A conserved RNA-protein complex component involved in physiological germline apoptosis regulation in C. elegans

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Summary

Two conserved features of oogenesis are the accumulation of translationally quiescent mRNA, and a high rate of stage-specific apoptosis. Little is understood about the function of this cell death. In C. elegans, apoptosis occurring through a specific ‘physiological’ pathway normally claims about half of all developing oocytes. The frequency of this germ cell death is dramatically increased if apoptosis is prevented car-1(RNAi) animals are characterized by a progressive oogenesis defect that leads rapidly to gonad failure. Elevated germ cell death similarly compensates for lack of the translational regulator CPB-3 (CPEB), orthologs of which function together with CGH-1 in diverse organisms. We conclude that CAR-1 is of critical importance for oogenesis, that the association between CAR-1 and CGH-1 has been conserved, and that the regulation of physiological germ cell apoptosis is specifically influenced by certain functions of the CGH-1/CAR-1 RNP complex. We propose that this cell death pathway facilitates the formation of functional oocytes, possibly by monitoring specific cytoplasmic events during oogenesis.

Key words: Germline, Oocyte, Caenorhabditis elegans, Drosophila, RNA binding, Apoptosis, Cytokinesis, P body

Introduction

Germ cells are a highly specialized lineage that is responsible for transmitting genetic information from one generation to the next, and, ultimately, for the development of an organism. The process by which a self-renewing germline stem cell population gives rise to oocytes involves the accumulation of cytoplasmic components, such as mRNAs and proteins, that will orchestrate oocyte and zygote development until embryonic transcription begins (Saffman and Lasko, 1999). C. elegans provides a valuable system with which to study germ cell development because of its genetic tractability and ‘production line’ mode of oogenesis (Fig. 1).

In metazoas as diverse as C. elegans and humans, one hallmark of oogenesis is that apoptosis occurs at a high frequency during or shortly after the late pachytene stage of meiosis (Baker, 1963; Borum, 1961; Gumienny et al., 1999; Pepling and Spradling, 2001). In mammals, developing oocytes transfer cytoplasmic components within cysts, then, around the time of birth, approximately two-thirds of these oocytes die as their cysts break down (Pepling and Spradling, 2001). About half of all developing oocytes undergo apoptosis in C. elegans, just before the survivors form discrete cells from a syncytium (Fig. 1) (Gumienny et al., 1999). The functions of developmental germ cell apoptosis are not well understood. In C. elegans, this cell death is referred to as physiological because the sacrificed nuclei do not seem to be of poor quality, and because their associated cytoplasm is provided to their surviving sisters (Gumienny et al., 1999). Although this suggests that the dying nuclei function in effect as nurse cells, the absence of physiological apoptosis does not significantly impair fertility under normal laboratory conditions (Gumienny et al., 1999).

It is not known how physiological germ cell death is regulated, but it is clear that this process is controlled differently from all other apoptosis in C. elegans, including a pathway that culs defective germ cell nuclei in response to genotoxic stress (Gartner et al., 2000; Gumienny et al., 1999; Hofmann et al., 2002). Whole-genome and other RNAi analyses have identified only five genes that specifically prevent the physiological apoptosis pathway from claiming the vast majority of developing oocytes (Lettre et al., 2004; Navarro et al., 2001), which suggests that this process is influenced by specific cues.

Another conserved feature of oogenesis is that many newly produced mRNAs are localized to cytoplasmic storage structures. In many species, the germ line is maintained from...
that we call CAR-1 (cytokinesis/apoptosis/RNA-binding) that associates with CGH-1 within a conserved RNP complex, and in cytoplasmic foci. CAR-1 and CGH-1 orthologs similarly associate in Drosophila oocytes. RNAi knockdown of CAR-1 causes defective embryonic cytokinesis, along with an increase in physiological apoptosis that partially compensates for an oogenesis defect that otherwise leads rapidly to gonad failure. Increased germ cell death plays a similar role after knockdown of the CPEB (cytoplasmic polyadenylation element binding protein) ortholog CBP-3, which interacts functionally with CGH-1 orthologs in other species. We conclude that CAR-1 has a conserved role in germ cell development, and that physiological germine apoptosis may enhance the efficiency of oogenesis, and can partially compensate for a lack of some functions of the CGH-1/CAR-1 complex.

Materials and methods

Strains

C. elegans strains were maintained using standard methods (Brenner, 1974). The wild-type strain Bristol N2 and the following mutant strains were used: glp-4(bn2), ced-1(e1735), ced-3(n717), ced-9(n1950gf) (Riddle et al., 1997); cep-1(+/h40) (Derry et al., 2001); cgh-1(ok492); gld-1(q485) (Francis et al., 1995); mpk-1(ga177) (Lackner and Kim, 1998). Fly stocks were raised at 25°C on standard cornmeal and agar medium.

Antibody production and immunofluorescent staining

Polyclonal antibodies were raised to a CAR-1 peptide (amino acids 246-265, plus an amino terminal cysteine; NH2-CKAEKTGRPDWKKRETQNQ-COOH) in two chickens (Cocalico Biologicals, Pennsylvania, USA). Immunostaining with affinity purified CAR-1 antibody (Sulfolink, Pierce) was reduced to background in car-1(RNAi) germlines (see Fig. S1 in the supplementary material). Rabbit anti-CGH-1 antibodies used for affinity purification were generated against the peptide NH2-CDKLYVADQQLVDADETTA-COOH, representing CGH-1 residues 411-431. Immunostaining was performed using rat anti-CGH-1 and rabbit anti-PGL-1 (Kawasaki et al., 1998), as described (Navarro et al., 2001). Carnoy’s fixative was used to prepare intact worms for staining with 4’,6-diamidino-2-phenylindole (DAPI) (Villeneuve, 1994). Nomarski and fluorescent images were obtained using an Axioskop 2 microscope coupled with an AxioCam digital camera (Zeiss). Confocal images were obtained using a Zeiss LSM 510 UV microscope.

Trailerhitch-specific polyclonal antibodies were raised in rabbits against His6-tagged full-length recombinant protein that was expressed in E. coli, and purified with Ni-NTA agarose chromatography (Qiagen) and preparative SDS-PAGE (Kitayama Labes, Nagano, Japan). Drosophila ovaries expressing EGFP-Me31B (Nakamura et al., 2001) were immunostained with anti-Trailerhitch antisera and mouse anti-GFP 3E6 (Wako Pure Chemicals, Osaka, Japan) (Kobayashi et al., 1999). Anti-rabbit IgG Alexa 568 and anti-mouse IgG Alexa 488 (Molecular Probes) were used as secondary antibodies. Fluorescent images of Drosophila were acquired using a Leica TCS SP2 AOPS laser confocal microscope.

Co-immunoprecipitation, western analysis and protein identification

C. elegans protein extracts were prepared from 500,000 synchronized hermaphrodites (approximately 12 hours after the L4/adult molt) by sonication in homogenization buffer [100 mM NaCl, 25 mM HEPES (pH 7.5), 0.25 mM EDTA, 2 mM DTT, 5 mM
Na$_2$VO$_4$, 0.1% NP40] supplemented with 1×’complete’ protease inhibitors (Roche) and 50 U/ml RNAsin (Promega), followed by 20 strokes in a glass homogenizer. Homogenates were centrifuged at 15,000 g for 20 minutes at 4°C, and the supernatant either used immediately for immunoprecipitation or snap frozen in liquid nitrogen and stored at –80°C. Protein lysates (1 mg) were preabsorbed against protein L or G for 1 hour at 4°C. Affinity purified CAR-1 or CGH-1 antibodies were added to the cleared lysate and incubated for 1 hour at 4°C, after which protein L or G Sepharose beads were added and incubated for an additional hour. The beads were washed five times in 200 mM NaCl, 50 mM Tris (pH 7.4), 0.05% NP40, then proteins were extracted by boiling in 2×SDS sample buffer. To investigate the requirement of RNA for co-immunoprecipitation, protein lysates were prepared as described except that 5 μg/ml RNase A was added in place of RNAsin, and samples were incubated at room temperature for 15 minutes before centrifugation. Western blotting was conducted according to standard procedures, using species-specific HRP-labelled secondary antibodies (KPL) at a dilution as described above, but using 5 mg of protein lysate and 25 μg rabbit anti-CGH-1 or IgG antibodies. Bound proteins were eluted by incubation with 0.2 mg/ml CGH-1 peptide for 1 hour at 4°C. Eluted proteins were resolved in a 10% polyacrylamide gel that was stained with Simply Blue (Invitrogen). Excised proteins were digested with trypsin and subjected to tandem mass spectrometry [Pathology Functional Proteomics Center (PFPC), Harvard Medical School]. Proteins were identified by searching the NCB Inr database using the Mascot program. Immunoprecipitation of Drosophila ovary extracts, western analysis and protein identification were conducted as described (Nakamura et al., 2004).

RNAi studies

For RNAi experiments hermaphrodites were injected with double-stranded RNA (~1 μg/μl), and allowed to lay eggs for 8 hours before being transferred to new plates. F1 adult hermaphrodites were examined for cell death at 24 and 48 hours after the L4/adult molt. For analysis of the car-1(RNAi) embryonic phenotype, gravid F1 hermaphrodites were dissected on glass slides. Comparable car-1(RNAi) phenotypes were obtained using dsRNA corresponding to two different regions of the gene.

Analysis of brood size and germ cell death

To measure brood size, L4 stage F1 RNAi hermaphrodites and age-matched N2 and ced-3 animals were individually distributed to NEM plates and transferred at ~12 hour intervals to fresh plates. Progeny were counted 30-40 hours after the removal of the adult. To measure germ cell death by counting corpses, car-1(RNAi) adults were grown at 25°C and immobilized in M9 containing 0.03% tetramisole. Germ cells undergoing apoptosis were then identified by Nomarski optics (Gumienny et al., 1999). In acridine orange (AO) staining experiments, hermaphrodites were placed on plates to which 500 μl of 100 mM AO was added. After these plates were incubated for 3-4 hours in the dark, animals were immobilized and viewed by fluorescent microscopy.

Results

CAR-1 associates with CGH-1 in a germline ribonucleoprotein complex

To identify proteins that might be involved in CGH-1 functions, we immunoprecipitated endogenous CGH-1 from adult C. elegans extracts. These high-stringency immunoprecipitations reproducibly identified a group of bound proteins (Fig. 2A; not shown) that we sequenced by mass spectroscopy. Among the most abundant CGH-1-associated proteins were three C. elegans Y-box proteins (CEY-2, -3, and -4), orthologs of which are involved in translational silencing of oocyte mRNAs in Xenopus and Drosophila (Mansfield et al., 2002; Richter and Smith, 1984; Sommerville, 1999). Y-box proteins also co-precipitated with the Xenopus CGH-1 ortholog Xp54 and have been purified within a germline RNP complex along with the Drosophila CGH-1 ortholog Me31B (Table 1).
Table 1. CGH-1 orthologs and associated proteins

<table>
<thead>
<tr>
<th>Protein type</th>
<th>C. elegans</th>
<th>Drosophila</th>
<th>Xenopus*</th>
<th>S. cerevisiae††</th>
<th>Homo sapiens‡‡</th>
</tr>
</thead>
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<tr>
<td>DEAD-box helicase</td>
<td>CGH-1</td>
<td>Me31B</td>
<td>Xp54</td>
<td>Dhh1</td>
<td>RCK</td>
</tr>
<tr>
<td>Sm-like domain</td>
<td>CAR-1*</td>
<td>Tral*</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>CPEB family</td>
<td>CPB-3‡</td>
<td>Orb†</td>
<td>CPEB*</td>
<td>–</td>
<td>CPEB1†</td>
</tr>
<tr>
<td>Y-box</td>
<td>CEY-2, 3, 4*</td>
<td>Yps*</td>
<td>FRGY2*</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Cap-binding</td>
<td>?</td>
<td>eIF4E*</td>
<td>eIF4E‡</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>eIF4E-binding</td>
<td>–</td>
<td>Cup*</td>
<td>Maskin³</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5'-3' mRNA decay machinery</td>
<td>?</td>
<td>Exu*</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Evidence indicates that these proteins interact either physically or functionally with CGH-1 or its ortholog in the species shown.

*Proteins that have been isolated in a RNP complex along with a CGH-1 ortholog.
†Colocalization with proteins that interact with CGH-1 orthologs.
‡Predicted functional interaction with CGH-1 (see text).
††This work.
‡‡See Cougot et al., 2004; Eystathioy et al., 2003; Ingelfinger et al., 2002; Wilczynska et al., 2005. Human cytoplasmic RNA degradation particles that contain RCK have been designated as cdc1 or GW bodies, but for simplicity we refer to them here as P bodies.
?
—, No clear ortholog has been described.

Colocalization of CAR-1 and CGH-1

We next investigated whether CAR-1 is distributed similarly to CGH-1 within the germline and embryo. In adult hermaphrodites, the CAR-1 and CGH-1 proteins were detectable by western blotting exclusively in the germline (Fig. 2D), consistent with previous antibody staining and northern blot evidence that CGH-1 expression is germline specific (Navarro et al., 2001). Throughout the gonad CGH-1 associates with P granules, as revealed by its overlapping localization with the constitutive P granule component PGL-1 (Fig. 3E-G; not shown) (Navarro and Blackwell, 2005; Navarro et al., 2001). During the syncytial oogenesis stages (Fig. 1), P granules are localized to the perinuclear region, where each is associated with a cluster of nuclear pores (Pitt et al., 2000). After entry into meiosis, many newly synthesized mRNAs appear to pass through P granules on their way to the central core (Schisa et al., 2001). In parallel, CGH-1 staining levels increase dramatically in response to meiosis entry, and CGH-1 particles that are independent of P granules then accumulate within the core (Fig. 3I; not shown) (Navarro and Blackwell, 2005; Navarro et al., 2001).

Immunostaining of L4 and adult germ lines with CAR-1 antisera revealed a pattern remarkably similar to that of CGH-1 (Fig. 3). CAR-1 levels were modest in the proliferating stem cells at the distal end of the gonad, then increased upon entry into meiosis (Fig. 3A). Throughout the gonad some CAR-1 staining was localized to perinuclear particles, where it overlapped substantially with staining for CGH-1 and PGL-1 (Fig. 3C-G; not shown). After germ cells entered meiosis, CAR-1 appeared in cytoplasmic granules within the syncytial gonad core, in parallel with CGH-1 (Fig. 3A, H–I). Within the core, most CAR-1 particles colocalized with CGH-1, although some distinct CAR-1 and CGH-1 foci were also present. The distribution of CGH-1 appeared normal in car-1(RNAi) animals, but CAR-1 localization was highly abnormal in cgh-1(RNAi) hermaphrodites (not shown, and K. Oegema, personal communication) and in the predicted null deletion mutant cgh-
Importance of CAR-1 for oogenesis

Importance of CAR-1 for oogenesis

Without CGH-1, CAR-1 was appropriately associated with P granules in the mitotic region, but in meiotic cells it was no longer detected at P granules, but accumulated in large irregularly shaped aggregates within the core (Fig. 3K-M). By contrast, antibody staining indicated that PGL-1 localization was not severely disrupted in cgh-1(ok492) hermaphrodites (Navarro et al., 2001). Taken together, the data strongly support the idea that CAR-1 is functionally associated with CGH-1.

The distribution of CAR-1 staining was also strikingly similar to that of CGH-1 during embryonic development. During the earliest embryonic stages, both CAR-1 and CGH-1 were associated with P granules (Fig. 4A-F; not shown). CAR-1 and CGH-1 were then also present in somatic cells, where they were diffusely distributed throughout the cytoplasm and also colocalized in foci (Fig. 4A-F; not shown). After the four-cell stage, CAR-1 and CGH-1 disappeared in parallel from somatic cells, but were maintained until approximately the 200-cell stage in the germline (Fig. 4G-O), where they remained associated with P granules (not shown). The strong similarity between the CAR-1 and CGH-1 staining patterns in both the germline and the early embryo remarkably parallels their physical interaction (Fig. 2A,C).

CAR-1/CGH-1 association is conserved

Independently of this C. elegans work, sequencing of additional proteins in the Drosophila Me31B complex (Nakamura et al., 2001; Nakamura et al., 2004) revealed that its most abundant component is the CAR-1 ortholog Trailerhitch (Tral) (Table 1; not shown). In accordance with the interaction observed between CGH-1 and CAR-1 (Fig. 2A,C), endogenous Tral and Me31B coimmunoprecipitated from a Drosophila ovarian extract, and was dependent upon RNA (Fig. 5A). To examine the distribution of Tral in Drosophila ovaries, a strain expressing a GFP-Me31B fusion protein (Nakamura et al., 2001) was immunostained with a Tral-specific antibody (Fig. 5B-D). Me31B forms cytoplasmic particles that contain other members of the Me31B complex, along with specific mRNAs that are translationally regulated by this complex (Nakamura et al., 2001; Nakamura et al., 2004). Tral colocalized with GFP-Me31B in these cytoplasmic particles (Fig. 5B-D). The finding that Drosophila Me31B and Tral are present in the same complex and colocalize in the germline suggests that a functional association between CAR-1 and CGH-1 has been conserved.

Fig. 3. CAR-1 localization in the germline. Extruded gonads from one-day-old hermaphrodites were analyzed by immunostaining for CAR-1 (A) and DAPI staining for DNA (B). The distal region is to the bottom left and the proximal region at the top right. CAR-1 levels are low in the distal mitotic zone but increase as the germ cells enter meiosis (see detail). (C-J) CAR-1 localization during the pachytene stage. Extruded gonads were stained for CAR-1, CGH-1 and PGL-1. (C) Perinuclear localization of CAR-1; (D) merge of CAR-1 with CGH-1 and DAPI staining. (E-G) Merged CAR-1 (green) and CGH-1 (red) staining (E); merged CAR-1 (green) and PGL-1 (red) staining (F); PGL-1 staining alone (G); images are from the area surrounding the nucleus that is indicated in D. The extent of overlap between CGH-1, CAR-1 and PGL-1 staining was reproducibly comparable to that shown, but among individual foci the degree of overlap and the relative orientation of CGH-1- and CAR-1-stained foci varied. (H-J) A cross-section through the germline core, shown to highlight cytoplasmic CAR-1 (H) and CGH-1 (I) foci; a merged image with DAPI staining is shown in J. (K-N) Specific mislocalization of CAR-1 in the cgh-1(ok492) gonad, revealed by antibody staining. In the mitotic region (K), CAR-1 is present at low levels in perinuclear foci that colocalize with PGL-1 (L), as in wild type (not shown), but its localization becomes dramatically altered within the transition zone (TZ). (K-N) DAPI staining; (K-M) CAR-1 staining (green); (L) merge, including PGL-1 (red), which corresponds to the boxed region in K. Within the pachytene region of cgh-1(ok492) germlines, CAR-1 localization is highly abnormal (M), but the levels and localization of PGL-1 antibody staining are not detectably altered (N). (C-L,N) Single plane confocal images; (M) a confocal z-series projection.
**car-1 insufficiency increases physiological germ cell apoptosis**

To investigate whether CAR-1 and CGH-1 are involved in similar processes, we assayed whether germline apoptosis is elevated in *car-1(RNAi)* adult hermaphrodites. At 24 and 48 hours after the L4 molt, acridine orange (AO) staining and the counting of germ cell corpses indicated that two- to threefold more dying germ cells were present in *car-1(RNAi)* animals than in wild type (Fig. 6A,B), similar to the increase seen in *cgh-1(RNAi)* hermaphrodites (Navarro et al., 2001). This cell death required the caspase ortholog *ced-3*, demonstrating that it was apoptotic (Fig. 6A). *car-1* RNAi similarly increased the number of corpses present in the engulfment-defective mutant *ced-1(e1735)* (Hedgecock et al., 1983), indicating that this increase derived from elevated germ cell death, not impaired engulfment by the sheath cells (see Table S1 in the supplementary material).

In *C. elegans*, physiological germline apoptosis occurs only during oogenesis and is induced by a specific pathway that does not require either the p53 ortholog CEP-1 or the proapoptotic protein EGL-1, each of which is needed for genotoxic stress to induce germ cell death (Gumienny et al., 1999; Hofmann et al., 2002). No apoptotic cells were detected in *car-1(RNAi)* males or larval stage hermaphrodites (not shown), and *car-1* knockdown increased germ cell death comparably in wild type, *cep-1* and *ced-9(n1950gf)* mutants (Fig. 6A; not shown). In *ced-9(n1950gf)* animals, EGL-1 fails to trigger apoptosis, so that only the physiological germ cell pathway is still active (Gumienny et al., 1999; Schumacher et al., 2005). We conclude that CAR-1 and CGH-1, two germline proteins that are present in the same RNP, are each important for limiting the frequency of germ cell death through the physiological pathway.

**Oogenesis and embryogenesis abnormalities in car-1(RNAi) animals**

In young *car-1(RNAi)* hermaphrodites, the gonad generally appeared normal; in adults it contained small spherical extracellular bodies that we call anucleate cytoplasmic spheres (ACS) (Fig. 6C). The ACS did not stain with DAPI, were of similar size (3-8 μm), and accumulated primarily within the proximal gonad independently of germ cell apoptosis (see Table S2 in the supplementary material; not shown). They did not appear in males, had a granular appearance like that of oocyte cytoplasm, and stained brightly for the yolk receptor RME-2, an oocyte-specific marker (Grant and Hirsh, 1999) (see Fig. S5 in the supplementary material). Finally, no ACS were detected when *car-1* RNAi was performed in either of the two mutants in which developing oocytes fail to exit pachytene [*mpk-1(ga117) and *gld-1(q485)*; see Table S2 in the supplementary material]. Together, these results suggest that the ACS arose from oocytes that had progressed beyond the pachytene stage.

Despite their elevated levels of germ cell death, and in contrast to *cgh-1(RNAi)* animals (Navarro et al., 2001), *car-1* RNAi similarly increased the number of corpses present in the engulfment-defective mutant *ced-1(e1735)* (Hedgecock et al., 1983), indicating that this increase derived from elevated germ cell death, not impaired engulfment by the sheath cells (see Table S1 in the supplementary material). In *C. elegans*, physiological germline apoptosis occurs only during oogenesis and is induced by a specific pathway that does not require either the p53 ortholog CEP-1 or the proapoptotic protein EGL-1, each of which is needed for genotoxic stress to induce germ cell death (Gumienny et al., 1999; Hofmann et al., 2002). No apoptotic cells were detected in *car-1(RNAi)* males or larval stage hermaphrodites (not shown), and *car-1* knockdown increased germ cell death comparably in wild type, *cep-1* and *ced-9(n1950gf)* mutants (Fig. 6A; not shown). In *ced-9(n1950gf)* animals, EGL-1 fails to trigger apoptosis, so that only the physiological germ cell pathway is still active (Gumienny et al., 1999; Schumacher et al., 2005). We conclude that CAR-1 and CGH-1, two germline proteins that are present in the same RNP, are each important for limiting the frequency of germ cell death through the physiological pathway.

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Importance of CAR-1 for oogenesis

car-1(RNAi) hermaphrodites and males were fertile. However, car-1(RNAi) hermaphrodites consistently produced fewer progeny than wild type did (N2, 257.2±15.2, versus car-1(RNAi), 198.1±34.4; P<0.05; Fig. 7A). In both wild type and ced-3(n717) backgrounds essentially all of these car-1(RNAi) embryos failed to hatch, and instead arrested development with a profound cytokinesis defect in which cleavage furrows began to form but subsequently regressed, so that the first embryonic

Fig. 6. Increased physiological apoptosis in car-1(RNAi) hermaphrodites. (A) The number of germ cells that stained with AO was counted in one gonad arm per animal (n>25) at 24 and 48 hours after the L4 molt. This cell death was not observed in the ced-3(n717) background and occurred at similar levels in car-1(RNAi) and ced-9(n1950gf);car-1(RNAi) hermaphrodites. Error bars represent one standard deviation; asterisks denote P<0.05 by t-test. (B) Merged Nomarski and AO staining images of representative wild-type (N2) and car-1(RNAi) animals. AO-positive cells appear to vary in size because they are detected at different stages of death. White arrowheads indicate some of the apoptotic cells. (C) Presence of anucleate cytoplasmic spheres (ACS) in the car-1(RNAi) hermaphrodite gonad. A late one-day-old gonad is shown. White arrowheads indicate ACS, which accumulate in the proximal gonad and have a granular appearance similar to oocytes. Oocytes at the –1, –2 and –3 positions are indicated.

Fig. 7. Enhancement of oogenesis by physiological apoptosis. (A) Apoptosis facilitates progeny production in car-1(RNAi) and ced-3(RNAi) hermaphrodites. Brood size was only modestly reduced by RNAi depletion of either car-1 or ced-3 in the N2 background, but was dramatically decreased when RNAi was performed in ced-3(n717) animals. In this representative experiment, N2, n=10; ced-3(RNAi), n=21; car-1(RNAi), n=19; ced-3, n=10; ced-3;ced-3(RNAi), n=20; ced-3;ced-3(RNAi), n=21. Among multiple experiments, the brood size of ced-3(n717) varied between 92% and 98% of wild type, always within the range of statistical insignificance. Importantly, in each experiment car-1 and ced-3 RNAi resulted in consistent reductions in brood size in the wild-type and ced-3 backgrounds. Error bars represent one standard deviation; asterisks denote P<0.05 by t-testing the results of car-1 or ced-3 RNAi depletion with either N2 or ced-3(n717) controls. (B-F) Nomarski images of ced-3 and RNAi hermaphrodites. (B) One-day-old ced-3(n717);car-1(RNAi) hermaphrodites accumulate abnormal oocytes at the proximal gonad end. DAPI staining (insert) reveals that these proximal oocytes are arrested in diakinesis. A white bar indicates the approximate region from which the DAPI image was obtained from a fixed whole animal. (C) A one-day-old ced-3(n717) hermaphrodite gonad, depicted as in B, is indistinguishable from wild type. (D) A four-day-old ced-3(n717) hermaphrodite gonad. Compared with in the gonad in C, the pachytene region extends further proximally. Note that only three oocytes are present. (E) One-day-old ced-3(n717);ced-3(RNAi) hermaphrodite gonads have an extended pachytene region (see DAPI staining insert) and fewer oocytes than normal. (F) A three-day-old ced-3(n717);ced-3(RNAi) hermaphrodite gonad. The extended pachytene region is maintained but abnormal oocytes accumulate proximally, many of which are in diakinesis (not shown). Oocytes are outlined by dashed lines in B,D-F.
cell division was generally not completed (Fig. 8) (Gonczy et al., 2000; Piano et al., 2002).

Enhancement of oogenesis efficiency by physiological apoptosis

It is possible that car-1(RNAi) hermaphrodites generate fewer progeny than normal simply because the regulation of physiological apoptosis is perturbed, so that too many of their oocytes are killed. If this is so, car-1(RNAi) hermaphrodites should produce more progeny if this apoptosis were prevented. In striking contrast, although wild-type and caspase-defective ced-3(n717) animals produced comparable numbers of progeny (N2, 257.2±15.2, versus ced-3, 252.1±34.2), in the ced-3 background the brood size of car-1(RNAi) hermaphrodites was reduced dramatically [ced-3;car-1(RNAi), 60±28.6 versus car-1(RNAi); 198.1±34.4; Fig. 7A]. car-1 RNAi thus decreased the brood size of wild-type animals by 23% and that of ced-3 animals by 76%, indicating that ced-3 animals are differentially sensitive to the effects of car-1 RNAi. The elevated oocyte killing that normally occurs in response to car-1 RNAi therefore actually increases the number of progeny that can be produced.

In one-day-old ced-3;car-1(RNAi) animals, the proximal gonad region was filled with a disorganized array of abnormal oocytes that were arrested in diakinesis (Fig. 7B), in striking contrast to either car-1(RNAi) or ced-3 animals (Fig. 6C, Fig. 7C). This accumulation of defective oocytes did not involve accelerated oocyte production, because similar total combined numbers of eggs and oocytes were produced by ced-3 and ced-3;car-1(RNAi) animals during the first 16 hours of adulthood (see Fig. S6 in the supplementary material). When car-1 RNAi was performed in the N2 background, a less severe version of this phenotype eventually appeared in older animals that had ceased to produce progeny (not shown). It is possible that a lack of car-1 leads to the production of individual defective oocytes that are culled by the cell death mechanism, which would thus fulfill a ‘quality control’ function. This model predicts that abnormal car-1(RNAi) oocytes would be generated from the beginning of adulthood. By contrast, during the first 12 hours of adulthood ced-3 and ced-3;car-1(RNAi) animals produced comparable numbers of progeny, and abnormal oocytes began to appear in only a small minority of ced-3;car-1(RNAi) gonads (not shown). It was only as oogenesis continued that ced-3;car-1(RNAi) animals produced progeny at a decreased rate; the animals eventually progressed to gonad failure before sperm were depleted. Taken together, the data suggest that the increased cell death occurring in car-1(RNAi) animals does not involve the elimination of individual defective cells, but instead promotes the production of functional oocytes.

To investigate whether germ cell death similarly facilitates oogenesis in a context where viable progeny are generated, we examined cpb-3(RNAi) animals. cpb-3 is expressed primarily during oogenesis (Luitjens et al., 2000), and its depletion by RNAi increases the frequency of physiological germ cell death similarly to cgh-1 or car-1 depletion (not shown) (Lettre et al.,

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**Fig. 8.** Cytokinesis defects in car-1(RNAi) embryos. Selected frames from a time-lapse Nomarski video recording (not shown) obtained from wild-type (A-H) and car-1(RNAi) (I-P) embryos. The relative time of each exposure is indicated in minutes. Anterior is to the left; black dots represent centrosomes; black arrowheads indicate cleavage furrows; white arrows indicate nuclei in the car-1(RNAi) embryo. Pronuclei appear in the anterior (maternal) and posterior (paternal) regions of the embryo (A, I). The maternal pronucleus migrates to the posterior of the embryo and associates with the paternal pronucleus (B, J). Pronuclear move towards the middle of the embryo before fusing (C, K). Wild-type embryos then initiate the first mitotic division: (C) late prophase; (D) anaphase; (E) appearance of cleavage furrows (arrowheads). The cell cycle continues, producing embryos of two (F), four (G) and six (H) cells. Defects in car-1(RNAi) embryos become apparent during the first cell division. Pronuclear fusion is delayed (K), and cleavage furrows begin to form (not shown) but subsequently regresses, giving rise to a one-cell embryo containing two nuclei (N). During the next cell cycle, cytokinesis is re-initiated and cleavage furrows develop (arrowheads, O), but again these regress coincident with the reappearance of the nuclei. Embryonic cell cycles that are coupled with abnormal cytokinesis continue, resulting in multinucleated cells (P; arrowheads, nuclei).
In contrast to car-1(RNAi) animals, cpb-3(RNAi) hermaphrodites are not only fertile but also give rise to 100% viable progeny (Fig. 7A, not shown) (Lettre et al., 2004). Interestingly, CPB-3 is the closest C. elegans ortholog of the RNA-binding CPEB translational regulators (Luitjens et al., 2000), which associate physically and functionally with CGH-1 orthologs in Drosophila, Xenopus and humans (Table 1) (Mansfield et al., 2002; Minshall and Standart, 2004; Sommerville, 1999). This last evidence suggests that CPB-3 might be functionally associated with the C. elegans CGH-1/CAR-1 complex, even though we did not detect it under our immunoprecipitation conditions.

cpb-3(RNAi) animals produced only slightly fewer progeny than did wild type, but in the ced-3 background their brood size was significantly reduced (Fig. 7A). In one-day-old ced-3;cpb-3(RNAi) adults, the pachytene region of the gonad was abnormally extended towards the proximal end (Fig. 7E; not shown), a pattern similar to that observed in old ced-3 animals after sperm depletion (Fig. 7D; not shown) (Gumienny et al., 1999). In addition to this apparent failure to exit pachytene appropriately, in old ced-3;cpb-3(RNAi) animals abnormal small oocytes that were arrested in diakinesis accumulated at the proximal gonad end (Fig. 7F; not shown). These defects are similar to those seen in ced-3;car-1(RNAi) hermaphrodites (Fig. 7B). These cpe-3 experiments reveal a second example in which a progressively worsening oogenesis defect is partially suppressed by an increase in physiological apoptosis, and suggest that CAR-1 and CPB-3 may function in overlapping processes.

**Discussion**

**Localization of CAR-1 to a conserved germline RNP**

The RNA helicase CGH-1 and its orthologs function during germ cell development in C. elegans, Drosophila and Xenopus (Ladomery et al., 1997; Minshall and Standart, 2004; Nakamura et al., 2001; Navarro et al., 2001). We now show that, in C. elegans, CGH-1 associates physically and functionally with the previously uncharacterized, predicted RNA-binding protein CAR-1. We have identified a conserved RNP complex that contains CGH-1 and CAR-1, along with Y-box proteins (Fig. 2A,C; Table 1). CAR-1 and CGH-1 colocalize in the germline and early embryo, and, in meiotic cells, the localization of CAR-1 depends upon CGH-1 (Figs 3, 4). The respective *Drosophila* CGH-1 and CAR-1 orthologs Me31B and Tral similarly interact in a RNA-dependent manner, and colocalize in the germline (Fig. 5B-D). In C. elegans, physiological cell death is dramatically increased by knockdown of CGH-1, CAR-1 or CBP-3, orthologs of which are functionally and physically associated with components of the CGH-1 complex in other species (Table 1). A functional association among CGH-1, CAR-1 and, possibly, CPEB orthologs thus may be a conserved aspect of germline development.

The *S. cerevisiae* CGH-1 ortholog Dhh1 is a characteristic component of P bodies and is required for their mRNA degradation function (Coller and Parker, 2004; Sheth and Parker, 2003). Similarly, the CGH-1 ortholog RCK is typically found in mammalian P bodies (Cougot et al., 2004). Although this suggests that the CAR-1/CGH-1 foci we have described may correspond to a type of P body, it seems unlikely that mRNA degradation is their major function during oogenesis, in which mRNAs accumulate. In yeast, Dhh1-containing P bodies accumulate deadenylated mRNAs and increase in size if their degradative apparatus is blocked (Sheth and Parker, 2003), and in metazoans deadenylation is the major mechanism for restricting maternal mRNA translation (Johnstone and Lasko, 2001). If germline CGH-1/CAR-1 foci are involved in storage or translational regulation but not degradation of maternal mRNA, it could explain why they increase in intensity and appear in the gonad core in parallel with newly synthesized mRNA (Fig. 3A,J) (Gibert et al., 1984; Navarro and Blackwell, 2005; Navarro et al., 2001; Schisa et al., 2001), and why physical association between CGH-1 orthologs and the mRNA decapping/degradation machinery has not been detected in the metazoan germline (Table 1).

In general, RNA helicases are each found in a specific set of RNA-protein complexes, where they facilitate RNA-RNA or RNA-protein interactions (Rocak and Linder, 2004). Our finding that CGH-1 is required for CAR-1 localization specifically after meiosis entry (Fig. 3K) suggests that one function of this helicase might be to facilitate the formation of CGH-1/CAR-1 RNP particles as these proteins and newly synthesized mRNA accumulate to high levels within the gonad core. In the embryo, CGH-1 and CAR-1 persist until the approximate 200-cell stage in the germline, but begin to disappear after the four-cell stage in somatic cells (Fig. 4), a pattern remarkably similar to that of a major maternal mRNA subset (Seydoux and Fire, 1994). We speculate that some CGH-1/CAR-1 foci might function as degradative P bodies in the embryo, where maternal mRNAs must be disposed of in a regulated fashion.

**CAR-1 and CPB-3 are required for normal oogenesis**

car-1 is required for oogenesis, as is shown by evidence that, in car-1(RNAi) hermaphrodites, ACS are produced, physiological apoptosis is elevated, and brood size is dramatically reduced and abnormal oocytes are formed if this cell death is prevented (Fig. 6, Fig. 7A,B). ACS were not detected in cpb-3(RNAi) or cgh-1(RNAi) animals, although in the latter case misshapen oocyte fragments may appear in the proximal gonad (not shown). It is striking that a lack of CAR-1, an apparent RNA-binding protein, results in two phenotypes that are consistent with cytoskeletal or membrane abnormalities (ACS production and a cytokinesis defect). In car-1(RNAi) embryos, maternally derived PAR-1 and PAR-3 (Kemphues et al., 1988) are appropriately localized to the posterior and anterior, respectively (not shown), indicating that many aspects of maternal gene expression are intact. car-1 knockout therefore does not globally perturb mRNA metabolism or translation, but affects a more specific cellular process or a mRNA subset. In *Xenopus* eggs, the CAR-1 ortholog RAP55 was recently found within a large RNP that is distinct from the CGH-1/CAR-1 complex, and that is required for the centrosome-independent pathway of mitotic spindle assembly (Blower et al., 2005), suggesting that CAR-1 might be involved in localizing or regulating specific mRNAs in multiple contexts in germ cells.

Our evidence that cpb-3 decreases brood size is consistent with the hypothesis that CPB-3 is important for oogenesis. No other function has been defined for CPB-3, and RNAi experiments suggest that it does not function redundantly with...
the three other *C. elegans* CPEB-related proteins (Luitjens et al., 2000). In some species, CPEB proteins regulate oocyte maturation through stimulating readenylation and translation of specific mRNAs (Cao and Richter, 2002), but none of the *C. elegans* CPEB proteins have been implicated in this process (Lettre et al., 2004; Luitjens et al., 2000). Interestingly, oocytes are unable to exit pachytene in mice that lack CPEB (Tay and Richter, 2001). The pachytene region is extended in the *ced-3; cpb-3(RNAi)* hermaphrodite germline (Fig. 7E), suggesting that CPB-3 is involved in pachytene exit, and that this function for CPEB proteins might be conserved in some metazoa.

**CGH-1, CAR-1 and physiological apoptosis**

Little is understood about the regulation or functions of developmental germ cell death (see Introduction). In *car-1(RNAi)* and *cpb-3(RNAi)* animals, an increase in cell death partially compensates for an oogenesis defect, as indicated by the markedly increased severity of their germline abnormalities in the *ced-3* background (Fig. 7). However, in *ced-3;car-1(RNAi)* and *ced-3;cpb-3(RNAi)* hermaphrodites, abnormal small oocytes appear only rarely during the first 12 hours of adulthood (Fig. 7B,F; not shown), suggesting that these oocytes do not derive from individual abnormal cells that would otherwise be 'culled' by apoptosis. One possibility is that the consequences for oogenesis of lacking either CAR-1 or CPB-3 are initially not as severe because germ cell components have been accumulated during larval stages, but that they become catastrophic after these stores have been depleted. Physiological apoptosis may then sustain the process of oogenesis by increasing the supply or facilitating the organization of important cytoplasmic constituents. It is consistent with this model that the dying nuclei normally appear to function as nurse cells (Gumienny et al., 1999), and that the frequency of physiological apoptosis increases over time in both wild-type and *car-1(RNAi)* animals (Fig. 6A). This cell death pathway may thus be regulated by a cytoplasmic ‘checkpoint’, which functions in parallel to the p53-dependent mechanisms that trigger cell death in response to genotoxic stress.

In *C. elegans*, whole genome RNAi screening and our experiments have identified six genes that specifically limit the frequency of physiological germ cell death (Lettre et al., 2004; Navarro et al., 2001) (this work). These genes encode a predicted E3 ubiquitin ligase (R05D3.4), a kinase (PMK-3), and four predicted RNA-binding proteins: CGH-1, CAR-1, CPB-3, and the zinc finger protein T02E1.3a. Although this list is unlikely to be complete, the small number of genes it includes suggests that the physiological apoptosis pathway responds to specific cues. It is remarkable that two of these proteins (CGH-1 and CAR-1) associate with each other, and that a third (CPB-3) is functionally associated with CGH-1 orthologs in other species (Table 1). This suggests that the regulation of physiological apoptosis may be influenced specifically by certain functions of the CGH-1/CAR-1 complex. Thus, lack of CGH-1, CAR-1 or CPB-3 may lead to inappropriate metabolism or regulation of particular mRNAs, resulting in oogenesis abnormalities that can be compensated for by increased oocyte death. One intriguing possibility is that the effects of *cgfh-1* RNAi on physiological germ cell death might derive from the mislocalization of CAR-1 (Fig. 3K-M). The sterility and cytokinesis defects seen in *cgf-1(RNAi)* and *car-1(RNAi)* animals, respectively, presumably stem from additional requirements for CGH-1 and CAR-1 function.

In species as diverse as *C. elegans* and mice, around the time of pachytene exit it is decided whether each oocyte will survive or die (Gumienny et al., 1999; Pepling and Spradling, 2001). This process occurs approximately as cytoplasmic communications among oocytes end. It has been proposed that a function of developmental germ cell apoptosis is to maintain mitochondrial genome integrity by eliminating unfit mitochondria (Krackauer and Mira, 1999; Pepling and Spradling, 2001). In developing *Drosophila* oocytes, mitochondria that are preserved for the germline in the next generation appear to localize to the Balbiani body (Cox and Spradling, 2003), an oocyte organelle associated with numerous mRNAs (Matova et al., 1999). It is intriguing that in *Xenopus* oocytes the CGH-1 ortholog Xp54 is highly enriched in the Balbiani body, and that, in *Drosophila*, proteins and mRNAs that associate with the CGH-1 ortholog Me31B interact transiently with this structure (Cox and Spradling, 2003; Smillie and Sommerville, 2002). These associations suggest the exciting possibility that a specific connection between the CGH-1/CAR-1 complex and the regulation of developing germ cell survival may be conserved.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/22/4975/DC1

**References**


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