, because when wild-type sperm were provided by mating, these *ife-1* mutant hermaphrodites became fertile and produced viable embryos that developed into adult worms (Amiri et al., 2001; Henderson et al., 2009). Although defective, *ife-1(RNAi)* adult males contained highly condensed sperm nuclei (Amiri et al., 2001), suggesting that meiosis and spermatogenesis still occurred in the absence of *ife-1*. Moreover, when *ife-1(RNAi)* hermaphrodites were masculinized by a *fem-3* gain-of-function mutation and forced not to switch from spermatogenesis to oogenesis, even *ife-1(RNAi)* adult hermaphrodites produced mature-looking sperm (Amiri et al., 2001). These results suggest that depletion of IFE-1, a germline-specific homolog of eIF4E, causes a defect in sperm function (motility, fertility, etc.) rather than in spermatogenesis.

To further understand the molecular function of IFE-1 in regards to sperm activity, comparative proteomic analysis was performed between the *ife-1* mutant and wild-type control with the aim of identifying proteins that were down-regulated in the *ife-1* mutant. We noticed that another mutant allele, *ok1978*, also displayed temperature-sensitive sterility (Table 1). *ok1978* is a DNA rearrangement consisting of a 1047 bp deletion and an 8 bp insertion, which removes the stop codon and the 3' UTR of *ife-1*, intergenic sequence, and the 5' portion of the downstream gene, F53A2.7 (Fig. 1A). It appears that the stop codon and/or the 3' UTR of *ife-1* have a critical effect on the expression of *ife-1*, because the phenotype of *ok1978* was very similar to that of null allele, *bn127*, especially at 25°C (Table 1). In other words, *ok1978* hermaphrodite worms grown at 25°C were 100% sterile and produced unfertilized oocytes similar to *bn127* hermaphrodite worms (Table 1, Fig. 1B). Their adult gonads often contained unfertilized endomitotic oocytes but seldom contained sperm even when not aged yet (Fig. 2). In contrast, wild-type N2 adult hermaphrodite gonads retained mature sperm in the proximal region for at least 2 days after they started laying eggs at 25°C (Fig. 2). Furthermore, when mated with wild-type males, the *ok1978* hermaphrodites be-

came fertile and produced viable embryos at 25°C (100%, n = 10). However, *ok1978* adult males could not produce outcross progeny at 25°C (0%, n = 10), although their gonads contained highly condensed mature-looking sperm nuclei as observed in the N2 adult male gonads (Fig. 2).

In this study, *ok1978* mutants were used for analysis because their brood size was much larger, and their sterility and embryonic lethality were significantly lower than those of the *bn127* mutants at permissive temperatures (Table 1). These features could provide a significant advantage when propagating a large number of worms for proteomic analysis. One concern was that *ok1978* mutation also affects the structure of the downstream gene, F53A2.7. Therefore, the effect of depletion of F53A2.7 was examined by RNAi, and it was confirmed that RNAi of F53A2.7 did not cause any recognizable defects in the germline development at both 20°C and 25°C (data not shown). These results indicate that *ok1978* mutation phenotypically affects only *ife-1* (F53A2.6) gene as a reduction-of-function type mutation, and thus it was used in this study.

Comparative proteomic analysis between *fem-3(q20)* and *ife-1(ok1978); fem-3(q20)* worms

Although it has been previously attempted (Chu et al., 2006), it is fairly difficult to obtain a large worm population consisting of 100% males because males cannot produce progeny by themselves. On the other hand, it is easy to obtain a large population of 100% hermaphrodite worms since they can produce progeny by self-fertilization and the produced progeny are all hermaphrodites. Nevertheless, because hermaphrodites produce sperm only during the L4 larval stage, and especially in the *ife-1* mutants, their defective sperm seem to be immediately washed off and not retained in the spermatheca after the onset of oogenesis (Fig. 2), it is not easy to find the best timing for harvesting when a majority of the population are producing/retaining sperm. To circumvent this problem, we introduced another mutation, *fem-3(q20)*. When up-shifted to a restrictive temperature, *fem-3(q20)* worms continuously produce sperm without switching to the oogenesis in the adult stage. Thus, a double-mutant strain, YHS15: *ife-1(ok1978); fem-3(q20)*, was constructed and used for the following comparative proteomic analysis with the control strain, JK816: *fem-3(q20)* (Fig. 3A; also see "Materials and Methods").

Hermaphrodite worm populations of *fem-3(q20)* and *ife-1(ok1978); fem-3(q20)* were propagated at permissive 16°C, synchronized at the L1 larval stage by bleaching and starvation, up-shifted to restrictive 25°C, grown to the young adult stage and then harvested. Whole-worm protein lysates were prepared from the worm pellets, labeled with Cy3 and Cy5 fluoro-

A

B

Fig. 4. RNAi analysis of sperm-specific genes that were down-regulated in the *ife-1(ok1978)* mutant. RNAi-treated worms were placed on plates and development of laid eggs was monitored after incubation for 4 days and 5 days at 20°C. *gsp-3(RNAi)*, but not RNAi of *msp* genes, produced abundant unfertilized oocytes and/or early-arrested embryos, which did not hatch after 5 days, as was observed for the *ife-1(ok1978)* mutants that were grown at 25°C. Scale bar, 100 μ m.

Fig. 3. (A) Schematic of the comparative proteomic analysis between *fem-3(q20)* and *ife-1(ok1978); fem-3(q20)* mutants. *fem-3(q20)* gain-of-function (*gf*) mutation was introduced into both strains as a genetic background because *fem-3(q20)gf* mutant hermaphrodites are masculinized and constitutively produce sperm at 25°C, while normal hermaphrodites produce sperm during only the L4 larval stage and switch to produce oocytes throughout the adult stage. (B) Comparison of GSP-3 and MSP spots between *fem-3(q20)* and *ife-1(ok1978); fem-3(q20)* whole-worm protein preparations. The whole-worm proteins were separated on 2D-DIGE gel and quantified. The differentially expressed protein spots were then identified by mass spectrometry.

cent dyes, and applied to 2D-DIGE analysis as described previously (Na et al., 2009). Protein spots significantly down-regulated in the *ife-1(ok1978); fem-3(q20)* protein preparation compared to the *fem-3(q20)* control were further analyzed by MALDI-TOF mass spectrometry for protein identification. Through this analysis, 16 differentially expressed proteins were identified (Table 2, Fig. 3B).

RNAi analysis of down-regulated genes

A majority of the *C. elegans* genes that show germline-enriched and/or sex-biased expression profiles have been identified through a genome-wide microarray gene expression analysis (Reinke et al., 2004). According to this annotation, 4 of the above 16 proteins were encoded by genes that were either "male enriched" or "male-germline enriched" (Table 2). To identify a down-regulated target gene that was responsible for the fertilization defect of the *ife-1* mutants, we examined whether any of these genes showed the same phenotype as *ife-1* mutants when depleted by RNAi (Table 3). Since the major sperm proteins (MSPs), which were one of the 4 down-regulated proteins, are encoded by a family of over 50 almost identical genes called *msp* genes (Ward et al., 1988), we tested some of the *msp* genes for RNAi. We found that RNAi for *gln-2*, *lbp-9*, and four *msp* genes did not cause significant sterility (Table 3, Fig. 4). On the other hand, RNAi of *gsp-3* produced a similar phenotype as the *ife-1* mutants (Table 3, Fig. 4). 56% and 63% of *gsp-3(RNAi)* hermaphrodites produced only unfertilized oocytes or early-stage arrested embryos, both of which failed to hatch at 20°C and 25°C, respectively. Even when they produced fertilized embryos that developed into adult worms, they also pro-

duced a significant fraction of unfertilized oocytes. Furthermore, when mated with N2 males, all of the *gsp-3(RNAi)* hermaphrodites became fertile at both 20°C and 25°C ($n = 10$ for each temperature). Similar results for *gsp-3(RNAi)* were also reported by others (Chu et al., 2006; Hanazawa et al., 2001).

DISCUSSION

In this study, we analyzed the proteome of the *ife-1* mutant and demonstrated that comparative proteomic analysis is a useful tool to identify targets of translational regulation when combined with genetics. Through this comparative analysis, we identified 4 types of male or male-germline enriched genes that were down-regulated in *ife-1(ok1978)* mutants. Among these genes, *gsp-3* appeared to be the most critical target of IFE-1 regulation in sperm function, because RNAi of *gsp-3* phenocopied the *ife-1*-like phenotype (Table 3, Fig. 4). *gsp-3* encodes one of four yeast Glc7-like protein phosphatases (Chu et al., 2006). Targeted disruption of their mouse homolog, PP1 γ , caused male infertility in mutant mice due to defects in spermiogenesis and meiosis (Varmuza et al., 1999). Therefore, their function in sperm may have remained conserved throughout evolution. Among the four *C. elegans* Glc7-like protein phosphatases, GSP-3 and GSP-4 seem to function redundantly for sperm, because both *gsp-3(RNAi)* and *gsp-4(RNAi)* caused hermaphrodite sterility that could be rescued by mating with wild-type males (Boag et al., 2003; Hanazawa et al., 2001). Interestingly, both *gsp-3(RNAi)* hermaphrodites and *gsp-4(RNAi)* hermaphrodites contained mature-looking sperm, suggesting that their sperm were defective in function (motility or fertility) rather than in spermatogenesis itself as was observed for the *ife-1* mutants. We consider that both *gsp-3* and *gsp-4* were positively regulated by IFE-1 since GSP-3 and GSP-4 proteins are nearly identical and therefore likely shared the same 2D-gel spot that was down-regulated in the *ife-1* mutant. One clear difference in the phenotypes between the *ife-1* mutants and *gsp-3/4(RNAi)* was the temperature sensitivity. That is, although the *ife-1* mutant phenotype was temperature-sensitive, the *gsp-3/4(RNAi)* phenotype was not. One possible explanation for this observation is that IFE-1 activity is essential only at elevated temperatures for efficient expression of GSP-3/4, but at low temperatures, other activities can somehow compensate for the absence of IFE-1 and GSP-3/4 are expressed above a threshold level. However, further analysis is required to confirm this hypothesis.

Another intriguing candidate target of IFE-1 regulation in sperm function was the Major Sperm Protein (MSP). MSPs are a family of proteins conserved among nematodes. This family consists of closely related, small, basic proteins that make up 15% of sperm protein (Ward et al., 1988). They are encoded by a multigene family of over 50 genes, including many pseudogenes (Ward et al., 1988). At the transcriptional level, at least some of the *msp* genes seem to be regulated by ELT-1, which is a germline-enriched GATA transcription factor (Shim, 1999). MSP functions not only as a cytoskeletal protein essential for sperm locomotion (Italiano et al., 1996), but also as a crucial signal for oocyte maturation and gonadal sheath contraction (Miller et al., 2001). Thus, MSP is a key sperm protein essential for fertilization. So far, studies on MSPs have been achieved either by using an *in vitro* reconstitution system (Italiano et al., 1996), or by exogenously adding purified MSPs to feminized worms, which do not possess their own sperm (Miller et al., 2001). Construction of knockout worms, in which all the *msp* genes are disrupted, is practically impossible because of the large number of *msp* genes in the *C. elegans* genome. In this

sense, a true knockout phenotype of all *msp* genes has not yet been observed. Although the RNAi of *msp* genes did not display any significant defects, this result does not necessarily indicate that MSPs are dispensable for sperm function, because RNAi phenotype is often not as strong as knockout phenotype, and some genes are even reported to be insensitive to RNAi. If the *ife-1* mutant phenotype is rescued by exogenously added MSPs or by transgenic expression of an *msp* gene, the *ife-1* mutant would be perceived as a useful genetic resource, where MSP activities are grossly repressed.

IFE-1 is one of the five *C. elegans* homologs of eIF4E, the mRNA 5' cap-binding protein, which is essential for initiation of translation (Jankowska-Anyszka et al., 1998). IFE-1 is unique among the five homologs, because only IFE-1 can localize to P granules, the germline-specific granules of *C. elegans*, by associating with PGL-1, which is a constitutive protein component of P granules (Amiri et al., 2001; Kawasaki et al., 2004). It is well known that eIF4E is negatively regulated by its association with eIF4E-binding proteins (4E-BPs), which, when bound to eIF4E, prevent it from binding to eIF4G, thereby inhibiting translation initiation (Sonenberg, 1996). By identifying IFE-1 targets in this study, it has become possible to discern whether PGL-1 functions as a negative regulator like 4E-BPs or as a positive regulator of IFE-1 in the translational control of sperm activity.

ACKNOWLEDGMENTS

We thank Yonsei Proteome Research Center (YPRC) for 2D-DIGE and MALDI-TOF mass spectrometry analyses, Dr. Yuji Kohara for yk cDNA clones, and Dr. Susan Strome for a worm strain SS712. We also thank Dr. Brett Keiper and Dr. Susan Strome for useful comments. This work was supported by the faculty research fund of Konkuk University in 2007 to I.K. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources.

REFERENCES

- Ahn, D.H., Singaravelu, G., Lee, S., Ahnn, J., and Shim, Y.H. (2006). Functional and phenotypic relevance of differentially expressed proteins in calcineurin mutants of *Caenorhabditis elegans*. *Proteomics* 6, 1340-1350.
- Amiri, A., Keiper, B.D., Kawasaki, I., Fan, Y., Kohara, Y., Rhoads, R.E., and Strome, S. (2001). An isoform of eIF4E is a component of germ granules and is required for spermatogenesis in *C. elegans*. *Development* 128, 3899-3912.
- Boag, P.R., Ren, P., Newton, S.E., and Gasser, R.B. (2003). Molecular characterisation of a male-specific serine/threonine phosphatase from *Oesophagostomum dentatum* (Nematoda: Strongylida), and functional analysis of homologues in *Caenorhabditis elegans*. *Int. J. Parasitol.* 33, 313-325.
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71-94.
- Chu, D.S., Liu, H., Nix, P., Wu, T.F., Ralston, E.J., Yates, J.R., and Meyer, B.J. (2006). Sperm chromatin proteomics identifies evolutionarily conserved fertility factors. *Nature* 443, 101-105.
- Hanazawa, M., Mochii, M., Ueno, N., Kohara, Y., and Iino, Y. (2001). Use of cDNA subtraction and RNA interference screens in combination reveals genes required for germ-line development in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 98, 8686-8691.
- Henderson, M.A., Cronland, E., Dunkelbarger, S., Contreras, V., Strome, S., and Keiper, B.D. (2009). A germline-specific isoform of eIF4E (IFE-1) is required for efficient translation of stored mRNAs and maturation of both oocytes and sperm. *J. Cell Sci.* 122, 1529-1539.
- Italiano, J.E., Roberts, T.M., Stewart, M., and Fontana, C.A. (1996). Reconstitution in vitro of the motile apparatus from the amoeboid sperm of *Ascaris* shows that filament assembly and bundling

- move membranes. *Cell* **84**, 105-114.
- Jankowska-Anyska, M., Lamphear, B.J., Aamodt, E.J., Harrington, T., Darzynkiewicz, E., Stolarski, R., and Rhoads, R.E. (1998). Multiple isoforms of eukaryotic protein synthesis initiation factor 4E in *Caenorhabditis elegans* can distinguish between mono- and trimethylated mRNA cap structures. *J. Biol. Chem.* **273**, 10538-10542.
- Kawasaki, I., Shim, Y.H., Kirchner, J., Kaminker, J., Wood, W.B., and Strome, S. (1998). PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in *C. elegans*. *Cell* **94**, 635-645.
- Kawasaki, I., Amiri, A., Fan, Y., Meyer, N., Dunkelbarger, S., Motohashi, T., Karashima, T., Bossinger, O., and Strome, S. (2004). The PGL family proteins associate with germ granules and function redundantly in *Caenorhabditis elegans* germline development. *Genetics* **167**, 645-661.
- Maeda, I., Kohara, Y., Yamamoto, M., and Sugimoto, A. (2001). Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. *Curr. Biol.* **11**, 171-176.
- Miller, M.A., Nguyen, V.Q., Lee, M.H., Kosinski, M., Schedl, T., Caprioli, R.M., and Greenstein, D. (2001). A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. *Science* **291**, 2144-2147.
- Na, K., Lee, E.Y., Lee, H.J., Kim, K.Y., Lee, H., Jeong, S.K., Jeong, A.S., Cho, S.Y., Kim, S.A., Song, S.Y., et al. (2009). Human plasma carboxylesterase 1, a novel serologic biomarker candidate for hepatocellular carcinoma. *Proteomics* **9**, 3989-3999.
- Reinke, V., San Gil, I., Ward, S., and Kazmer, K. (2004). Genome-wide germline-enriched and sex-biased expression profiles in *Caenorhabditis elegans*. *Development* **131**, 311-323.
- Saffman, E.E., and Lasko, P. (1999). Germline development in vertebrates and invertebrates. *Cell. Mol. Life Sci.* **55**, 1141-1163.
- Shim, Y.H. (1999). *elt-1*, a gene encoding a *Caenorhabditis elegans* GATA transcription factor, is highly expressed in the germ lines with *msp* genes as the potential targets. *Mol. Cells* **9**, 535-541.
- Sonenberg, N. (1996). mRNA 5' cap-binding protein eIF4E and control of cell growth. In *Translational Control*, J.W.B. Hershey, M.B. Mathews, and N. Sonenberg, eds. (Cold Spring Harbor, New York, USA: Cold Spring Harbor Laboratory Press), pp. 245-269.
- Varmuza, S., Jurisicova, A., Okano, K., Hudson, J., Boekelheide, K., and Shipp, E.B. (1999). Spermiogenesis is impaired in mice bearing a targeted mutation in the protein phosphatase 1 γ gene. *Dev. Biol.* **205**, 98-110.
- Ward, S., Burke, D.J., Sulston, J.E., Coulson, A.R., Albertson, D.G., Ammons, D., Klass, M., and Hogan, E. (1988). Genomic organization of major sperm protein genes and pseudogenes in the nematode *Caenorhabditis elegans*. *J. Mol. Biol.* **199**, 1-13.