

# Combinatorial RNA interference indicates GLH-4 can compensate for GLH-1; these two P granule components are critical for fertility in *C. elegans*

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## SUMMARY

We report that four putative germline RNA helicases, GLHs, are components of the germline-specific P granules in *Caenorhabditis elegans*. GLH-3 and GLH-4, newly discovered, belong to a multi-gene *glh* family. Although GLHs are homologous to *Drosophila* VASA, a polar granule component necessary for oogenesis and embryonic pattern formation, the GLHs are distinguished by containing multiple CCHC zinc fingers. RNA-mediated interference (RNAi) reveals the GLHs are critical for oogenesis. By RNAi at 20°C, when either loss of GLH-1 or GLH-4 alone has no effect, loss of both GLH-1 and GLH-4 results in 97% sterility in the *glh-1/4(RNAi)* offspring of injected

hermaphrodites. *glh-1/4(RNAi)* germlines are under-proliferated and are without oocytes. *glh-1/4(RNAi)* animals produce sperm; however, spermatogenesis is delayed and the sperm are defective. P granules are still present in *glh-1/4(RNAi)* sterile worms as revealed with antibodies against the remaining GLH-2 and GLH-3 proteins, indicating the GLHs function independently in P granule assembly. These studies reveal that *C. elegans* can use GLH-1 or GLH-4 to promote germline development.

Key words: Germ granules, RNA helicases, CCHC zinc fingers, Glycine-rich repeats, Combinatorial RNA-mediated interference

## INTRODUCTION

Germ granules are cytoplasmic, non-membranous RNA/protein complexes aggregated in the primordial germ cells of many higher eucaryotes (Eddy, 1975). In the nematode *Caenorhabditis elegans*, germ granules are called P granules (Strome and Wood, 1982, 1983; Wolf et al., 1983). P granule components are candidate cytoplasmic determinants, specifying germ cell identity in the embryo and gametogenesis in the next generation. P granules are dispersed throughout the cytoplasm in the mature oocyte, segregate to the posterior cortex after fertilization and continue to segregate into the P germline lineage in the early embryo until they surround the nucleus of the P<sub>4</sub> germ cell progenitor at the 16-24 cell stage. P granules remain in the germline throughout the life of the worm, surrounding each germ cell nucleus during mitotic proliferation and gametogenesis.

In *Drosophila melanogaster* germ granules are called polar granules. Transplantation of cytoplasm containing polar granules can induce germ cells to ectopically form at the anterior of the fly embryo; genetically-marked anterior pole cells can become primordial germ cells when transplanted into foster mothers (Illmensee and Mahowald, 1975). Mutations that affect pole cells include ones in the 'posterior group' genes: *vasa*, *tudor*, *staufen*, *valois*, *mago-nashi*, *oskar* and *nanos*

(reviewed by St Johnston and Nüsslein-Volhard, 1992). Because of maternal effects, mutations in many of these genes have a *grandchildless* phenotype. Ectopic *osk* expressed at the anterior pole is sufficient to recruit VASA and TUDOR; these three polar granule components must be functional to form pole cells (Ephrussi and Lehmann, 1992). Thus, one or more of these components may be the 'germline determinants' in the fly.

VASA is a germline-specific, ATP-dependent DEAD-box RNA helicase (Hay et al., 1988; Lasko and Ashburner, 1988). VASA regulates the translation of at least two mRNAs, *gurken* and *nanos* (Styhler et al., 1998; Gavis et al., 1996). A null *vasa* mutation causes a range of defects in oogenesis (Styhler et al., 1998). Germline RNA helicases are conserved components of germ granules, with VASA homologues reported in several species, including *Xenopus*, mouse, and *Caenorhabditis* (Komiya et al., 1994; Fujiwara et al., 1994; Rousell and Bennett, 1993; Gruidl et al., 1996).

The germline helicase (*glh*) genes, *vasa* homologues, encode P granule components in *C. elegans* (Rousell and Bennett, 1993; Gruidl et al., 1996; this report). GLH-1 and GLH-2 contain the eight conserved motifs characteristic of DEAD-box RNA helicases and, like VASA, they contain N-terminal glycine-rich repeats. The glycine repeats in the GLHs differ from VASA; they contain no charged amino acids. GLH-1 and GLH-2 also differ from VASA and most other RNA helicases

in containing multiple CCHC zinc fingers similar to those in CNBP (cellular nucleic acids binding protein) and in HIV retroviral nucleocapsid binding (NC) protein. The zinc fingers in the CNBP and NC proteins provide specificity to RNA binding (Pellizzoni et al., 1998; Schmalzbauer et al., 1996). We previously reported a role for GLH-1 or GLH-2 in germline development. Injecting either a *glh-1* or a *glh-2* antisense RNA, both >2 kb non-gene-specific fragments, resulted in approx. 10% sterile offspring; GLH-1 and GLH-2 were both eliminated in these affected F<sub>1</sub> progeny. The small percentages of sterile worms had disorganized gonads and produced no oocytes (Gruidl et al., 1996).

Both protein and RNA P granule components have been reported. Three of these components, GLH-1, GLH-2 and PGL-1, a novel protein with RGG RNA binding motifs, are constitutively present in P granules. Other P granule components include PIE-1 (Mello et al., 1996), MEX-1 (Guedes and Priess, 1997), MEX-3 (Draper et al., 1996), POS-1 (Tabara et al., 1998) and GLD-1 (Jones et al., 1996). While these proteins are novel, each contains known RNA binding motifs. These non-constitutive proteins associate with P granules during early embryogenesis and disappear in the daughters of the germline precursor P<sub>4</sub> cell. *nos-2* RNA, the *C. elegans nanos* equivalent, is the first mRNA reported that is associated, also transiently, with P granules in embryos (Subramaniam and Seydoux, 1999). Mutant analyses have determined the products of each of these genes are critical for embryonic lineage identity or germline function.

We report here that two additional putative RNA helicases, GLH-3 and GLH-4, are P granule constituents and that all four GLHs assemble independently in P granules. Based on RNA interference (RNAi), we report GLH-1 and GLH-4 have overlapping functions. GLH-4 is able to substitute for GLH-1 at the permissive temperature of 20°C but not at the restrictive temperatures of 25–26°C.

## MATERIALS AND METHODS

### Strains

N2 variety Bristol nematodes were used unless stated otherwise. The *him-8(e1489)* strain was provided by D. Shakes (College of William and Mary). *fog-2(q71)* was received from T. Schedl (Washington University). *glh-3(um1)* was isolated in our laboratory; *glh-1(bn103)* was isolated by J. Kirchner in the Strome laboratory, Indiana University. The *glh* mutant isolation is a collaborative effort between the two laboratories. In these studies worms were grown at 20°C and 25°C using standard techniques (Brenner, 1974). (*C. elegans* is ideally grown at 20°C; growth at 25–26°C is used to reveal temperature-sensitive effects.)

### Genomic analyses

Searches of the *C. elegans* genomic sequence for genes that contain the eight conserved DEAD-box helicase motifs and CCHC zinc fingers revealed three previously unidentified genes: *glh-3* (cosmid B0414), *glh-4* (cosmid T12F5), and *glh-4b* (cosmid T08D2). *glh-3* (YK40E10) and *glh-4* (YK114D5) cDNAs were obtained from the EST project (Y. Kohara, Nat. Inst. Genetics, Mishima, Japan). To determine the 5' ends of both genes, RT-PCRs were performed using internal *glh*-specific primers and a primer corresponding to the trans-spliced SL1 leader sequence found on the 5' ends of many *C. elegans* RNAs (Krause and Hirsh, 1987). The sequences of the *glh-3* and *glh-4* cDNAs were completed using multiple primers.

### RNA analyses: northern blot analyses and in situ hybridizations

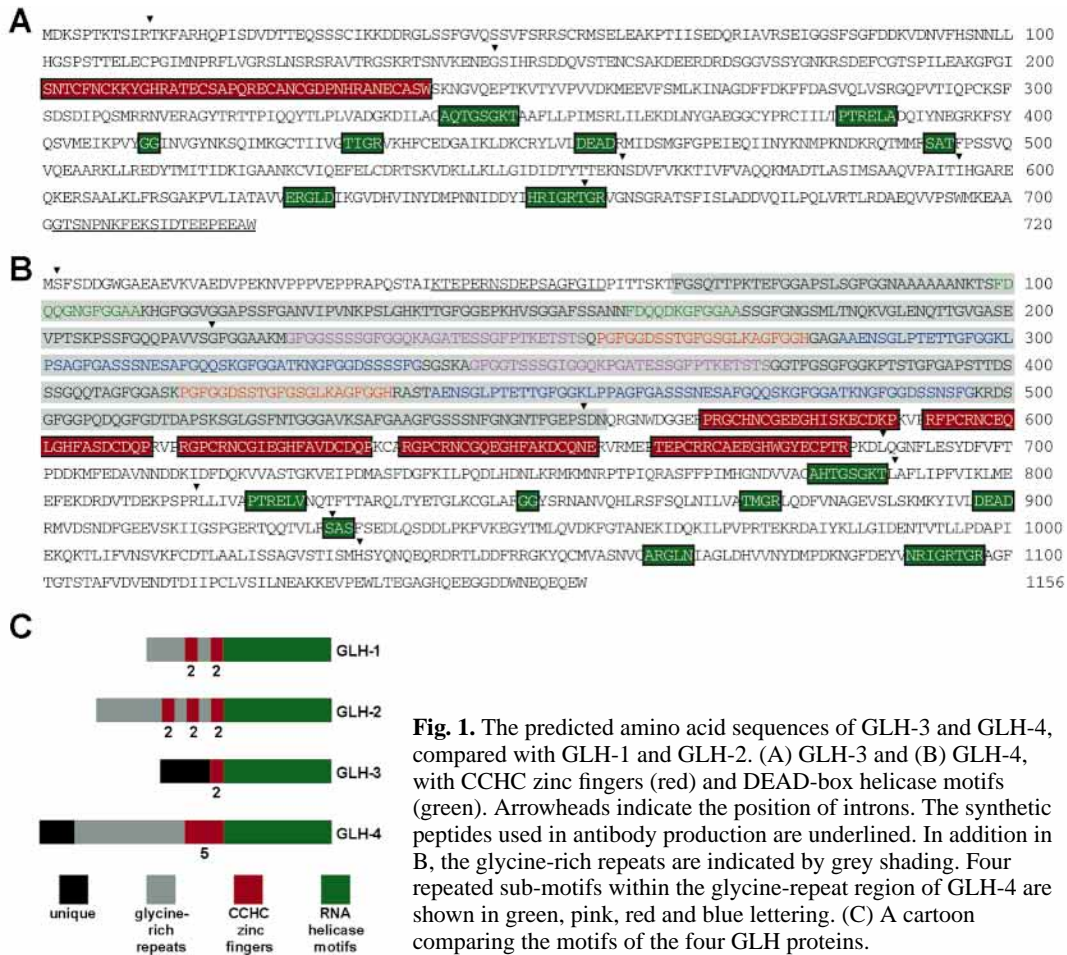
Northern blot analyses were performed using 3 µg of poly(A)<sup>+</sup> RNA/lane with equal amounts of *glh*-specific probes. RNAs from N2 and germline-defective *C. elegans* strains *glp-4(bn-2)* and *glp-1(q231)* (Beanan and Strome, 1992; Austin and Kimble, 1987) were isolated as described in (Roussell and Bennett, 1993). The temperature-sensitive *glp-4* and *glp-1* strains are essentially normal at 15°C but have very few germ cell nuclei at 25°C. *glh-3*- and *glh-4*-specific probes were made from their cDNAs and labeled with <sup>32</sup>P by random priming. The *glh-3* probe is a 459 bp subclone from nt 106 to nt 565; the *glh-4* probe is a 443 bp subclone from nucleotide 3139 through the poly(A) tail (23 As). These same probes were used for in situ hybridizations on splayed adult worms. For in situ hybridizations, single stranded probes were prepared by in vitro transcription using <sup>35</sup>S-labeled rCTP (New England Nuclear) of linearized templates with T7 or T3 polymerase. Fixations and hybridizations were as described by Gruidl et al. (1996). After washing, slides were briefly incubated with 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) in PBS. Images were collected by a SPOT-2 CCD camera from a Zeiss Axioplan microscope.

### Protein analyses: antibody production, western blot analyses and immunocytochemistry

Peptides from the C terminus of GLH-3 (21aa) and the N terminus of GLH-4 (18aa) (underlined in Fig. 1) were synthesized on a RaMPS multiple peptide synthesis system (DuPont) and conjugated to keyhole limpet hemocyanin according to the manufacturer's directions (Pierce Chemical). Antibodies were generated using standard protocols; rabbits were injected with 100 µg of conjugated peptide, followed by multiple boosts. The anti-GLH-4 peptide antibodies were effective for western blot analyses, but ineffective for immunocytochemistry. To generate more robust GLH-4 antibodies, 100 µg of a 722 amino acid (aa) fusion protein from aa 435 to the C terminus were also injected along with the conjugated N-terminal GLH-4 peptide. These anti-GLH-4 antibodies were affinity purified as described by Gruidl et al. (1996). Unexpectedly, the anti-GLH-3 antibodies cross-reacted to the similarly sized GLH-1 protein, as determined with in vitro-expressed GLH proteins (not shown). (While their C-terminal amino acids are different, both GLH-1 and GLH-3 end with EExW.) To remove this cross-reactivity, anti-GLH-3 antibodies (100 µl diluted 1:15 in PBST, PBS with the addition of 0.2% Tween-20, and 0.66% BSA) were pre-absorbed overnight at 4°C against approx. 25 µg of baculovirally produced GLH-1, GLH-2 and GLH-4 (PAS, unpublished results) spotted onto nitrocellulose. Western blot analyses were done essentially as described by Gruidl et al. (1996) with protein from N2 or *glh-3(um1)* worms. Secondary antibodies for western analyses were conjugated to horseradish peroxidase (Southern Biotech Assoc.). Protocols used for immunocytochemistry are essentially as described by Strome and Wood (1983) or by Seydoux and Dunn (1997) with primary antibodies used at 1:10–1:100 dilution. Secondary antibodies used for immunocytochemistry were conjugated to Cy-3, Cy-5, Alexa Fluor™ 488 and Alexa Fluor™ 546 fluorochromes (Jackson ImmunoResearch Laboratories and Molecular Probes) and were diluted 1:1000 for Cy and 1:2000 for Alexa Fluor™ secondaries. Dual or triple labelings were done as for single labeling except that primary antibodies were incubated together. After overnight incubation and washing, the secondary antibodies were added sequentially, each for 2 hours. Digital fluorescent images were captured as for the in situ hybridizations described above. Confocal images were collected with a BioRad MRC600 microscope and images were processed using Adobe Photoshop software.

### RNA interference studies

The *glh* gene-specific 'helicase' fragments were amplified separately from their cDNAs by PCR using 18 nt primers common to *glh-1-3*. These primers amplified DNA coding for aa 474–691 of GLH-3 and the corresponding regions in GLH-1 and GLH-2 (Fig. 1A; Roussell and Bennett, 1993; Gruidl et al., 1996). The *glh-4* helicase fragment was



**Fig. 1.** The predicted amino acid sequences of GLH-3 and GLH-4, compared with GLH-1 and GLH-2. (A) GLH-3 and (B) GLH-4, with CCHC zinc fingers (red) and DEAD-box motifs (green). Arrowheads indicate the position of introns. The synthetic peptides used in antibody production are underlined. In addition in B, the glycine-rich repeats are indicated by grey shading. Four repeated sub-motifs within the glycine-repeat region of GLH-4 are shown in green, pink, red and blue lettering. (C) A cartoon comparing the motifs of the four GLH proteins.

made using 18 nt primers amplifying DNA coding for aa 831-1079 of GLH-4 (Fig. 1B). The primers 5'ATGAGTAAAGGAGAAGAA3' and 5'AGAGCGGCCGCCACCGGT3' amplified green fluorescent protein DNA, a negative control, from the pJH4.52 vector (G. Seydoux, Johns Hopkins University). All primers used had T7 polymerase sites added to their 5' ends. dsRNA fragments were made by transcribing 5-8  $\mu$ l of the PCR mix with T7 polymerase (Ampliscribe<sup>TM</sup> from Epicentre). The dsRNA was not DNase-treated, as DNAsing dramatically decreased the *glh* RNAi effect (K. A. K., unpublished results). Worms used for injection were well-fed, staged young adults from a single N2 stock. Injection mixes consisted of equal concentrations of *glh* dsRNAs varying from 0.65  $\mu$ g/ $\mu$ l to 1.2  $\mu$ g/ $\mu$ l. Injected adults were purged of already-formed embryos from 18-24 hours to decrease the levels of GLH maternal proteins. Injected worms were put on bacterially seeded plates and transferred every 12 hours at either 20°C or 25°C. For the analyses in Table 1, worms were scored at 60 $\times$  magnification. They were considered sterile when they had a clear vulval area, indicating that no fertilized eggs were present in the uterus. Sterility was further verified on samples of worms by Nomarski optics (400 $\times$ ). Immunocytochemistry of RNAi-affected worms was as above. Germ cell nuclei were counted after staining splayed gonads with DAPI.

## RESULTS

### Two more proteins are members of the GLH family

*glh-3* and *glh-4* were found in searches of the *C. elegans* genomic sequence for genes that match *glh-1* and *glh-2* in the helicase region and that contain CCHC zinc fingers (GenBank

accession numbers: AF079509 for *glh-3*; AF079508 for *glh-4*). While *glh-3* and *glh-4* cDNA sequences match their genomic sequences, in the *glh-4* genomic DNA the initiating ATG is immediately followed by an intron of 48 nucleotides. In addition, a large approx. 1 kb intron is found within the *glh-4* 5'UTR. This organization differs from that predicted by the Genefinder program in ACeDB (<http://elegans.swmed.edu/genome.shtml#ACEDB>). All four *glhs* genes are located within 4.2 map units in the middle of Linkage Group I (LGI) (chromosome 1).

The GLH proteins share common features in addition to the helicase motifs (Fig. 1A,B). Fig. 1C compares the organization of the GLH proteins. In the N-termini of GLH-1, GLH-2 and GLH-4 there are multiple uncharged glycine-rich repeats (FGGGX<sub>n</sub>) (Gruidl et al., 1996; Fig. 1B); GLH-3 lacks glycine repeats (Fig. 1A). Similar FGG repeats, implicated as important for aggregation, are found in the plant lorricrins, in mammalian uterine trophinins and in nucleoporins (Steinert et al., 1991; Fukuda et al., 1995; Wu et al., 1995). By comparison, VASA contains five tandem charged RGG RNA binding motifs in its N terminus (Lasko and Ashburner, 1988). VASA binds RNA, albeit non-specifically (Liang et al., 1994). All four GLHs have multiple CCHC zinc fingers; VASA does not. GLH-3 has a pair of fingers; GLH-4 has five contiguous fingers. None of the fingers in GLH-3 and GLH-4 have matching amino acids (Fig. 1A,B); only in GLH-1 and GLH-2 are any of the fingers identical (Gruidl et al., 1996). The

predicted GLH-4 protein is the most divergent; GLH-4 retains complete conservation of only two helicase motifs, GG and DEAD (Fig. 1B). Therefore, while the GLHs share common features, each GLH is different.

### Another *glh-4*-like gene

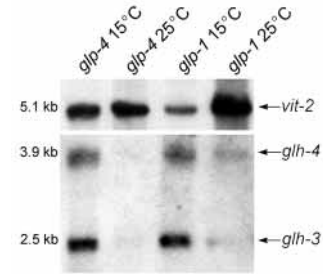
*glh-4b*, another *glh-4*-like gene, is also present in the *C. elegans* genome. *glh-4b* maps to the extreme right end of LGV. *glh-4* and *glh-4b* sequences are nearly identical (>95% alike). The differences between the two genes include several translational stops in *glh-4b*. A seventeen nucleotide deletion in *glh-4b* was confirmed by PCR on *C. elegans* genomic DNA with primers spanning the deletion (not shown). Of the six *glh-4*-like cDNAs available as ESTs, all have *glh-4*-specific nucleotides. Thus, *glh-4b* is likely a pseudogene.

### RNA studies indicate that *glh-3* and *glh-4* are germline specific

Northern blots of poly(A)<sup>+</sup> RNA probed with *glh-3*- and *glh-4*-specific fragments confirmed that the *glh-3* mRNA is 2.5–2.6 kb and that the *glh-4* mRNA is 3.9–4.0 kb (Fig. 2). Because gene-specific and full-length probes recognize single-sized messages, it seems unlikely that either gene is alternatively spliced. When the four *glhs* were compared in wild-type N2 poly(A)<sup>+</sup> RNA, *glh-1* mRNA was found to be >27-fold more abundant than *glh-4* mRNA, with *glh-2* levels 1.4-fold higher than those of *glh-4* levels and *glh-3* 1.3-fold higher than those of *glh-4* (not shown). When *glh-3* and *glh-4* were tested against the temperature-sensitive, germline-defective strains *glp-4(bn2)* and *glp-1(q231)*, both were dramatically reduced in the RNAs from worms grown at restrictive temperatures (Fig. 2), indicating these *glhs* are likely to be germline specific, as are *glh-1* and *glh-2* (Roussel and Bennett, 1993; not shown). This conclusion is supported for *glh-4* by microarray analysis (V. Reinke and S. Kim, Stanford University; S. Ward, University Arizona; personal communication). *glh-4* appears to be 'germline intrinsic' in these assays, both when using N2 RNA versus *glp-4* RNA that gave a ratio of 3:1 and *fem-1(lf)* (oocytes only) vs. *fem-3(gf)* (sperm only) RNAs in which *glh-4* had a ratio of 1:1.

In situ hybridization studies determined that *glh-3* and *glh-4* RNAs are found throughout the adult gonad and are germline specific to the levels we can detect (Fig. 3A,C,D). *glh-3* hybridization is consistently weaker in the distal mitotic region (Fig. 3A, arrow) and stronger in the proximal meiotic region. *glh-4* hybridization is weak, but uniform throughout the gonad (Fig. 3C,D). Consistent with germline specificity, there is no significant hybridization of either probe to body wall or somatic tissue, including the intestine (Fig. 3C, arrowhead). The hybridization to the male gonad is weak for both probes (not shown; Fig. 3F). In embryos both RNAs are uniformly present throughout the youngest embryos (Fig. 3A,E). *glh-3* RNA, like *glh-1* and *glh-2* RNAs (Gruidl et al., 1996), decreases to background levels after approximately the 24-cell stage (Fig. 3A). For *glh-4* RNA, hybridization is strongest immediately after fertilization and then decreases rapidly, even showing a considerable decrease in the 2-cell embryo (Fig. 3E, arrow). When Fig. 3D and 3E are compared, *glh-4* RNA appears more abundant in newly fertilized embryos than anywhere else in the gonad, including in the mature oocyte immediately preceding the spermatheca, the point of

**Fig. 2.** Northern blot analysis indicates *glh-3* and *glh-4* RNA levels are enriched in the germline. Two temperature-sensitive germline-defective strains were tested: *glp-4(bn2)*, lanes 1 and 2, and *glp-1(q231)*, lanes 3 and 4. The *vit-2* (*vitellogenin*) RNA is a somatic message, indicating that as much or more RNA was loaded in the 25°C lanes. Exposures were 1 week for the *glh* probes as compared to 1.5 hours for *vit-2*. The sizes for the *glh-3* and *glh-4* mRNAs are the same when wild-type poly(A)<sup>+</sup> RNA was used (not shown).



fertilization (Fig. 3D, arrow). The signal in newly fertilized eggs could represent zygotic *glh-4* transcription occurring shortly after fertilization, which would be unprecedented in *C. elegans*. Alternatively, the higher level seen in the one cell embryo may be an artifact resulting from variability of in situ hybridization signals.

### Western analyses determine the specificity of anti-GLH-3 and anti-GLH-4 antibodies

Polyclonal antibodies generated against GLH-3 and GLH-4 are specific. Anti-GLH-3 antibodies are negative against protein homogenates from *glh-3(um1)* (Fig. 4, lane 1), a strain presumed to be null because 1.8 kb out of 2.4 kb of the *glh-3* coding sequence is deleted, leaving multiple in-frame stop codons. The anti-GLH-3 antibodies are reactive to a 78 kDa protein in wild-type homogenates, the predicted size for GLH-3 (Fig. 4, lane 2). Anti-GLH-4 antibodies are specific by western blot analyses, recognizing a 120 kDa protein, the predicted size for GLH-4 (Fig. 4, lane 3).

### Immunocytochemistry reveals that all four GLHs are P granule components

When anti-GLH-3 and anti-GLH-4 antibodies were used for immunocytochemistry on *C. elegans* adults and embryos, both were found to be constitutive P granule components. Antibodies against each of these GLH proteins recognize P granules throughout the life of the worm: in the germline precursor cells of the embryo (Fig. 5A,D,E), in larvae (not shown), and surrounding the proliferating germ cells in the adult hermaphrodite and male gonads (Fig. 5F,H; not shown). Combinations of GLH antibodies were used to establish that the GLH proteins localize together in the embryonic P granules (Fig. 5E) as well as in the adult gonad (Fig. 5H).

### RNA interference suggests loss of GLH-1 and GLH-4 results in sterility

Individual and combinatorial loss of GLH function was assessed by RNA interference (RNAi). Injections of gene-specific dsRNA into a hermaphrodite produces a phenocopy in the F<sub>1</sub> progeny that closely matches that of the partial or complete loss-of-function phenotype (Fire et al., 1998; Montgomery et al., 1998). Injections of multiple dsRNAs have been reported to eliminate protein function for the corresponding genes (Subramaniam and Seydoux, 1999).

The most dramatic *glh(RNAi)* phenocopy results from injections with multiple dsRNA mixtures that include *glh-1*

**Table 1. RNAi with *glh-1* and *glh-4* ds RNAs results in essentially complete sterility at 20°C, while *glh-1(RNAi)* is most potent at 25°C**

Gene	20°C		25°C	
	No. of worms injected	Sterile worms in F <sub>1</sub> generation	No. of worms injected	Sterile worms in F <sub>1</sub> generation
<i>glh-1</i>	7	0% (0/360)	7	44% (1008/2306)
<i>glh-2</i>	19	0% (0/3014)	16	21% (133/623)
<i>glh-3</i>	14	0% (0/2371)	43	0% (0/4685)
<i>glh-4</i>	17	0% (0/798)	20	0% (0/876)
<i>glh-1/2</i>	16	4.4% (42/944)	20	23% (253/1084)
<i>glh-1/3</i>	10	0% (3/1340)	14	32% (285/900)
<i>glh-1/4</i>	60	97% (5368/5519)	69	33% (914/2774)
<i>glh-2/3</i>	20	0% (0/3313)	32	13% (172/1301)
<i>glh-2/4</i>	33	0% (0/1056)	19	15% (136/888)
<i>glh-3/4</i>	12	0% (0/1870)	10	0% (0/1136)
<i>glh-1/2/3</i>	44	4.9% (109/2234)	42	36% (1060/2945)
<i>glh-1/2/4</i>	7	93% (393/424)	36	38% (599/1584)
<i>glh-1/3/4</i>	10	92% (1376/1494)	10	31% (111/355)
<i>glh-2/3/4</i>	17	0% (0/approx. 1300)	39	0% (0/2015)
<i>glh-1/2/3/4</i>	74	93% (4078/4380)	53	14% (570/4027)

and *glh-4*. Loss of GLH-1 and GLH-4 causes 97% sterility in F<sub>1</sub> adults grown at 20°C (Table 1; Fig. 6A,C,E). Individual *glh(RNAi)* results in a temperature-sensitive effect for *glh-1* and *glh-2* and no discernible effect for *glh-3* or *glh-4* (Table 1). At 25°C, F<sub>1</sub> sterility is seen for individual *glh-1(RNAi)* and *glh-2(RNAi)*, rising to 44% and to 21% respectively from 0% for both at 20°C (Table 1). Although the helicase regions of the *glhs* are alike, we found that each *glh(RNAi)* is specific. Even with *glh-1* or *glh-2* that are 91% alike in the helicase region, only the cognate protein is lost, with the other GLH proteins still present (Fig. 6B,D; not shown). *glh(RNAi)* worms have fewer germ cell nuclei and show a delay in spermatogenesis.

All *glh(RNAi)* sterile worms, resulting from either single or combinatorial *glh(RNAi)*, appear to have the same defects (Fig. 6A,C,E); it is only the percentage of worms affected that differs (Table 1). *glh(RNAi)* adults have abnormal, shrunken gonads with germ cell nuclei that do not surround a central rachis as do those in wild type (compare size and morphology in Figs 6A,C and 5F,G). This is even the case when <10% of the worms are affected; thus, while the penetrance of RNAi in some combinations may be incomplete, the expressivity of the phenocopy has little variability. While these sterile hermaphrodite and male germlines undergo mitosis, they have fewer germ cell nuclei. Numbers of germ cell nuclei were compared between equivalent stages of *glh1/4(RNAi)* and N2 worms. L3 (third larval stage) *glh-1/4(RNAi)* worms averaged 45 germ cell nuclei/ gonad arm while L3 wild-type worms averaged 122 germ cell nuclei/gonad arm. The L4 (fourth larval stage) *glh-1/4(RNAi)* progeny averaged 82 germ cell nuclei/gonad arm while L4 wild-type worms averaged 223 germ cell nuclei/gonad arm. *glh1/4(RNAi)* young adults averaged 150 germ cell nuclei/gonad arm, compared to the corresponding wild-type N2 controls that averaged 576 germ cell nuclei/gonad arm. Overall, there is not a wide range of phenotypes in *glh(RNAi)* worms: 65% of *glh1/4(RNAi)* adults have 100-200 germ cell nuclei/ gonad arm, with 92% having 75-225 germ cells. The numbers of germ cell nuclei indicated above do not include sperm (or oocytes and embryos for N2).

Sperm counts for *glh-1/4(RNAi)* hermaphrodites averaged 86 sperm/gonad arm; in comparison, wild-type hermaphrodites normally produce about 160 sperm/gonad arm (Schedl, 1997). Although spermatogenesis is normally completed during the L4 stage in N2 worms, spermatogenesis is delayed in *glh-1/4(RNAi)* animals. Some young *glh(RNAi)* F<sub>1</sub> hermaphrodites, identified by fully formed vulvas, have spermatocytes along with sperm; others have not gone beyond the pachytene stage.

### ***glh(RNAi)* hermaphrodites lack oocytes**

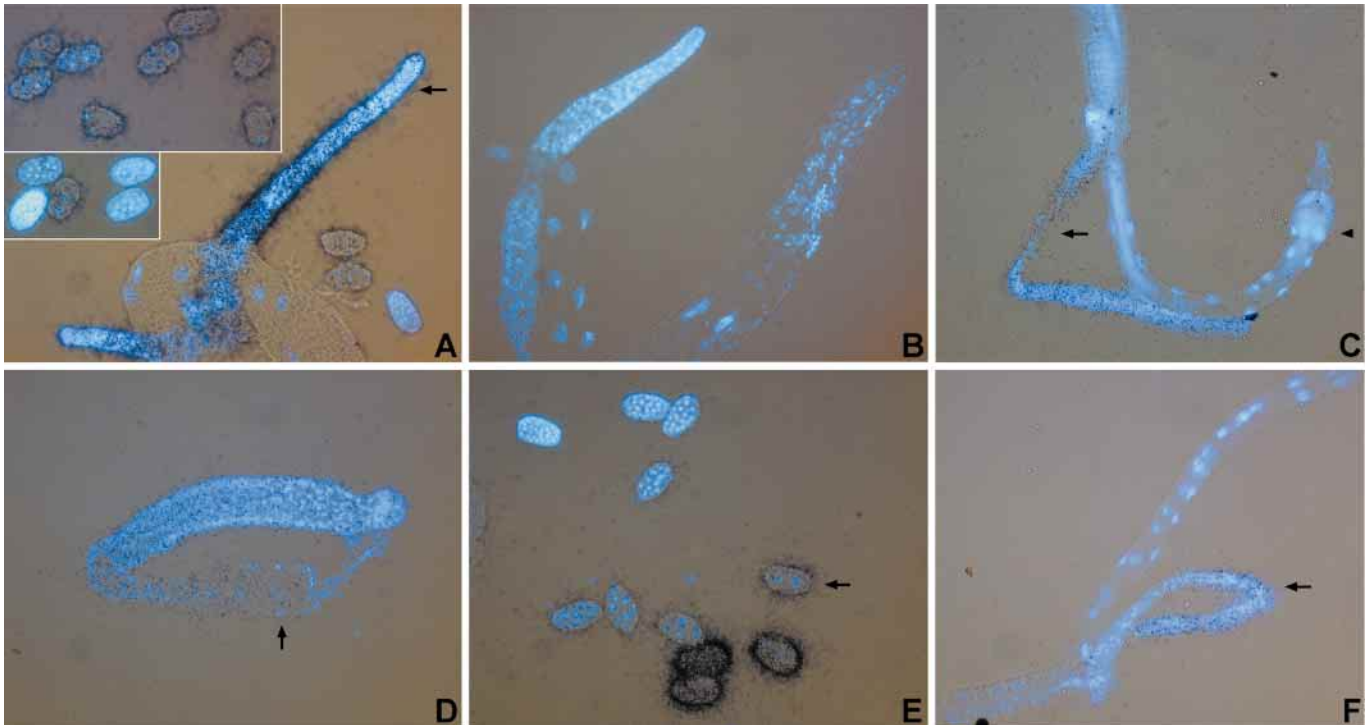
Notably, *glh(RNAi)* hermaphrodites fail to produce oocytes. These worms undergo the ‘switch’ from spermatogenesis to oogenesis; however, the germ cell nuclei appear to stop at pachytene, failing to undergo diakinesis. We verified that germ cells have entered meiosis using antibodies against GLD-1, a meiotic protein strongly expressed through the pachytene stage (Jones et al., 1996). GLD-1 is present in the proximal region of the *glh(RNAi)* gonad, as it is in wild type (not shown). In rare instances a few oocytes are seen in older *glh(RNAi)* F<sub>1</sub> adults, either indicating that small percentages of *glh(RNAi)* worms can complete oogenesis or that the RNAi effect has weakened over time. These oocytes are smaller than normal and appear unhealthy. Because no viable embryos are produced, the oocytes, the sperm, or both are defective.

### ***glh(RNAi)* males produce defective sperm**

To examine gametogenesis in *glh(RNAi)* worms, we first tested if *glh(RNAi)* sperm were capable of fertilizing normal oocytes. *glh* dsRNAs were injected into *him-8(e1489)* (high incidence of males) hermaphrodites to produce *glh(RNAi)* males. In *C. elegans* matings, wild-type male sperm are superior, out-competing those of the hermaphrodite, resulting in entirely out-crossed progeny (LaMunyon and Ward, 1995). In this experiment *him-8; glh-1/2/3/4(RNAi)* F<sub>1</sub> males and *him-8; glh-1/4(RNAi)* F<sub>1</sub> males were mated to *dpy-5(e61) unc-87(e1216)* hermaphrodites. Even though the males appeared to mate normally, at most 6% of the F<sub>2</sub> generation were cross progeny. In contrast, plates of control *him-8 × dpy5 unc-87* matings yielded essentially all cross progeny. To give the *glh1/4(RNAi)* sperm an opportunity to fertilize healthy oocytes without any wild-type sperm present, *glh1/4(RNAi)* males were crossed with *fog-2(q71)* hermaphrodites. *fog-2(q71)* hermaphrodites are defective in spermatogenesis, but produce normal oocytes, a feminization of gonad (*fog*) phenotype (Schedl and Kimble, 1988). These crosses resulted in reduced brood sizes, averaging 5% of the progeny produced by the controls; these numbers are consistent with those seen with the *dpy unc* crosses. While most *fog-2 × him-8; glh(RNAi)* matings resulted in no progeny, a few matings were successful and one produced 115 offspring; by comparison, the control matings of *fog-2* females with *him-8* males from non-injected mothers averaged 410 progeny. These results indicate the vast majority of the *glh(RNAi)* sperm are non-functional.

### **Feminized *glh(RNAi)* hermaphrodites lack oocytes**

To test whether *glh(RNAi)* worms would be capable of making oocytes if spermatogenesis were bypassed, *fog-2(q71); glh-1/4(RNAi)* hermaphrodites were examined. *fog-2* male siblings are normal and were mated with *fog-2* hermaphrodites that had been injected with *glh* dsRNAs, producing *fog-2; glh(RNAi)* F<sub>1</sub> progeny. We found that *fog-2; glh1/4(RNAi)* F<sub>1</sub> hermaphrodites



**Fig. 3.** In situ hybridizations indicate *glh-3* and *glh-4* accumulation is specific to the adult gonad and is not localized in the early embryo. (A) *glh-3* antisense RNA in the gonad of an adult hermaphrodite; arrow indicates the distal region of the gonad; insets: additional embryos from the same slide. (B) *glh-3* sense RNA in an adult hermaphrodite; DAPI (blue) stains nuclei. (C) *glh-4* antisense RNA in splayed young adult hermaphrodite; arrow indicates gonad; arrowhead indicates intestine, including pharynx. *vit-2* probes hybridize exclusively to the intestine (not shown). (D) *glh-4* antisense RNA in an isolated gonad showing mature oocyte (arrow). (E) *glh-4* antisense RNA in embryos; same slide as D. Arrow: 2-cell embryo. (F) *glh-4* antisense RNA in a splayed male; arrow indicates testis. The *glh-3* hybridizations were 8 day exposures; *glh-4* 18-20 days, except for C, which was 8 days.  $2 \times 10^6$  cpm of probe were used for each slide. Magnification: 400 $\times$ .

do not produce oocytes. So even in a feminized background, *glh(RNAi)* worms do not initiate oogenesis. Taken together, the result of the loss of GLH function appears to be a successive slowing down of germ cell proliferation, resulting in a failure to complete oogenesis, the last stage of gametogenesis.

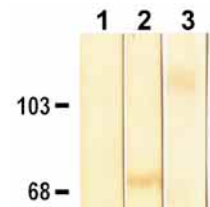
#### ***glh(RNAi)* reveals the GLHs independently localize to P granules**

Surprisingly, the GLHs appear to assemble autonomously in P granules. *glh-1/4(RNAi)* germlines still have GLH-2 and GLH-3 organized in P granules (Fig. 6D; not shown), implying these two GLHs are independent of GLH-1 and GLH-4. While the distribution of GLH-2 is like that of wild type (more proximal than distal, Fig. 6D), the perinuclear GLH-2 staining is less distinct in the *glh-1/4(RNAi)* germline than in wild type (Fig. 6D, compare inset 1 and inset 2), probably due to the disorder of the *glh-1/4(RNAi)* germline. All other *glh(RNAi)* combinations consistently indicate independence as well (not shown).

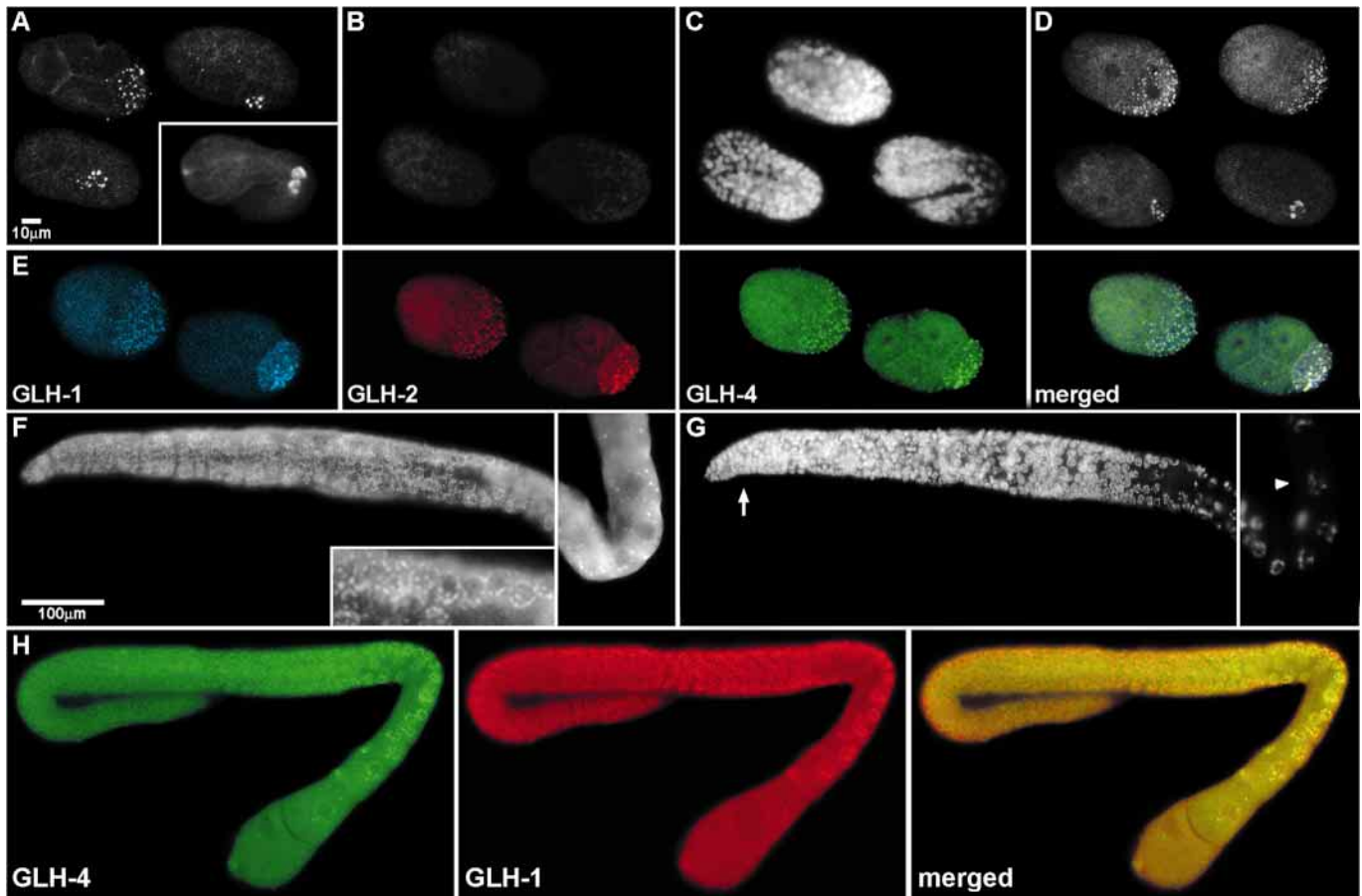
#### **GLH maternal protein precludes a *glh(RNAi)* embryonic phenocopy**

Based on the embryonic pattern phenotype of *vasa* mutants, it might have been expected that *glh(RNAi)* would result in embryonic defects; it does not. All the embryos are viable after *glh(RNAi)*; the F<sub>1</sub> worms counted in Table 1 reflect the entire broods produced by the injected worms after the 18-hour purge.

**Fig. 4.** Western blot analyses demonstrate the specificity of the anti-GLH-3 and anti-GLH-4 antibodies. *C. elegans* protein homogenates from staged adult worm populations and GIBCO-BRL high molecular mass pre-stained protein markers were run on 8% SDS/PAGE gels. Anti-GLH-3 antibodies are unreactive to protein isolated from the *glh-3(um1)* strain, lane 1, a predicted protein null (K. A. K. and K. L. B. unpublished results). The *glh-3(um1)* homogenates were intact as assessed with anti-GLH-4, anti-actin and anti-tubulin antibodies (not shown). Hyperimmune anti-GLH-3 serum reacts with a 78 kDa protein in wild-type homogenates, lane 2. Anti-GLH-4 peptide antibodies recognize a 120 kDa protein in wild-type homogenates, lane 3. Polyclonal sera against the GLH-4 fusion protein are also specific by western analysis (not shown).



For most maternally expressed genes in *C. elegans*, embryonic RNAi phenocopies are observed when the offspring produced in the first few hours after injection are discounted (purging). However, GLH maternal proteins are ubiquitous and rather abundant throughout the L4 larval and adult gonad (not shown; Fig. 5F,H). After an 18-hour purge, F<sub>1</sub> embryos of *glh-1/4(RNAi)* injected worms were tested against anti-GLH and anti-PGL-1 antibodies. The GLH and PGL proteins were still associated with embryonic P granules (Fig. 7A-C; not shown) at levels essentially similar to those found in wild-type embryos.



**Fig. 5.** GLH-3 and GLH-4 are P granule components. (A) GLH-3 localizes to P granules in embryos, including a four-cell embryo, a 24-cell embryo and a post-gastrulation embryo. (Inset) Two-fold embryo in which P<sub>4</sub> has divided into Z<sub>2</sub> and Z<sub>3</sub> daughters. (B) GLH-3 protein is absent in *glh-3(um1)*. (C) DAPI staining of B. (D) GLH-4 is in P granules in embryos; it is also found in somatic blastomeres, despite affinity purification. (E) GLH-1 (mouse antibody), GLH-2 (chicken antibody) and GLH-4 all localize to P granules in *glh-3(um1)* embryos. White indicates the GLHs localize together in the merged image. A-E are confocal images, except inset. (F) GLH-3 in an N2 hermaphrodite gonad surrounds each germ cell nucleus. (Inset) Proximal gonad, 3× magnification of F. (G) DAPI staining of gonad in F. Germ cell nuclei (arrow), and oocytes (arrowhead) are indicated. (H) GLH-4 and GLH-1 surround the germ cell nuclei in P granules in the hermaphrodite gonad; co-localization is indicated in yellow.

Therefore, while *glh(RNAi)* likely mimics the null phenotype in the adult worm, the perdurance of maternal GLH protein after RNAi prevents us from determining if the GLHs function in the embryo. By contrast, after RNAi the germline of the F<sub>1</sub> adult lacks zygotic GLH (Fig. 6B; not shown). The lack of zygotic GLH protein in the sterile *glh(RNAi)* adult is further indicated by examining PGL-1 localization. PGL-1 is dependent upon GLH-1 to localize to P granules (Kawasaki et al., 1998). In *glh-1/4(RNAi)* germlines PGL-1 is still present, but is no longer associated with P granules; PGL-1 is now diffuse in the cytoplasm (Fig. 7D). Thus, while the maternal contribution of GLH proteins is unaffected by *glh(RNAi)*, eliminating zygotic GLH protein appears sufficient to produce complete sterility.

#### Multiple dsRNAs suppress one another at high temperatures

Unexpectedly, when multiple dsRNAs are injected and the worms are grown at higher temperatures, the percentage of sterile *glh(RNAi)* F<sub>1</sub> worms is reduced (Table 1). For example, at 25°C, *glh-1/2(RNAi)*, *glh-1/3(RNAi)* and *glh-1/4(RNAi)* each causes less F<sub>1</sub> sterility than *glh-1(RNAi)* alone (Table 1); *glh-*

*2/3(RNAi)* and *glh-2/4(RNAi)* also results in less F<sub>1</sub> sterility than *glh-2(RNAi)* alone (Table 1). The significance of the reduction in sterility seen in multiple *glh(RNAi)* at 25°C is not likely to be a *glh*-specific effect, in light of the additional RNA tested. When the non-relevant green fluorescent protein (*gfp*) dsRNA is added to *glh-1* dsRNA, we also see a decrease in the percentages of F<sub>1</sub> sterility. While *glh-1(RNAi)* results in approx. 40% F<sub>1</sub> sterility, *gfp/glh-1(RNAi)* sterility is reduced to 6% in F<sub>1</sub> progeny at 25°C. This reduction-in-sterility effect is temperature dependent. At 20°C, the percentage of *gfp/glh-1/4(RNAi)* F<sub>1</sub> sterile progeny remains >95%. Whether this high temperature RNAi phenomenon is unique to the *glhs* remains to be tested. Others have observed that certain RNAs in pools cause a 'dampening' or suppressive effect upon RNAi, although temperature dependence was not examined in those studies (C. Echeverri, EMBL Heidelberg, personal communication).

#### DISCUSSION

All four GLHs are constitutive components of the nematode P

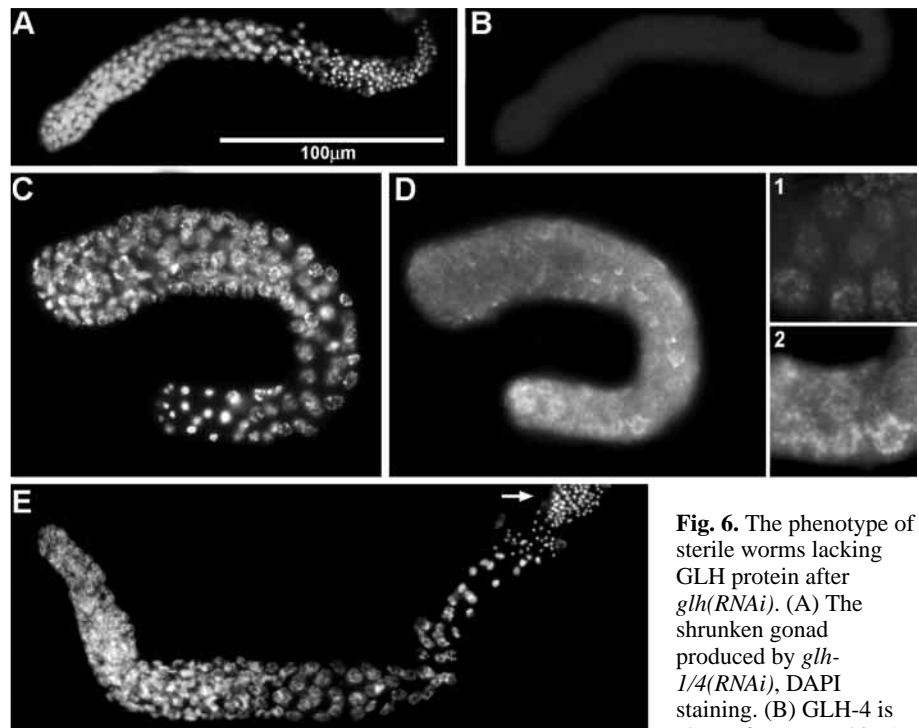
granules. Genetic screens in *C. elegans* for maternal effect steriles did not uncover *glh* mutations (Capowski et al., 1991), probably because multiple *glhs* have overlapping functions (this work). *vasa* is single copy in *Drosophila*, but may share partially redundant functions with the *spindle* (*spn*) genes (Styhler et al., 1998), including *spnE* (*homeless*), a germline-specific, putative DE-H-box RNA helicase (Gillespie and Berg, 1995). While no more than one *vasa* homologue has been reported in any vertebrate, there certainly could be multiple, undiscovered vertebrate *vasa*-like genes.

It is unlikely there are additional, unknown *glh* genes in *C. elegans* because the genomic sequence is essentially complete (The *C. elegans* Sequencing Consortium, 1998). The only other *glh*-like gene, *glh-4b*, has multiple in-frame stops and is most probably a pseudogene. This work has not ruled out that other RNA helicases, ones lacking zinc fingers and glycine repeats, may also be germline-specific components of the P granules.

Combinatorial RNAi indicates GLH-1 and GLH-4 can compensate for one another in germline development; GLH-2 or GLH-3 may contribute subtly or not at all to fertility. Multiple germline helicases with overlapping functions would likely provide a selective reproductive advantage to nematodes, protecting them against sterility. The *C. elegans* adult contains almost twice as many germline cells as somatic cells, and the parasitic nematode of swine *Ascaris suum* produces an estimated 250,000 eggs/day. While we have not found *glh-3* and *glh-4*-like genes in *Ascaris*, *glh-1* and *glh-2* homologues have been detected by northern hybridization, with both transcripts present in *Ascaris* ovarian poly(A)<sup>+</sup> RNA (Roussel and Bennett, 1993; K. L. B., unpublished results).

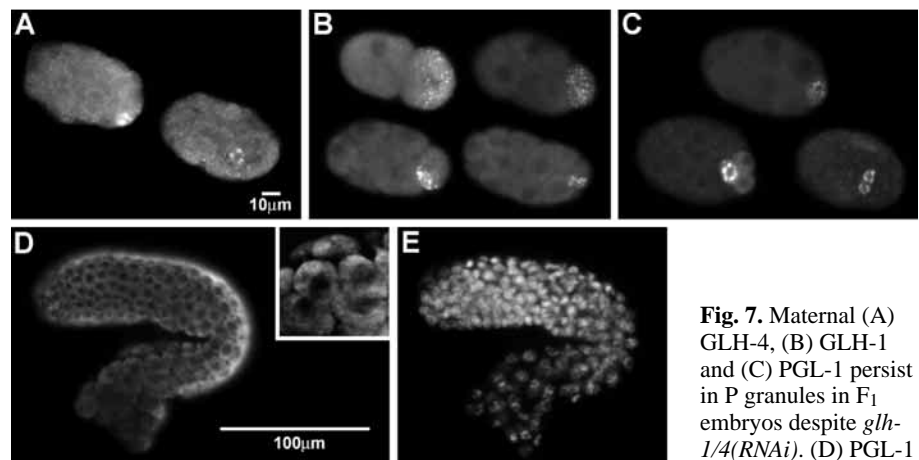
Can we be sure that RNAi is an accurate reflection of the loss of GLH function? The two loss-of-function *glh* mutant strains thus far identified both show similar phenotypes when compared to their RNAi results. The *glh-3* (*um1*) strain, like *glh-3*(RNAi) (Table 1), has a subtle or no mutant phenotype (K. A. K. et al., unpublished results), while in *glh-1*(*bn103*) approx. 40%-50% of the F<sub>1</sub> homozygous progeny that are produced from a heterozygous mother are sterile at 26°C (Kawasaki et al., 1998), mirroring the *glh-1*(RNAi) phenocopy at 25°C (Table 1). Those *glh-1*(RNAi) worms with a clear vulval region at 25°C produce 72% fewer germ cell nuclei than are produced by wild

type, while *glh-1*(*bn103*) sterile worms grown at 25°C have 71% fewer germ cell nuclei. These sterile *glh-1*(*bn103*) worms lack oocytes as do *glh-1*(RNAi) sterile worms grown at 25°C. Thus while we cannot guarantee that RNAi results in complete loss of GLH function, we detect no protein for GLH-1 or GLH-4 after *glh-1/4*(RNAi), and the phenocopy seen with RNAi accurately reflects partial, if not complete, loss of zygotic GLH function. Analyses of the *glh* mutant strains will determine if their phenotypes are more severe than *glh*(RNAi).



**Fig. 6.** The phenotype of sterile worms lacking GLH protein after *glh*(RNAi). (A) The shrunk gonad produced by *glh-1/4*(RNAi), DAPI staining. (B) GLH-4 is absent from gonad in A.

(C) DAPI staining of another *glh-1/4*(RNAi) gonad. (D) GLH-2 is present in P granules in the *glh-1/4*(RNAi) gonad in C. (Inset 1) GLH-2 in wild-type proximal gonad; (inset 2) GLH-2 in *glh-1/4*(RNAi) proximal gonad, 2× magnification of D. (E) *glh-1/2/3/4*(RNAi) results in the same phenocopy as *glh-1/4*(RNAi) in A. Germ cell nuclei and sperm, (arrow) are seen, but no oocytes (compare with wild type in 4G).



**Fig. 7.** Maternal (A) GLH-4, (B) GLH-1 and (C) PGL-1 persist in P granules in F<sub>1</sub> embryos despite *glh-1/4*(RNAi). (D) PGL-1 is diffuse in the

cytoplasm, no longer P granule associated, in the *glh-1/4*(RNAi) gonad. (Inset) *glh-1/4*(RNAi) proximal gonad, 2× magnification of D. (E) DAPI staining of gonad in D.



*glh-1/4(RNAi)* produces global changes in germline development. The germlines of hermaphrodites and males are under-proliferated. *glh-1/4(RNAi)* also causes defects in spermatogenesis, as does the knockout of the mouse VASA homologue (T. Noce, Mitsubishi Kasei Institute, Japan, personal communication). Most of the sperm produced by *glh-1/4(RNAi)* males are unable to fertilize healthy oocytes when mated to either *dpy-5 unc-87* or *fog-2* hermaphrodites. The most striking *glh-1/4(RNAi)* defect is the lack of oocytes. Even in a feminized background, where spermatogenesis is eliminated, *glh-1/4(RNAi)* worms do not produce oocytes. Our results imply that the *glhs* are necessary for the completion of gametogenesis. Therefore while the terminal phenocopy from loss of GLH-1 and GLH-4 is a halt in oogenesis, the defects in the germline begin much earlier in development and are cumulative.

How does the sterile defect produced by *glh-1/4(RNAi)* compare with that described for *pgl-1(bn101)*, a mutant in another constitutive P granule component? *pgl-1(bn101)* is a temperature-sensitive, maternal-effect sterile strain, as is *glh-1(bn103)* (Kawasaki et al., 1998). At 26°C, there are two classes of under-proliferated *pgl-1* germlines. 40% of *pgl-1* F<sub>1</sub> animals produce oocytes at restrictive temperatures; the rest do not. At 20°C, >95% of *glh-1/4(RNAi)* worms do not produce oocytes. Thus losing GLH-1 and GLH-4 at permissive temperatures results in a more severe phenotype than losing PGL-1 at restrictive temperatures; however the phenotype of the loss-of-function of either GLH or PGL is qualitatively similar in terms of the germline defects and the appearance of the gonads, suggesting that PGL and the GLHs function together in P granules.

Why does losing GLH-1 and GLH-4 by RNAi at 25°C result in fewer sterile worms than at 20°C? It is possible that the suggested dampening effect of multiple RNAs at high temperatures is partially responsible for the differences seen. However, we propose a lack of GLH-4 function at higher temperatures is more important than dampening and that the *glh-1/4(RNAi)* sterility at 25°C is largely or solely due to the loss of GLH-1 function in the F<sub>1</sub> generation. Therefore, we argue that the *glh-1/4(RNAi)* sterility of 33% is comparable to the 44% sterility seen with *glh-1(RNAi)* at 25°C (Table 1) and is not comparable to the 97% sterility that results from the elimination of these two putative helicases at permissive temperatures. We hypothesize that GLH-4 is involved in a heat-sensitive process and is not able to contribute to GLH function at 25-26°C (Table 1). This hypothesis is based on our RNAi results and preliminary findings with the *glh-1* loss-of-function strain, *glh-1(bn103)*. *glh-1(bn103)* is 100% sterile at 26°C, and approx. 10-15% sterile at 20°C in the F<sub>2</sub> generation (Kawasaki et al., 1998). Consistent with RNAi results, the *glh-1(lf)* worms are temperature sensitive and are 40% sterile in the F<sub>1</sub> generation. Therefore, we suggest that GLH-4 allows for near-normal germline development in *glh-1(bn103)* at permissive, but not at restrictive temperatures. Alternatively, the process of germline development could involve GLH-1 being part of a 'cold-sensitive' pathway and when GLH-1 is lost, GLH-4 function becomes necessary at 20°C. This interpretation is also consistent with the RNAi and *glh-1(lf)* mutant results.

RNAi results indicate that the GLH proteins independently associate in P granules. Immunocytochemistry studies demonstrate that in the severely-disrupted *glh-1/4(RNAi)*

germlines, GLH-1 and GLH-4 are missing, but GLH-2 and GLH-3 are still present in what appear to be defective P granules (Fig. 6D; not shown). In addition, the two *glh* mutant strains, in which the cognate GLH is eliminated, demonstrate autonomous assembly of GLHs in P granules. In the *glh-1(bn103)* and *glh-3(um1)* strains, all other GLHs localize to P granules (K. A. K. et al., unpublished results; Fig. 5E). Based on the information presented here, we predict GLH-1 and GLH-4 function in parallel pathways. The GLHs may self-assemble or assemble upon a common, as yet unidentified, germ granule scaffold protein. Alternatively there could be several different populations of P granules. While the GLHs may be independent of one another, genetic studies indicate GLH-1 interacts with PGL-1 (Kawasaki et al., 1998). This interaction is also seen by RNAi; when *glh-1* is missing, PGL-1 is no longer P-granule associated (Fig. 7D).

The interactions of GLH-1 and GLH-4 as revealed by RNAi are expected to be confirmed with the isolation of a *glh-4* null strain and the generation of the double mutant strain. Genetics may also reveal unexpected interactions between the GLHs, perhaps indicating roles for GLH-2 and GLH-3 not seen by RNAi.

How do the GLHs function? Because the numbers, arrangements and types of CCHC zinc fingers vary in the GLHs, each may bind a different set of RNAs, with the GLHs regulating translation of these specific RNAs at designated times during germline development. It is not yet known whether the GLHs bind specific RNAs, including *nos-2* (Subramaniam and Seydoux, 1999). While the biochemical mechanisms used by these putative RNA helicases remain to be determined, the results presented here allow us to conclude that the four GLHs are independent P granule components and that GLH-1 and GLH-4 are necessary in the larval through the adult stages for normal, robust germline proliferation, for oogenesis and for fertility.

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