**cgh-1**, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in *C. elegans*

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SUMMARY

A high frequency of apoptosis is a conserved hallmark of oocyte development. In *C. elegans*, about half of all developing oocytes are normally killed by a physiological germline-specific apoptosis pathway, apparently so that they donate cytoplasm to the survivors. We have investigated the functions of CGH-1, the *C. elegans* ortholog of the predicted RNA helicase ste13/ME31B/RCK/p54, which is germline-associated in metazoans and required for sexual reproduction in yeast. We show that CGH-1 is expressed specifically in the germline and early embryo, and is localized to P granules and other possible mRNA-protein particles. *cgh-1* is required for oocyte and sperm function. It is also needed to prevent the physiological germline apoptosis mechanism killing essentially all developing oocytes, making lack of *cgh-1* function the first stimulus identified that can trigger this mechanism. We conclude that *cgh-1* and its orthologs may perform conserved functions during gametogenesis, that in *C. elegans* certain aspects of oocyte development are monitored by the physiological germline apoptosis pathway, and that similar surveillance mechanisms may contribute to germline apoptosis in other species.

Key words: Germline, Apoptosis, RNA helicase, Oocyte, *Caenorhabditis elegans*, P granules, Germ plasm

INTRODUCTION

Germ cells are both pluripotent and immortal, because they give rise to all of the tissues in an animal, but to do so they must first form gametes. Gametogenesis involves exit from the mitotic cell cycle, entry into meiosis, and completion of complex differentiation programs (Saffman and Lasko, 1999). In diverse organisms, these programs direct a significant proportion of oocyte lineage cells to undergo apoptosis. In humans and other mammals, most developing oocytes die by apoptosis before birth, primarily during the late pachytene stage of meiotic prophase (Baker, 1963; Bakken and McClanahan, 1978; Borum, 1961; Morita and Tilly, 1999). This process is influenced by input from extrinsic survival factors, but its function and relationship to intrinsic oocyte characteristics remain unclear. In *Drosophila* and *Hydra*, nurse cells that are derived from the same precursor as oocytes die in order to supply the oocytes with cytoplasmic components (Foley and Cooley, 1998; McCall and Steller, 1998; Miller et al., 2000). In the nematode *C. elegans*, up to half of all developing oocytes normally undergo apoptosis, apparently so that their cytoplasm can be utilized by their sisters (Gumienny et al., 1999). As in mammals, in *C. elegans* physiological germ cell death occurs late during the pachytene stage of meiosis. This process is regulated by a mechanism that is genetically distinct from those that control other apoptotic events in *C. elegans*, including the germline genomic stability checkpoint (Gartner et al., 2000; Gumienny et al., 1999).

In many animals, germline specification and development involves characteristic RNA-protein particles. Throughout the *C. elegans* life cycle, P granules that contain proteins and mRNA are present specifically in germline cells (Pitt et al., 2000; Schisa et al., 2001; Seydoux and Fire, 1994; Strome and Wood, 1982; Subramaniam and Seydoux, 1999). In *Drosophila*, polar granules that are similar to P granules specify germ cell formation, and are segregated to the embryonic germline (Rongo et al., 1997; Saffman and Lasko, 1999; Wylie, 2000). Mammalian germ cells contain somewhat similar structures (Saffman and Lasko, 1999), and in mice an ortholog of the *Drosophila* polar granule protein VASA is required for spermatogenesis (Tanaka et al., 2000), suggesting that some germline RNA storage or metabolism mechanisms are conserved in mammals.

Among the RNA-associated proteins present in the metazoan germline is RCK/p54, a DEAD-box type predicted RNA helicase (Fig. 1; de la Cruz et al., 1999). This family
includes proteins that are involved in mRNA processing and metabolism, as well as VASA and other germline proteins, and many of its members can unwind double stranded (ds)RNA in an ATP-dependent manner. RCK/p54 is represented by a highly conserved ortholog in each eukaryote (Fig. 1B). In Drosophila and Xenopus, the RCK/p54 ortholog is expressed primarily in the germline and early embryo, and in Xenopus oocytes it is abundant in cytoplasmic complexes that contain stored mRNA (de Valoir et al., 1991; Ladomery et al., 1997). Remarkably, in Saccharomyces pombe and S. cerevisiae the RCK/p54 ortholog (ste13, dhh1; Fig. 1B) is essential for sexual reproduction but not growth (Maekawa et al., 1994; Moriya and Isono, 1999). S. pombe ste13 mutants die after failing to sporulate, and cannot initiate meiosis as diploids. The ste13 phenotype can be suppressed by the Drosophila RCK/p54 ortholog (ME31B) but not by vasa, suggesting that RCK/p54 proteins act through conserved mechanisms and may have conserved functions.

To investigate whether ste13/ME31B/RCK/p54 proteins are important for gamete formation in metazoans, we have studied the C. elegans ortholog, which we refer to as cgH-1 (conserved germline RNA helicase; Fig. 1). We show that CGH-1 is expressed specifically in the germline and early embryo, and is associated with germline cytoplasmic RNA-protein particles. Inhibition of cgH-1 expression by RNA interference (RNAi) results in production of non-functional sperm, and in apoptotic death of essentially all developing oocytes through the physiological germline pathway. When apoptosis is inhibited, cgH-1(RNAi) oocytes proliferate normally and continue to develop, but are incapable of being fertilized. Our findings indicate that cgH-1 is essential for RNA-associated gametogenesis functions that may be conserved between metazoans and fungi. They also reveal an association between the survival of immature oocytes and their eventual functional capability, and indicate that the physiological germline apoptosis mechanism can be triggered by certain abnormalities in oocyte development.

MATERIALS AND METHODS

Strains

Maintenance and genetic manipulation of C. elegans were carried out according to standard procedures. C. elegans variety Bristol, strain N2, was used as the wild type. The following mutations have been described previously: fem-1(hc17), fem-3(q20gf), glp-4(bn2), ced-1(e1735), ced-3(n717), ced-4(n1162), ced-9(n1950gf), him-8(e1489) and fog-2(q71) (Riddle et al., 1997, or in the references therein); unc-32(e189); rde-1(n219) (Tabara et al., 1999); mpk-1(ga117) (Lackner and Kim, 1998); and spo-11(ok79) (Dernburg et al., 1998).

Northern analysis and in situ hybridization

To prepare RNA, the fem-1, fem-3(q20gf), glp-4 and N2 strains were grown at 15°C for 5 days, embryos were extracted, then after 3 days at 25°C these were harvested as adults. Total RNA was fractionated in formaldehyde-agarose gels and blotted onto Hybond-N membranes at 25°C these were harvested as adults. Total RNA was fractionated and N2 strains were described previously:

Antibody production and immunofluorescent staining

Six polyclonal rat antisera were raised (Cocalico Biologicals) against a peptide that corresponds to CGH-1 residues 411 to 431 plus an amino terminal cysteine (NH2-CDPKLYVADQVLDADETTATCCOOG). These antisera (T1-T6) stained gonads similarly, and the corresponding preimmune sera each detected only a light diffuse background (not shown). No staining of either gonads or embryos was observed after CGH-1 antisera had been depleted of specific reactivity by the CGH-1 peptide (Sulfolink; Pierce). Affinity-purified sera T1 and T2 were indistinguishable in their staining (not shown).

To examine PGL-1 and CGH-1 expression in gonads, animals were opened on glass coverslips, which were inverted and placed on a polylysine-treated slide. Samples were immediately placed in liquid nitrogen and freeze-cracked, then fixed in 100% methanol at −20°C for 1 minute, followed by 1× PBS, 0.08 M Hepes (pH 6.9), 1.6 mM MgSO4, 0.8 mM EDTA, 3.7% paraformaldehyde for 30 minutes. They were then washed twice for 20 minutes in 1× PBS, 0.5% Tween 20 (PBT), and blocked for 30 minutes in 30% normal goat serum, 0.2% azide in PBT. For immunofluorescent staining of embryos, gravid adult hermaphrodites were opened directly on polylysine-coated slides and processed as above. When gonads were fixed with paraformaldehyde prior to or simultaneously with methanol (as above), CGH-1 staining was less granular than in Fig. 3 but was similarly in part co-localized with PGL-1. CGH-1 staining patterns were more diffuse if gonads were not freeze-cracked (not shown). Embryonic CGH-1 staining patterns were identical under each fixation condition. Intact larvae were stained using a whole-mount protocol (Finney and Ruvkun, 1990). Affinity purified CGH-1 antisera were used at 1:10 and 1:25 for gonad/animal and embryo staining, respectively. During incubation with a rhodamine-conjugated donkey anti-rat IgG (H+L) (Jackson) secondary antibody (at 1:100), nuclei were stained with 0.08 μg/ml DAPI. Samples were mounted using 10 μl Vectashield (Vector Lab). Staining with GLD-1, GLP-1, RME-2, GLH-(1-4), and phospho-histone H3 (Upstate) antibodies was performed as described (Grant and Hirsh, 1999; Jones et al., 1996; Kadyk and Kimble, 1998; Kuznicki et al., 2000). Images were obtained on an Axioskop 2 MOT microscope equipped with an AxioCam digital camera (Zeiss), then processed in Photoshop 5.5 (Adobe). Confocal images were obtained with a BioRad Radiance 2000 MP multiphoton microscope, and processed using Confocal Assistant 4.02 and Photoshop 5.5.

RNAi studies

dsRNA produced in vitro (RiboMax, Promega) was injected into young adults at 1 mg/ml (Fire et al., 1998). Injected animals were allowed to purge embryos for 6 hours and were then transferred to new plates. F1 offspring produced over the next 3 days were scored for fertility. Occasionally an injected animal remained fertile and produced only wild-type offspring, and was excluded from the analysis on the assumption that the injection had failed. We obtained indistinguishable RNAi effects using a full-length cgH-1 cDNA, and PCR fragments that extended 268, 567 and 999 bp 3′ from the translation initiation codon (not shown). All cgH-1 RNAi experiments described were performed at 25°C. At 20°C sterility was also observed, but some F1s eventually recovered from the RNAi effect (not shown). RNAi was performed on homoyzogotes in the ced-1(e1735), ced-3(n717), ced-4(n1162) and ced-9(n1950) (gf) strains. In mpk-1(ga117) and spo-11(ok79), RNAi was performed on heterozygotes and homozygous offspring were identified. In somatic cell death assays, to confirm that RNAi had been effective, ced-1::cgH-1(RNAi) embryos and L1 larvae were allowed to develop into adults after corpses had been counted.
To assess male fertility, we performed multiple matings of three \textit{cgh-1} (RNAi) or wild-type males with an N2 hermaphrodite, from which male cross progeny were scored, with a \textit{fog-2} (q71) animal, which does not self-fertilize, and with an \textit{rde-1} (ne219); unc-32(e189) homozygote, from which cross progeny were non-unc. Production of \textit{cgh-1} (RNAi) males is described in the text. No cross progeny were produced in these experiments, or when six \textit{cgh-1} (RNAi) males were placed with an N2 hermaphrodite (not shown). Sperm were prepared and activated in vitro by pronase as described previously (L’Hernault and Roberts, 1995).

**Cell death assays**

Cell corpses were counted in animals that had been anesthetized in 10 \mu{l} M9 plus 30 mM NaCl, using Nomarski optics (Gumienny et al., 1999). To stain with Acridine Orange (AO), animals were incubated in 200 \mu{l} of 100 mM AO in M9 for 2 hours in the dark, fed for 1 hour to decrease background gut fluorescence, then observed as above. Results were processed using Excel (Microsoft).

**RESULTS**

**Germline development in \textit{C. elegans}**

In \textit{C. elegans}, gametes develop in an assembly line fashion as they move from the distal end of each of two gonad arms towards the uterus (Fig. 2A) (Schedl, 1997). Germline cells located at the distal end constitute a proliferating stem cell population. These developing gametes are within a syncytium in which each is only partially enclosed by a plasma membrane, but they will be referred to as cells in keeping with convention. As they move proximally, they pass through a “transition zone” in which they enter the prophase of meiosis I. In hermaphrodites, numerous pachytene stage cells are located between the transition zone and the gonad loop. As germ cells move through the loop, they become more fully cellularized and initiate oocyte growth. These developing oocytes then complete oogenesis prior to fertilization, after which they finish meiosis. The adult germline derives from two embryonic precursors (Z2 and Z3), which do not begin to proliferate until the L1 larval stage. Meiotic cells first appear in L3 animals, within the proximal gonad. In L4 hermaphrodites, in each gonad arm the first approximately 40 germline cells to enter meiosis differentiate into about 160 sperm, after which a switch in sexual fate occurs so that adults produce only oocytes.

**Germline and early embryonic expression of \textit{cgh-1}**

The predicted CGH-1 protein consists primarily of a conserved helicase region of 372 residues (Fig. 1B). This region contains elements that are characteristic of predicted RNA helicases, including the DEAD box and other motifs associated with their ATPase, RNA binding, and helicase activities (de la Cruz et al., 1999). Within the helicase region, CGH-1 is about 70% identical to its yeast, human and \textit{Drosophila} orthologs (Fig. 1B). In contrast, CGH-1 is less than 38% identical to its next closest relative, the DEAD box helicase and translation factor eIF-4A, suggesting that preservation of a helicase function is insufficient to account for its evolutionary conservation. The CGH-1 helicase region is slightly less closely related to those of the germline helicases VASA and DEADSouth, and the GLH (germ line helicase) proteins (not shown; Kuznicki et al., 2000; MacArthur et al., 2000). CGH-1 also lacks additional apparent functional domains that are present in the \textit{C. elegans} GLH proteins (Kuznicki et al., 2000).

The \textit{cgh-1} mRNA is expressed in the germline during larval and adult stages (Fig. 2B-F). Northern blotting indicated that levels of a single \textit{cgh-1} transcript were reduced significantly in \textit{glp-4(bn2)} adult hermaphrodites, in which the germline is underproliferated (12% of wild type; Fig. 2B) (Beanean and Strome, 1992). The \textit{cgh-1} transcript was expressed at high levels (76% of wild type) in \textit{fem-1(hc71)} mutants, which produced only oocytes (Kimble et al., 1984), and at lower levels (48% of wild type) in \textit{fem-3} gain-of-function (gf) hermaphrodites, which produced only sperm (Barton et al., 1987). By in situ hybridization, \textit{cgh-1} mRNA appeared to be restricted to the two germline precursors Z2 and Z3 in wild-type L1 stage hermaphrodites (Fig. 2C), and was detectable specifically in the gonad at low levels into the L3 stage (Fig. 2D). \textit{cgh-1} expression was significantly higher during the early L4 stage, however, when numerous meiotic cells are present (Fig. 2E). In adults it remained gonad-specific and was not apparent in the somatically derived uterus (Fig. 2F).

We investigated CGH-1 protein expression using rat antisera against a carboxyl-terminal CGH-1 peptide (Fig. 3). An affinity purified antiserum identified a single 48 kDa species by western blotting, compared with the predicted CGH-1 molecular mass of 48.7 kDa (not shown). In intact hermaphrodite larvae and adults, CGH-1 was detectable specifically in the gonad in germline but not somatic cells (not shown).
shown). Paralleling its mRNA expression, CGH-1 levels were barely detectable in L1 and L2 animals, higher during the L3 stage, and significantly increased in L4 and adult gonads. CGH-1 levels remained modest in proliferating cells at the distal gonad end, where it co-localized with the P granule components PGL-1 (Fig. 3A-C) and the GLH helicases (not shown). In the transition zone, where cells enter meiosis (Fig. 2A), CGH-1 appeared in additional cytoplasmic granules within the central gonad core (Fig. 3A-C). We refer to these as CGH-1 somatic granules because they appeared to be maintained in the cytoplasm throughout oogenesis and into early embryogenesis, during which they were not segregated to the germline (see below). CGH-1 remained at high levels in association with each granule type in later stage meiotic cells and oocytes (Fig. 3A-I). In contrast, in the male gonad, CGH-1 was readily detectable only in cells that were entering meiosis, in which it was also co-localized with P granules (Fig. 4A-D).

CGH-1 remained present in somatic and P granules in the early embryo (Fig. 5). Embryonic P granules can be detected by PGL-1 staining, and are initially segregated by four cell divisions to a single germline precursor (P4), which forms the two germline precursors Z2 and Z3 at around the 100 cell stage (Schedl, 1997) (Fig. 5B,F,J,N). In the germline, these PGL-1 particles were associated with co-localized CGH-1 staining through the 100 cell stage (Fig. 5A-P). CGH-1 staining then disappeared from Z2 and Z3 by the 200 cell stage (not shown). CGH-1 was also readily detectable in somatic granules...
RNA helicase required for oocyte survival

Throughout the embryo through the four-cell stage (Fig. 5A-H; not shown). The latter CGH-1 staining then began to diminish, and was barely detectable at the 28 cell stage and absent in 50-100 cell embryos (Fig. 5I-P; not shown). No CGH-1 staining was detected at later stages (not shown), consistent with embryonic CGH-1 expression being maternally derived.

cgh-1 is essential for hermaphrodite and male fertility

We inhibited cgh-1 expression using RNAi (Fire et al., 1998). Young adult hermaphrodites that were injected with cgh-1 dsRNA became sterile within 24 hours (not shown). During this time, they generally produced up to 20 F1 offspring that matured to adulthood, followed by 2-3 embryos with heterogeneous defects that did not hatch. We studied the role of cgh-1 during development by analyzing the mature F1 offspring, which we refer to as cgh-1(RNAi) animals. CGH-1 was not detectable by antibody staining in cgh-1(RNAi) hermaphrodites (Fig. 3J) or males (Fig. 4E), indicating a profound decrease in its expression. The gonad appeared normal in early L4 stage cgh-1 (RNAi) animals, but in most young adults its distal region was distended, and abnormal oocytes were visible proximally (Fig. 6B; not shown). cgh-1(RNAi) hermaphrodites were otherwise normal in appearance, and lacked evident defects in their somatic gonad cells, but were unable to self-fertilize (Table 1). They were also sterile when mated with wild type males, even when RNAi was performed in a strain that produces only oocytes (fog-2(q71); not shown) (Schedl and Kimble, 1988), indicating that cgh-1 is required to form functional oocytes.

A normal number of sperm was present in the cgh-1(RNAi) hermaphrodite spermatheca (Fig. 6B; not shown), raising the question of whether cgh-1 is involved in spermatogenesis. We produced cgh-1(RNAi) F1 males by introducing cgh-1 dsRNA into RNAi resistant rde-1/rde-1 hermaphrodites (Tabara et al., 1999), then mating these injected animals with wild type males. Each injected P0 animal produced numerous hermaphrodite and male F1 rde-1/+; cgh-1(RNAi) offspring which became sensitive to RNAi upon zygotic expression of rde-1 (Tabara et al., 1999). rde-1/+; cgh-1(RNAi) hermaphrodites were sterile and indistinguishable from cgh-1(RNAi) hermaphrodites produced from wild type animals (Table 1; not shown). rde-1/+; cgh-1(RNAi) males were normal in appearance and produced sperm. Their germline cells did not express detectable CGH-1 (Fig. 4E,F), but contained PGL-1 in P granules (not shown). These cgh-1(RNAi) males were sterile, however, suggesting that their sperm function was abnormal. These effects were also observed in cgh-1(RNAi) males that were produced by injected him-8(e1489) hermaphrodites, or by injected wild type animals that were crossed with males (not shown). Primary spermatocytes and spermatozids prepared from rde-1/+; cgh-1(RNAi) males appeared normal, but upon activation most of their mature...
sperm extended abnormally short pseudopods, a defect that would result in decreased motility and could account for their sterility (Fig. 6K,L).

cgh-1 is required for oocyte survival

Although cgh-1(RNAi) hermaphrodites generally did not lay oocytes, in their gonads the germline differentiation markers GLP-1, GLD-1, and RME-2 were present in appropriate regions, indicating that oogenesis had not been arrested at a particular stage (Fig. 6C-H). PGL-1 and the four GLH proteins were also present in P granules in cgh-1(RNAi) hermaphrodite germline cells (Fig. 6I,J; see below). In C. elegans, germline proliferation correlates with presence of cells that stain with an antibody to phosphorylated histone H3, which is required for mitotic chromosome condensation (Kadyk and Kimble, 1998; Wei et al., 1999). Cells expressing this phosphoepitope were present within the mitotic regions of nearly 50% of one day old cgh-1(RNAi) hermaphrodite gonads, indicating that some proliferation was continuing (Table 2; not shown). cgh-1(RNAi) gonads contained an approximately normal number of germ cells (not shown), however, suggesting that cgh-1(RNAi) hermaphrodites might be producing oocytes that were dying at an elevated rate. Consistent with this idea, in cgh-1(RNAi) gonads chromosome morphologies appeared generally normal at each oogenesis stage, except that numerous cells with condensed chromosomes were present near the loop (not shown).

We investigated whether germline cell death was increased in cgh-1(RNAi) animals by counting cell corpses and staining with AO, which accumulates specifically in apoptotic cells (Gumienny et al., 1999). At 24 and 48 hours after the L4 stage, numbers of corpses and AO-positive cells were elevated three- to five-fold in cgh-1(RNAi) hermaphrodite gonads (Fig. 7A-D; Table 3). This cell death required ced-3 and ced-4, which encode the C. elegans caspase and Apaf-1 orthologs respectively (Hengartner, 2000), demonstrating that it was apoptotic (Fig. 7E,F; Table 3). cgh-1 RNAi comparably increased germline corpse numbers in ced-1 mutants, in which corpse engulfment is defective (Hengartner, 2000) (Fig. 7K,L; Table 3), suggesting that the elevated numbers of dead cells in cgh-1(RNAi) gonads was the result of increased apoptosis and not impaired engulfment. In contrast, interference with cgh-1 did not significantly increase corpse numbers in the late embryonic and L1 larval head (Table 4), indicating that cgh-1 is not broadly required for somatic cell survival. Given that up to half of oocyte precursors are normally sacrificed in wild type hermaphrodites (Gumienny et al., 1999), the sharply elevated numbers of dead cells in cgh-1(RNAi) animals suggested that essentially all of the oocytes that they produce die by apoptosis.

Several lines of evidence indicate that the increased apoptosis in cgh-1(RNAi) hermaphrodites was induced by the physiological germline pathway. As in wild type animals (Gumienny et al., 1999), in cgh-1(RNAi) adults cell death was centered around the gonad loop, where cells exit the pachytene stage (Fig. 2A), although some earlier and later stage meiotic

<table>
<thead>
<tr>
<th>Table 1. Sterility in cgh-1(RNAi) animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0 strain</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>TE injected</td>
</tr>
<tr>
<td>cgh-1(RNAi)</td>
</tr>
<tr>
<td>Uninjected</td>
</tr>
<tr>
<td>cgh-1(RNAi)</td>
</tr>
</tbody>
</table>

Six hours after dsRNA injection, control and cgh-1(RNAi) P0 animals were transferred to new plates and kept at 25°C. Individual homozygous rde-1(ne219); unc-32(e189); unc-19(e189)/+ injected or control hermaphrodites were then crossed with N2 males, so that their F1 progeny* were rde-1(ne219); unc-32(e189)/+. Each set of F1 progeny was scored for fertility after 3 days. The number of sterile animals over the total scored is indicated in parentheses.
RNA helicase required for oocyte survival

Fig. 5. Presence of CGH-1 in the embryo. Embryos of the 2-cell (A-D), late 4-cell (E-H), approximately 50-cell (I-L) and approximately 100-cell (M-P) stages were stained with anti-CGH-1 (A,E,I,M), anti-PGL-1 (B,F,J,N) and DAPI (D,H,L,P) and examined by fluorescence microscopy. C, G, K and O are merged images. Examination of four-cell embryos by confocal microscopy indicated that, in general, each PGL-1 particle co-localized with a CGH-1 particle (not shown).

Table 2. Decreased germline proliferation in cgh-1(RNAi) animals

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Early L4</th>
<th>1 day</th>
<th>2 days</th>
<th>3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>5.1±3.7</td>
<td>4.9±2.8</td>
<td>4.2±2.9</td>
<td>3.4±3.1</td>
</tr>
<tr>
<td>cgh-1(RNAi)</td>
<td>4.5±2.5</td>
<td>2.5±3.2</td>
<td>1.7±2.9</td>
<td>0±0</td>
</tr>
<tr>
<td>ced-3(n717)</td>
<td>4.7±2.6</td>
<td>4.0±1.9</td>
<td>3.0±2.7</td>
<td>ND</td>
</tr>
<tr>
<td>cgh-1(RNAi) ced-3(n717)</td>
<td>4.8±2.2</td>
<td>4.1±2.4</td>
<td>3.7±2.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

Gonads were prepared and stained for phospho-histone H3 at the indicated time after hermaphrodites were scored as early L4s. The average number of positively staining cells per gonad is shown along with the standard deviation. The number of completely negatively staining gonads over the total is indicated in parentheses. ND: not done.

Table 3. Germline cell death in cgh-1(RNAi) animals

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>No. of AO positive cells/arm</th>
<th>n</th>
<th>Germline corpses/arm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>52</td>
<td>3.2±1.8</td>
<td>53</td>
<td>4.2±2.5</td>
</tr>
<tr>
<td>cgh-1(RNAi)</td>
<td>84</td>
<td>10.1±3.9</td>
<td>66</td>
<td>21.9±7.3</td>
</tr>
<tr>
<td>ced-1(e1735)</td>
<td>ND</td>
<td>ND</td>
<td>35</td>
<td>19.7±5.2</td>
</tr>
<tr>
<td>ced-3(n717); cgh-1(RNAi)</td>
<td>ND</td>
<td>ND</td>
<td>32</td>
<td>76.4±10.5</td>
</tr>
<tr>
<td>ced-3(n717)</td>
<td>108</td>
<td>0.1±0.4</td>
<td>108</td>
<td>0.2±0.8</td>
</tr>
<tr>
<td>ced-3(n717); cgh-1(RNAi)</td>
<td>67</td>
<td>1.1±2.1</td>
<td>67</td>
<td>1.5±2.1</td>
</tr>
<tr>
<td>ced-4(n162)</td>
<td>47</td>
<td>0±0</td>
<td>47</td>
<td>0.1±0.5</td>
</tr>
<tr>
<td>ced-4(n1162); cgh-1(RNAi)</td>
<td>41</td>
<td>0.0±0.2</td>
<td>41</td>
<td>0.2±0.6</td>
</tr>
<tr>
<td>ced-9(n1950)(gf)</td>
<td>50</td>
<td>3.9±2.4</td>
<td>50</td>
<td>6.8±2.7</td>
</tr>
<tr>
<td>ced-9(n1950)(gf); cgh-1(RNAi)</td>
<td>63</td>
<td>12.4±5.2</td>
<td>64</td>
<td>20.1±5.8</td>
</tr>
<tr>
<td>mpk-1(ga117)</td>
<td>29</td>
<td>0±0</td>
<td>29</td>
<td>0.1±0.6</td>
</tr>
<tr>
<td>mpk-1(ga117); cgh-1(RNAi)</td>
<td>29</td>
<td>0.2±0.8</td>
<td>29</td>
<td>0.3±0.9</td>
</tr>
<tr>
<td>spo-11(ok79)</td>
<td>69</td>
<td>3.5±3.5</td>
<td>43</td>
<td>4.7±1.8</td>
</tr>
<tr>
<td>spo-11(ok79); cgh-1(RNAi)</td>
<td>28</td>
<td>10.9±6.0</td>
<td>27</td>
<td>18.7±6.9</td>
</tr>
</tbody>
</table>

Two days after hermaphrodites of the indicated genotypes were scored as L4 larvae, acridine orange (AO) positive cells and corpses were counted within their gonads. n, number of gonad arms scored (one per animal). ND, not done.
cells were also dying (Fig. 7C,D). Germline cell death was also not observed during spermatogenesis in cgh-1(RNAi) larvae or males (not shown), as would be predicted for the physiological pathway. Physiological germline apoptosis requires mitogen-activated-protein (MAP) kinase signalling (Gumienny et al., 1999), a pathway which is involved in germ cell development in many species, and in *C. elegans* is required for progression beyond pachytene (Church et al., 1995). Accordingly, cgh-1 RNAi did not cause germ cell death or obviously affect the pachytene arrest in the MAP kinase mutant mpk-1 (Fig. 7G,H; Table 3). Physiological germ cell death does not require meiotic recombination or unrepaired DNA (Gartner et al., 2000), and cell death associated with cgh-1 RNAi was unaffected by the spo-11 mutation, which prevents meiotic recombination (Table 3; Dernburg et al., 1998). Finally, and most importantly, physiological germline cell death is regulated differently from all other *C. elegans* apoptosis in that it is not dependent upon inhibition of the anti-apoptotic protein CED-9 by the EGL-1 protein (Gumienny et al., 1999). Accordingly, in cgh-1(RNAi) animals the frequency of germline cell death was not decreased by the ced-9(n1950gf) mutation (Fig. 7I,J; Table 3), which is resistant to EGL-1 and inhibits other apoptotic events (Gartner et al., 2000).

These findings raise questions of whether the apoptosis observed in cgh-1(RNAi) oocytes is associated with an underlying functional defect, and of whether this defect involves a catastrophic impairment of oocyte proliferation, physiology, or development, or a more subtle abnormality. In cgh-1(RNAi) animals, over time fewer germline cells stained positively for phosphorylated histone H3, suggesting that their proliferation eventually ceased (Table 2). This proliferation decrease did not occur in a ced-3 background, indicating that it depended entirely upon the apoptotic signal. Small oocytes accumulated proximally in ced-3; cgh-1(RNAi) and ced-4; cgh-1(RNAi) hermaphrodites (Fig. 7E,F). ced-3; cgh-1(RNAi) animals failed to produce offspring when mated with wild type males, however (not shown), indicating that these oocytes were defective. ced-3; cgh-1(RNAi) oocytes were very similar in appearance to the small oocytes that are characteristic of ced-3 animals but were present in elevated numbers, presumably because
RNA helicase required for oocyte survival

they were not fertilized (Figs 7E,F and 8A,B) (Gumienny et al., 1999). ced-3; cgh-1(RNAi) oocytes did not undergo non-apoptotic cell death (Figs 7F, 8B; Table 3), they appropriately reached the diakinesis stage of meiosis (Fig. 8C,D), and contained P granules (Fig. 8E,F), ced-3; cgh-1(RNAi) oocytes also expressed the yolk receptor RME-2, although they were partially defective in placing it on their surface (Fig. 8G,H). In some cgh-1(RNAi) animals, a small number of oocytes survived and reached a nearly normal size, and moved their nuclei to one side or assumed a spherical shape (not shown). These events are characteristic of late oocyte development and meiotic maturation, respectively (Schedl, 1997), although in the cgh-1(RNAi) gonad their progression did not appear to be organized normally (not shown). We conclude that when apoptosis does not occur, oocytes produced by cgh-1(RNAi) animals develop appropriately in many respects, but cannot fulfill their reproductive function.

DISCUSSION

cgh-1 function in the germline

Previous studies have implicated cgh-1 orthologs in yeast sporulation (Maekawa et al., 1994; Moriya and Isono, 1999), and we have now determined that cgh-1 is required for oogenesis and spermatogenesis in the metazoan C. elegans. CGH-1 appears to be expressed specifically in the germline and early embryo, and is the first protein to be found in P granules or other germline mRNA-protein particles that corresponds to a yeast gene with a specialized sporulation function. Perhaps paralleling the requirement for ste13 for meiosis entry (Maekawa et al., 1994), CGH-1 levels increase as C. elegans germ cells enter meiosis (Figs 3A and 4A). Our findings suggest that CGH-1 is involved in ancestral mechanisms that are essential for gametogenesis in diverse eukaryotes.
Various observations suggest that CGH-1 and its orthologs may regulate metabolism or translation of some mRNAs that are stored in developing germ cells. Its *Xenopus* ortholog p54 is highly abundant among proteins that are complexed with stored oocyte mRNA, including Y-box proteins that can regulate translation (Ladomery et al., 1997; Sommerville, 1999). The *Drosophila* CGH-1 ortholog ME31B (Fig. 1B) associates with a related Y-box protein, co-localizes with mRNAs that are transported from nurse cells to the oocyte, and is required to prevent these mRNAs from being translated prematurely (A. Nakamura and S. Kobayashi, personal communication). In the *C. elegans* germline, CGH-1 is present not only in P granules, but also in somatic granules that appear in the gonad core coincidentally with entry into meiosis (Fig. 3A). Accumulation of these granules parallels an increase in mRNA transcription, and appearance of newly transcribed RNA in the gonad core (Gibert et al., 1984; Schisa et al., 2001). Finally, CGH-1 disappears from embryonic cells (Fig. 5A,E,I,M) roughly in parallel to Class II maternal mRNAs, which are rapidly lost from somatic cells but persist longer in the germline precursor (Schisa et al., 2001; Seydoux and Fire, 1994).

The germline is the only stem cell population in *C. elegans*, but some *cgh-1* orthologs (Fig. 1B) function in other contexts that share similarities with stem cells. *S. pombe* ste13 is dispensable for proliferation, but essential for entry into G0 upon starvation (Maekawa et al., 1994). Like sexual reproduction, this is a quiescent state from which a proliferating cell population re-emerges. Human RCK/p54 is present at some 11q23 lymphoma chromosomal translocation breakpoints, and is overexpressed in some tumors and cell lines, suggesting that it can contribute to transformed cell survival or proliferation (Akao et al., 1995; Lu and Yunis, 1992; Nakagawa et al., 1999). In mammals RCK/p54 is normally expressed in oocytes, but the RCK/p54 protein is also present in somatic tissues that are subject to turnover (Akao et al., 1995; Paynton, 1998). The specialized post-transcriptional regulatory mechanisms in which *cgh-1* orthologs function thus may be important in multiple self-renewing cell types.

**cgh-1** and apoptotic surveillance of oocytes

In *C. elegans*, physiological germline apoptosis is regulated differently from all other somatic or germline apoptotic events that have been described (Gartner et al., 2000; Gumienny et al., 1999). This process occurs with characteristic timing, primarily late during the pachytene stage of meiotic prophase. Physiological germline apoptosis also normally spares the cytoplasm associated with the sacrificed oocytes, so that it is available to their sister cells, suggesting that it may maintain oocyte homeostasis (Gumienny et al., 1999). How this pathway selects developing oocytes for death is an important issue for investigation. This mechanism kills essentially all developing oocytes if CGH-1 is absent, making CGH-1 the first example of a germline function that influences this specialized apoptosis pathway. The conservation of CGH-1 (Fig. 1B) suggests that elucidation of its functions will provide important insights into how physiological germline apoptosis is controlled not only in *C. elegans*, but also in other organisms.

The association of CGH-1 with P granules raises the question of whether abnormalities in constitutive P granule components might also trigger physiological germline apoptosis. Like *cgh-1(RNAi)* animals (Fig. 6B), PGL-1 or GLH-1 mutant animals (Fig. 8) show a perinuclear accumulation of DAPI, anti-GLH-1 or anti-RME-2 staining, in both oocytes and somatic cells, suggesting that these proteins are involved in the regulation of oocyte homeostasis.

**Table 4. Effects of cgh-1 (RNAi) on somatic cell death**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Embryonic corpses</th>
<th>n</th>
<th>Corpses in L1 larvae</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ced-1</em></td>
<td>36.7±7.3</td>
<td>33</td>
<td>26.7±6.3</td>
<td>16</td>
</tr>
<tr>
<td><em>ced-1; cgh-1 (RNAi)</em></td>
<td>38.0±7.7</td>
<td>30</td>
<td>29.4±5.9</td>
<td>18</td>
</tr>
</tbody>
</table>

Corpses were counted in the heads of pretzel stage embryos and L1 larvae of the indicated genotypes. L1 larvae that were scored contained four germ line cells. n, number of animals scored.
helical loss-of-function phenotypes can include the presence of abnormal oocytes, but in smaller numbers (Kawasaki et al., 1998; Kuznicki et al., 2000). These P granule mutant or RNAi phenotypes are also characterized by severe decreases in germ line proliferation, however, that are associated with decreased gonad size. In animals that lack GLH proteins, proliferation defects are also observed during larval stages, and sperm number is reduced (Kuznicki et al., 2000). In contrast, germ cell numbers and proliferation are normal until the young adult stage in cgh-1(RNAi) animals (Table 2; not shown). While it will be of interest to determine whether P granule defects might stimulate physiological apoptosis, their impairment of germ proliferation is likely to involve other mechanisms.

The oocytes produced by ced-3; cgh-1(RNAi) animals could not be fertilized (not shown), indicating that cgh-1 is essential for oocyte function. cgh-1(RNAi) oocytes developed normally in some respects, however. They expressed oogenesis genes appropriately (Figs 6C-J and 8E-H), and did not undergo non-apoptotic cell death when apoptosis was prevented (Figs 7F and 8B; Table 3). Instead, ced-3; cgh-1(RNAi) oocytes proliferated and reached the diakinesis stage appropriately, contained P granules, and did not appear obviously different from ced-3 oocytes (Figs 7E,F, 8A-F; Table 2). They did not place RME-2 on their surface with normal efficiency, however (Fig. 8G,H). This defect could indicate a membrane trafficking abnormality, as could the failure of cgh-1(DNAi) sperm to extend their pseudopods normally (Fig. 6K,L). We conclude that aside from triggering physiological apoptosis, lack of cgh-1 did not catastrophically impair oocyte viability, proliferation, or development. Confirmed or apparent apoptosis occurs in Drosophila oocytes that lack MEI1B, or the related helicases vasa or Abstrakt, or that are nutritionally deprived (A. Nakamura and S. Kobayashi, personal communication; Drummond-Barbosa and Spradling, 2001; Irion and Leptin, 1999). Mouse spermatocytes that lack vasa also undergo apoptosis (Tanaka et al., 2000). As more is understood about how germline cell death is regulated in these organisms, it will be interesting to determine whether these last examples of germ cell death represent apoptosis triggered by a physiological germline pathway, or general apoptotic responses to cell abnormalities or stress.

C. elegans oocytes that are sacrificed physiologically do not seem to represent a distinct fate (Gumienny et al., 1999), suggesting that cgh-1(RNAi) oocytes have not simply become nurse cells. Instead, it appears likely that physiological germline apoptosis can be triggered by abnormalities in metabolism or translation of mRNAs that are regulated by cgh-1. This cell death signal might derive from inappropriate expression of particular oocyte or maternal genes, but it is also possible that cgh-1 regulates genes that directly influence oocyte survival. In either case, our findings identify a connection between the survival of immature oocytes and their eventual functional capability. We conclude that the physiological germline apoptosis pathway responds to aspects of oocyte development that may include accumulation or organization of germ line cytoplasm components, and that along with its apparent role in maintaining oocyte homeostasis (Gumienny et al., 1999), this pathway can function as a surveillance mechanism that eliminates defective oocytes.

Some aspects of cell death in the oocyte lineage are strikingly similar among species, suggesting that this surveillance mechanism may be a conserved function of physiological germline apoptosis. Drosophila nurse cell apoptosis is regulated distinctly from embryonic cell death (Foley and Cooley, 1998), and may be related to mechanisms that eliminate defective egg chambers (Chao and Nagoshi, 1999). As in C. elegans, in prenatal and young mammals most oocyte apoptosis appears to occur during the late pachytene stage (Baker, 1963; Bakken and McClanahan, 1978; Borum, 1961). Mammalian oocyte apoptosis is likely to be influenced by multiple intrinsic and extrinsic inputs (Krakauer and Mira, 1999; Morita and Tilly, 1999; Perez et al., 2000), but our findings suggest a paradigm by which the potential competence of a developing oocyte might influence whether it is chosen for death or survival. It will be of considerable importance to determine whether such surveillance mechanisms exist in mammals, in which the reproductive cost of each defective oocyte is particularly high.

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