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

Peter R. Boag[1], Akira Nakamura[2] and T. Keith Blackwell[1,*]

[1]Joslin Diabetes Center and Department of Pathology, Harvard Medical School, One Joslin Place, Boston, MA 02215, USA
[2]Laboratory for Germline Development, RIKEN Center for Developmental Biology, Kobe, Hyogo 650-0047, Japan
*Author for correspondence (e-mail: keith.blackwell@joslin.harvard.edu)

Accepted 24 August 2005

Development 132, 4975-4986
Published by The Company of Biologists 2005
doi:10.1242/dev.02060

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 by a lack of the RNA helicase CGH-1, orthologs of which are involved in translational control in oocytes and decapping-dependent mRNA degradation in yeast processing (P) bodies. Here, we describe a predicted RNA-binding protein, CAR-1, that associates with CGH-1 and Y-box proteins within a conserved germline RNA-protein (RNP) complex, and in cytoplasmic particles in the gonad and early embryo. The CGH-1/CAR-1 interaction is conserved in *Drosophila* oocytes. When *car-1* expression is depleted by RNA interference (RNAi), physiological apoptosis is increased, brood size is modestly reduced, and early embryonic cytokinesis is abnormal. Surprisingly, 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 metazoa 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 culls 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 germline is maintained from

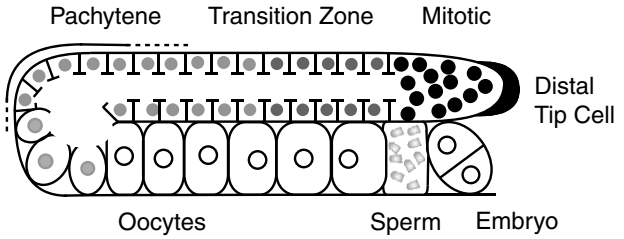

Fig. 1. A *C. elegans* adult hermaphrodite gonad arm, within which germ cells develop in an assembly-line fashion (Hubbard and Greenstein, 2000; Schedl, 1997). The somatic distal tip cell maintains a population of self-renewing germline stem cells. The first germ cells to enter meiosis form approximately 160 sperm during the L4 stage, then, during adulthood, exclusively oocytes are produced. As germ cells move away from the distal tip, they enter meiotic prophase I in the transition zone, then progress to the pachytene stage. The germline initially consists of a syncytium, in which germ cells are only partially enclosed in a membrane and share a common cytoplasmic core. The gonad bend or 'loop' region, where germ cell deaths normally occur, is indicated by a line outside of the gonad. After exit from pachytene, developing oocytes begin to form discrete cells that enlarge as they move through the loop. During diakinesis, a signal from sperm directs oocytes to undergo maturation. Oocytes are then fertilized as they move through the spermatheca into the uterus.

one generation to the next by ribonucleoprotein (RNP) particles that are referred to as germ plasm (Houston and King, 2000; Rongo et al., 1997; Saffman and Lasko, 1999; Wylie, 2000). For example, *C. elegans* germline cells are distinguished by P granules that are present throughout the life cycle (Strome and Wood, 1982). In essentially all metazoa, additional regulatory complexes that are distinct from germ plasm maintain some oocyte mRNAs in a deadenylated and translationally quiescent state until these mRNAs are to be translated (Chang et al., 2004; Cao and Richter, 2002; Johnstone and Lasko, 2001).

In *C. elegans*, each of these conserved aspects of oogenesis appears to involve the DEAD box RNA helicase CGH-1. When CGH-1 is lacking, physiological apoptosis is dramatically elevated and non-functional gametes are formed (Navarro et al., 2001). CGH-1 is germline specific and associates with P granules, but it also accumulates in additional cytoplasmic foci in oocytes. In both *Drosophila* and *Xenopus*, the CGH-1 ortholog and associated proteins bind and restrict the translation of maternal mRNAs (Ladomery et al., 1997; Minshall et al., 2001; Nakamura et al., 2001; Nakamura et al., 2004; Wilhelm et al., 2003; Wilhelm et al., 2000). Although this evidence predicts a translational regulatory function for CGH-1, in *S. cerevisiae* and human cells, the CGH-1 ortholog is an important functional component of cytoplasmic organelles called processing bodies (P bodies), in which mRNAs that are translationally inhibited and deadenylated are decapped, then degraded (Cougot et al., 2004; Sheth and Parker, 2003). mRNAs can be sequestered transiently in these dynamic structures if this degradation is blocked, leading to the suggestion that in the metazoan germline mRNA may be stored in P body-like structures (Coller and Parker, 2004; Sheth and Parker, 2003; Teixeira et al., 2005).

We have now identified a germline RNA-binding protein 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 germline 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(+/w40)* (Derry et al., 2001); *cgh-1(ok492)*; *gld-1(q485)* (Francis et al., 1995); *mpk-1(ga117)* (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; NH_2-CKAEGKTGRPDWKKERETNQE-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 NH_2-CDPKLYVADQQLVDAADETTA-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 His_6-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 AOBS 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_2VO_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 of 1/2500. For mass spectroscopy, CGH-1 immunoprecipitations were conducted essentially 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 NGM 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.

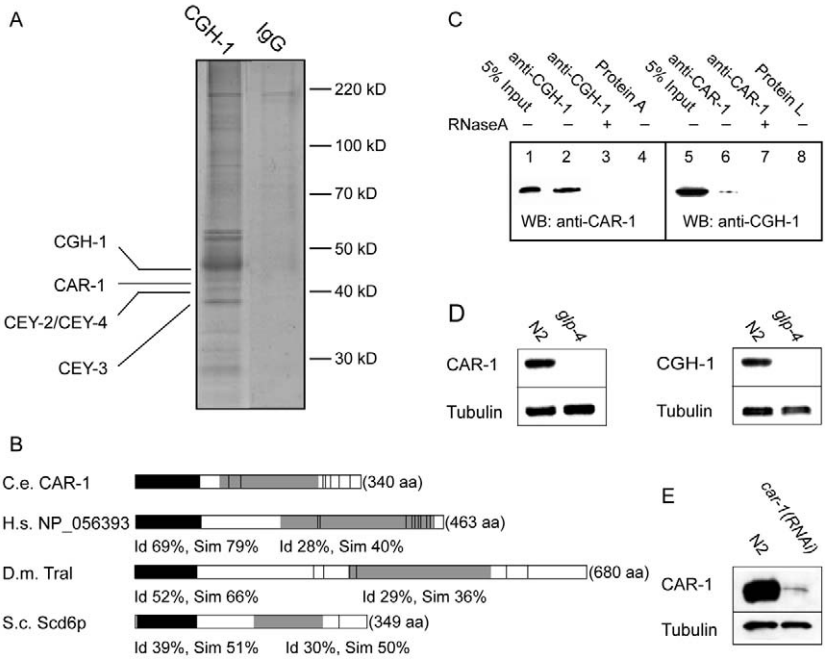

Fig. 2. CAR-1 and CGH-1 associate together in a RNA-dependent complex. (A) Immunoprecipitation of endogenous CGH-1-associated proteins from one-day-old adult hermaphrodites, identified by mass spectroscopy. (B) Each CAR-1 ortholog includes a Sm-like domain within a characteristic conserved region (black) and a variable number of Arginine-Glycine-Glycine (RGG) triplets or functional equivalents (vertical bars) (Birney et al., 1993). An additional conserved region (shaded) contains a FDF domain, a sequence motif of unknown function (Anantharaman and Aravind, 2004). The percentage identity (Id) and similarity (Sim) to *C. elegans* CAR-1 within these regions was determined by pair-wise BLAST. CAR-1, *C. elegans* (NP_493254); Hs, *H. sapiens* (NP_056393); Tral, *D. melanogaster* (AAF49905); Scd6p, *S. cerevisiae* (NP_015454). (C) Interaction between CAR-1 and CGH-1 requires RNA. Co-immunoprecipitation of CAR-1 and CGH-1 from *C. elegans* extracts (lanes 2 and 6) was abolished by RNase A treatment (lanes 3 and 7). Lanes 4 and 8 show control incubations. (D) CAR-1 and CGH-1 are detected specifically in the germline. Lysates from one-day-old adult wild-type worms (N2) and germline-deficient *glp-4(bn2)* hermaphrodites (Beanan and Strome, 1992) were analyzed by western blotting with CAR-1 and CGH-1 antisera, using Tubulin as a control. The CAR-1 antibody recognized a single germline-specific species of 45 kDa, larger than the predicted size of 37.6 kDa. (E) Diminished CAR-1 protein levels in *car-1(RNAi)* worms. Protein extracts from wild-type (N2) or *car-1(RNAi)* one-day-old adults were analyzed by western blotting.

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-precipitate 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

Protein type	*C. elegans*§	*Drosophila*¶	*Xenopus***	*S. cerevisiae*††	*Homo sapiens*‡‡ (HeLa cells)
DEAD-box helicase	CGH-1	Me31B	Xp54	Dhh1	RCK
Sm-like domain	CAR-1*	Tral*	?	?	?
CPEB family	CPB-3‡	Orb†	CPEB*	–	CPEB1†
Y-box	CEY-2, 3, 4*	Yps*	FRGY2*	?	?
Cap-binding	?	eIF4E*	eIF4E†	?	?
eIF4E-binding	–	Cup*	Maskin†	–	–
	–	Exu*	–	–	–
5′-3′ mRNA decay machinery	?	?	?	Lsm*, Dcp1*, Dcp2*, Xrn1*	hDcp1a†, hDcp1b†, hDcp2†, hLsm†, hCcr4†

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.
¶Mansfield et al., 2002; Nakamura et al., 2001; Nakamura et al., 2004; Wilhelm et al., 2003; Wilhelm et al., 2000.
**Ladomery et al., 1997; Minshall and Standart, 2004; Stebbins-Boaz et al., 1999.
††Sheth and Parker, 2003; Tharun et al., 2000.
‡‡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 dcp1 or GW bodies, but for simplicity we refer to them here as P bodies.
?, Unambiguous ortholog in genome, but no functional information available.
–, No clear ortholog has been described.

(Ladomery et al., 1997; Nakamura et al., 2001; Wilhelm et al., 2000). This suggests that the *C. elegans* complex within which the Y-box proteins and CGH-1 associate has been conserved.

In this study, we have analyzed the previously uncharacterized protein CAR-1, which we identified in the CGH-1 immunoprecipitates (Fig. 2A). Essentially all eukaryotes encode a CAR-1 ortholog (Fig. 2B; see Fig. S2 in the supplementary material), but the functions of these proteins have not been described. Each CAR-1 protein typically contains two RNA-associated motifs: a Sm-like domain (see Fig. S3 in the supplementary material), and a variable number of arginine-glycine-glycine (RGG) motifs at the carboxyl terminus. Sm-like domains are ancient protein-protein and protein-RNA interaction modules that are found in prokaryotes and eukaryotes (Salgado-Garrido et al., 1999; Seraphin, 1995). RGG clusters may form a 'RGG box' RNA-binding domain and are found in numerous proteins, including the P granule proteins PGL-1 and PGL-3 (Kawasaki et al., 2004; Kawasaki et al., 1998).

In general, protein complexes in which CGH-1 or CAR-1 orthologs have been found are stabilized by RNA. Interactions among several *Drosophila* Me31B complex components are RNA dependent (Nakamura et al., 2001; Nakamura et al., 2004), and the *Pleurodeles waltl* (newt) CAR-1 ortholog RAP55 and *Xenopus* CGH-1 ortholog Xp54 are each present in RNAse-sensitive oocyte RNP complexes (Lieb et al., 1998). In yeast, mRNA is essential for the formation and structural integrity of P bodies (Sheth and Parker, 2003; Teixiera et al., 2005). We tested whether the interaction between CGH-1 and CAR-1 also requires RNA, by immunoprecipitating these endogenous proteins from *C. elegans* extracts in the presence or absence of RNase A. Reciprocal western blotting of these immunoprecipitations showed that the interaction between CGH-1 and CAR-1 was abolished by RNase A (Fig. 2C), indicating that RNA is required for CAR-1 and CGH-1 to be present together within a RNP complex.

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 germlines 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-J). 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-*

1(ok492) (see Fig. S4 in the supplementary material; Fig. 3K-M). 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 (Fig. 3N), as reported previously for *cgh-1(RNAi)* animals (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).

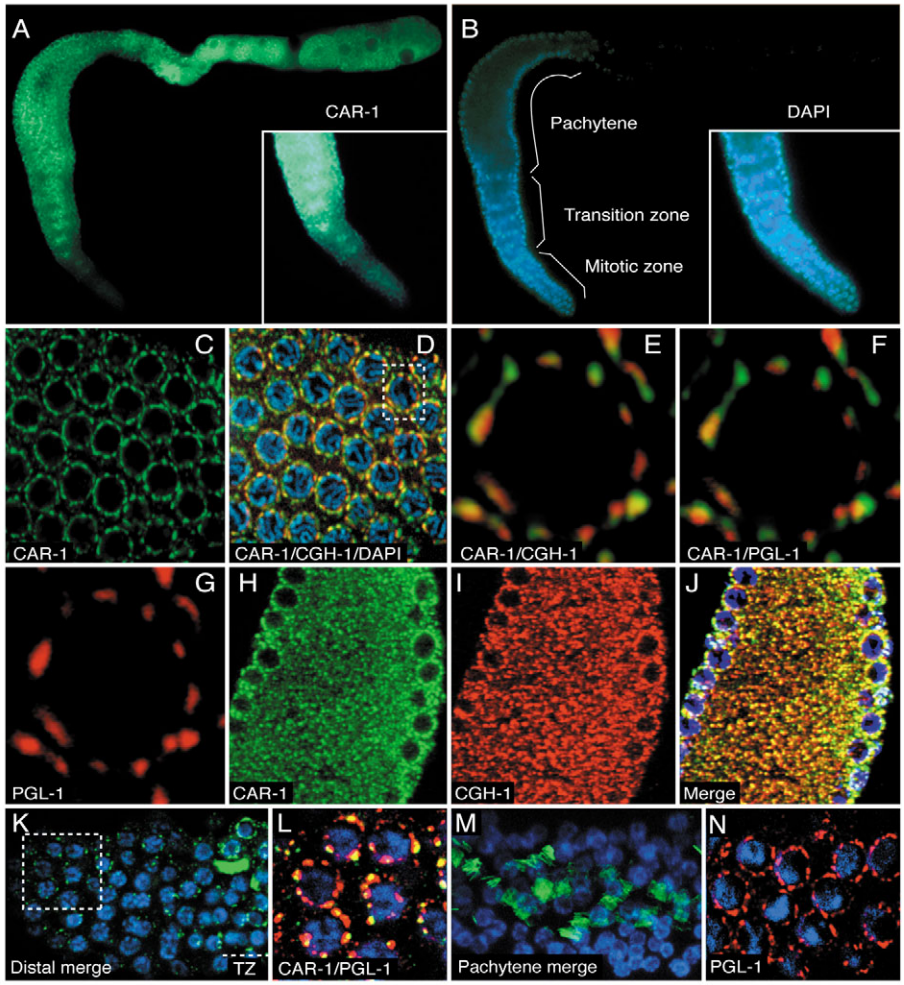

Fig. 3. CAR-1 localization in the germline. Extruded gonads from one-day-old hermaphrodites were analyzed by immunostaining for CAR-1 (A) and DAPI staining for DNA (B). The distal region is to the bottom left and the proximal region at the top right. CAR-1 levels are low in the distal mitotic zone but 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/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.

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-*

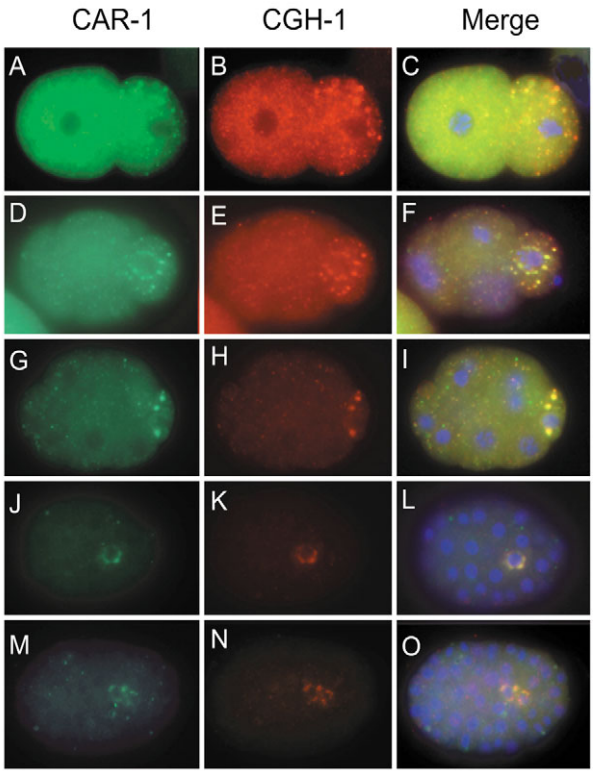

Fig. 4. CAR-1 colocalizes with CGH-1 in the embryo. Two-cell (A-C), four-cell (D-F), 16-cell (G-I), ~50-cell (J-L) and ~100-cell (M-O) embryos were immunostained for CAR-1 (A,D,G,J,M) and CGH-1 (B,E,H,K,N). Merged images that include DAPI staining are also shown (C,F,I,L,O). Anterior is to the left.

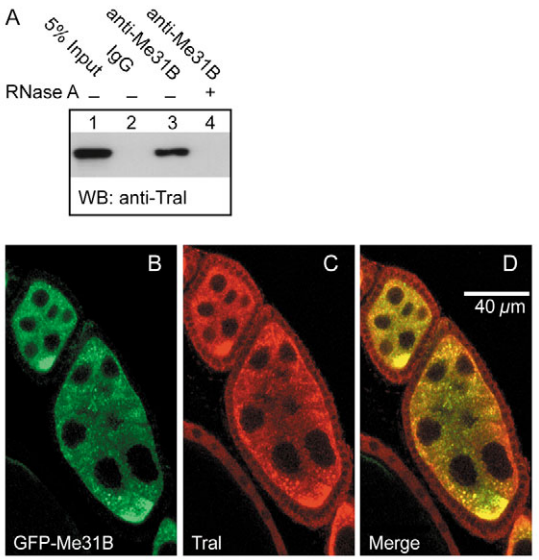

Fig. 5. *Drosophila* Tral and Me31B form a RNA-dependent complex and colocalize in the germline. (A) RNA-dependent interaction between *Drosophila* Tral (CAR-1 ortholog) and Me31B (CGH-1 ortholog). Extracts from *Drosophila* ovaries were immunoprecipitated with a Me31B antibody (Nakamura et al., 2004), and analyzed for the presence of Tral by western blotting. Co-immunoprecipitation of Tral with Me31B (lane 3) was abolished by RNase A treatment (lane 4). Lane 2 shows protein extract incubated with control IgG. (B-D) Extensive colocalization (D) of Me31B (B) and Tral (C) in cytoplasmic granules in the *Drosophila* germline. A stage-7 egg chamber expressing GFP-Me31B was stained with a Tral-specific antibody.

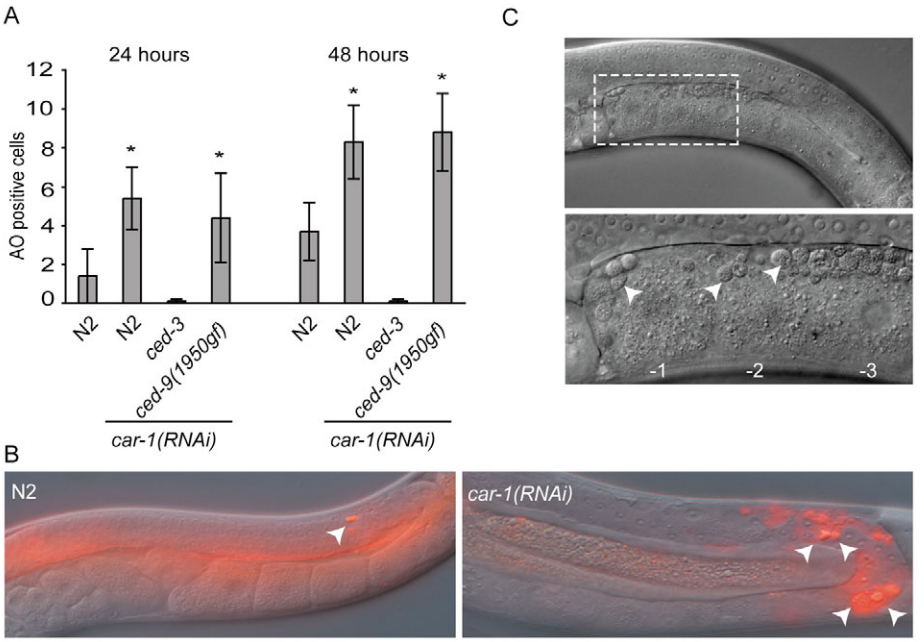

Fig. 6. Increased physiological apoptosis in *car-1(RNAi)* hermaphrodites. (A) The number of germ cells that stained with AO was counted in one gonad arm per animal (n>25) at 24 and 48 hours after the L4 molt. This cell death was not observed in the *ced-3(n717)* background and occurred at similar levels 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.

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

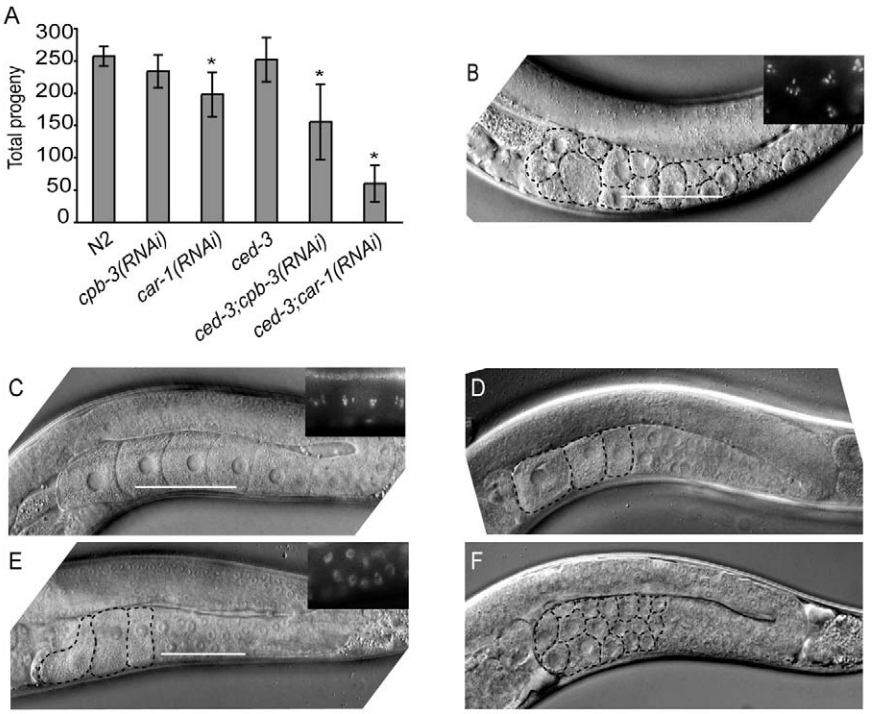

Fig. 7. Enhancement of oogenesis by physiological apoptosis. (A) Apoptosis facilitates progeny production in *car-1(RNAi)* and *cpb-3(RNAi)* hermaphrodites. Brood size was only modestly reduced by RNAi depletion of either *car-1* or *cpb-3* in the N2 background, but was dramatically decreased when RNAi was performed in *ced-3(n717)* animals. In this representative experiment, N2, *n=10*; *cpb-3(RNAi)*, *n=21*; *car-1(RNAi)*, *n=19*; *ced-3*, *n=10*; *ced-3;cpb-3(RNAi)*, *n=20*; *ced-3;cpb-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 *cpb-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-test comparing the results of *car-1* or *cpb-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);cpb-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);cpb-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.

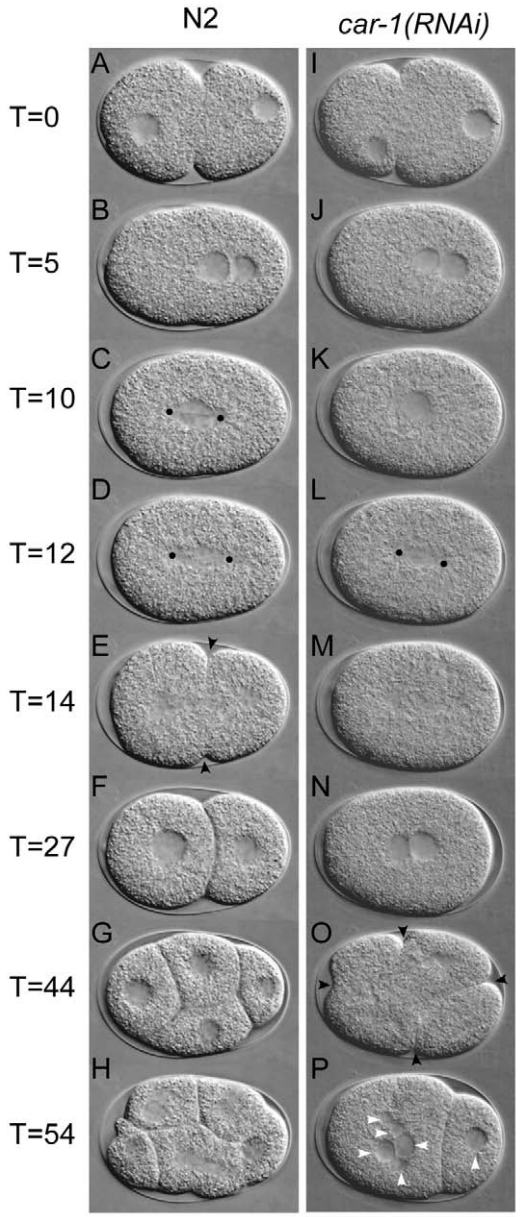

Fig. 8. Cytokinesis defects in *car-1(RNAi)* embryos. Selected frames from a time-lapse Nomarski video recording (not shown) obtained from wild-type (A-H) and *car-1(RNAi)* (I-P) embryos. The relative time of each exposure is indicted in minutes. Anterior is to the left; black dots represent centrosomes; 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). Pronuclei 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).

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.,

2004). 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 *cpb-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 metazoa 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* knockdown 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 in *ced-3(n717)* animals reveals 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 thus may 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 *cgh-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 *cgh-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 (Krakauer 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.

We thank the Caenorhabditis Genetics Center, Yuji Kohara and Susan Strome for reagents, Benedikt Kessler and Eric Spooner for mass spectroscopy, and David Greenstein for the outcrossed *cgh-1(ok492)* strain DG1764. We are also grateful to Roy Parker for helpful discussions, and to Karen Oegema and John White for communicating unpublished results and critically reading this manuscript, for which we also thank Amy Walker. P.R.B. was supported by a Myra Reinhard Family Foundation fellowship. This work was supported by funding from the NIH to T.K.B.

Supplementary material

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

References

Anantharaman, V. and Aravind, L. (2004). Novel conserved domains in proteins with predicted roles in eukaryotic cell-cycle regulation, decapping and RNA stability. *BMC Genomics* **5**, 45.

Baker, T. G. (1963). A quantitative and cytological study of germ cells in human ovaries. *Proc. R. Soc. London B Biol. Sci.* **158**, 417-433.

Beanan, M. J. and Strome, S. (1992). Characterization of a germ-line proliferation mutation in *C. elegans*. *Development* **116**, 755-766.

Birney, E., Kumar, S. and Krainer, A. R. (1993). Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. *Nucleic Acids Res.* **21**, 5803-5816.

Blower, M. D., Nachury, M., Heald, R. and Weis, K. (2005). A Rae1-containing ribonucleoprotein complex is required for mitotic spindle assembly. *Cell* **121**, 223-234.

Borum, K. (1961). Oogenesis in the mouse. A study of the meiotic prophase. *Exp. Cell Res.* **24**, 495-507.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Cao, Q. and Richter, J. D. (2002). Dissolution of the maskin-eIF4E complex by cytoplasmic polyadenylation and poly(A)-binding protein controls cyclin B1 mRNA translation and oocyte maturation. *EMBO J.* **21**, 3852-3862.

Chang, P., Torres, J., Lewis, R. A., Mowry, K. L., Houliston, E. and King, M. L. (2004). Localization of RNAs to the mitochondrial cloud in *Xenopus* oocytes through entrapment and association with endoplasmic reticulum. *Mol. Biol. Cell* **15**, 4669-4681.

Coller, J. and Parker, R. (2004). Eukaryotic mRNA decapping. *Annu. Rev. Biochem.* **73**, 861-890.

Cougot, N., Babajko, S. and Seraphin, B. (2004). Cytoplasmic foci are sites of mRNA decay in human cells. *J. Cell Biol.* **165**, 31-40.

Cox, R. T. and Spradling, A. C. (2003). A Balbiani body and the fusome mediate mitochondrial inheritance during *Drosophila* oogenesis. *Development* **130**, 1579-1590.

Derry, W. B., Putzke, A. P. and Rothman, J. H. (2001). *Caenorhabditis elegans* p53: role in apoptosis, meiosis, and stress resistance. *Science* **294**, 591-595.

Eystathioy, T., Jakymiw, A., Chan, E. K., Seraphin, B., Cougot, N. and Fritzler, M. J. (2003). The GW182 protein colocalizes with mRNA degradation associated proteins hDcp1 and hLSm4 in cytoplasmic GW bodies. *RNA* **9**, 1171-1173.

Francis, R. X., Barton, M. K., Kimble, J. and Schedl, T. (1995). *gld-1*, a tumor suppressor gene required for oocyte development in *Caenorhabditis elegans*. *Genetics* **139**, 579-606.

Gartner, A., Milstein, S., Ahmed, S., Hodgkin, J. and Hengartner, M. O. (2000). A conserved checkpoint pathway mediates DNA damage-induced apoptosis and cell cycle arrest in *C. elegans*. *Mol. Cell* **5**, 435-443.

Gibert, M. A., Starck, J. and Beguet, B. (1984). Role of the gonad cytoplasmic core during oogenesis of the nematode *Caenorhabditis elegans*. *Biol. Cell* **50**, 77-85.

Gonczy, P., Echeverri, G., Oegema, K., Coulson, A., Jones, S. J., Copley, R. R., Duperon, J., Oegema, J., Brehm, M., Cassin, E. et al. (2000). Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. *Nature* **408**, 331-336.

Grant, B. and Hirsh, D. (1999). Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol. Biol. Cell* **10**, 4311-4326.

Gumienny, T. L., Lambie, E., Hartwieg, E., Horvitz, H. R. and Hengartner, M. O. (1999). Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. *Development* **126**, 1011-1022.

Hedgecock, E. M., Sulston, J. E. and Thomson, J. N. (1983). Mutations affecting programmed cell, deaths, in, the nematode *Caenorhabditis elegans*. *Science* **220,** 1277-1279.

Hofmann, E. R., Milstein, S., Boulton, S. J., Ye, M., Hofmann, J. J., Stergiou, L., Gartner, A., Vidal, M. and Hengartner, M. O. (2002). *Caenorhabditis elegans* HUS-1 is a DNA damage checkpoint protein required for genome stability and EGL-1-mediated apoptosis. *Curr. Biol.* **12**, 1908-1918.

Houston, D. W. and King, M. L. (2000). Germ plasm and molecular determinants of germ cell fate. *Curr. Top. Dev. Biol.* **50**, 155-181.

Hubbard, E. J. and Greenstein, D. (2000). The *Caenorhabditis elegans* gonad: a test tube for cell and developmental biology. *Dev. Dyn.* **218**, 2-22.

Ingelfinger, D., Arndt-Jovin, D. J., Luhrmann, R. and Achsel, T. (2002). The human LSm1-7 proteins colocalize with the mRNA-degrading enzymes Dcp1/2 and Xrnl in distinct cytoplasmic foci. *RNA* **8**, 1489-1501.

Johnstone, O. and Lasko, P. (2001). Translational regulation and RNA localization in *Drosophila* oocytes and embryos. *Annu. Rev. Genet.* **35**, 365-406.

Kawasaki, I., Shim, Y. H., Kirchner, J., Kaminker, J., Wood, W. B. and Strome, S. (1998). PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in *C. elegans. Cell* **94**, 635-645.

Kawasaki, I., Amiri, A., Fan, Y., Meyer, N., Dunkelbarger, S., Motohashi, T., Karashima, T., Bossinger, O. and Strome, S. (2004). The PGL family proteins associate with germ granules and function redundantly in *Caenorhabditis elegans* germline development. *Genetics* **167**, 645-661.

Kemphues, K. J., Priess, J. R., Morton, D. G. and Cheng, N. S. (1988). Identification of genes required for cytoplasmic localization in early *C. elegans* embryos. *Cell* **52**, 311-320.

Kobayashi, S., Amikura, R., Nakamura, A. and Lasko, P. F. (1999). Techniques for analyzing protein and RNA distribution in *Drosophila* ovaries and embryos at structural and ultrastructural resolution. In *Advances in Molecular Biology: a Comparative Methods Approach to the Study of Oocytes and Embryos* (ed. J. D. Richter), pp. 426-445. New York: Oxford University Press.

Krakauer, D. C. and Mira, A. (1999). Mitochondria and germ-cell death. *Nature* **400**, 125-126.

Lackner, M. R. and Kim, S. K. (1998). Genetic analysis of the *Caenorhabditis elegans* MAP kinase gene *mpk-1*. *Genetics* **150**, 103-117.

Ladomery, M., Wade, E. and Sommerville, J. (1997). Xp54, the *Xenopus* homologue of human RNA helicase p54, is an integral component of stored mRNP particles in oocytes. *Nucleic Acids Res.* **25**, 965-973.

Lettre, G., Kritikou, E. A., Jaeggi, M., Calixto, A., Fraser, A. G., Kamath, R. S., Ahringer, J. and Hengartner, M. O. (2004). Genome-wide RNAi identifies p53-dependent and -independent regulators of germ cell apoptosis in *C. elegans*. *Cell Death Differ.* **11**, 1198-1203.

Lieb, B., Carl, M., Hock, R., Gebauer, D. and Scheer, U. (1998). Identification of a novel mRNA-associated protein in oocytes of *Pleurodeles waltl* and *Xenopus laevis*. *Exp. Cell Res.* **245**, 272-281.

Luitjens, C., Gallegos, M., Kraemer, B., Kimble, J. and Wickens, M. (2000). CPEB proteins control two key steps in spermatogenesis in *C. elegans*. *Genes Dev.* **14**, 2596-2609.

Mansfield, J. H., Wilhelm, J. E. and Hazelrigg, T. (2002). Ypsilon Schachtel, a *Drosophila* Y-box protein, acts antagonistically to Orb in the oskar mRNA localization and translation pathway. *Development* **129**, 197-209.

Matova, N., Mahajan-Miklos, S., Mooseker, M. S. and Cooley, L. (1999). *Drosophila* quail, a villin-related protein, bundles actin filaments in apoptotic nurse cells. *Development* **126**, 5645-5657.

Minshall, N. and Standart, N. (2004). The active form of Xp54 RNA helicase in translational repression is an RNA-mediated oligomer. *Nucleic Acids Res.* **32**, 1325-1334.

Minshall, N., Thom, G. and Standart, N. (2001). A conserved role of a DEAD box helicase in mRNA masking. *RNA* **7**, 1728-1742.

Nakamura, A., Amikura, R., Hanyu, K. and Kobayashi, S. (2001). Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during Drosophila oogenesis. *Development* **128**, 3233-3242.

Nakamura, A., Sato, K. and Hanyu-Nakamura, K. (2004). *Drosophila* cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. *Dev. Cell* **6**, 69-78.

Navarro, R. E. and Blackwell, T. K. (2005). Requirement for P granules and meiosis for accumulation of the germline RNA helicase CGH-1. *Genesis* **42**, 172-80.

Navarro, R. E., Shim, E. Y., Kohara, Y., Singson, A. and Blackwell, T. K. (2001). *cgh-1*, a conserved predicted RNA helicase required for gametogenesis and protection from physiological germline apoptosis in *C. elegans*. *Development* **128**, 3221-3232.

Pepling, M. E. and Spradling, A. C. (2001). Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Dev. Biol.* **234**, 339-351.

Piano, F., Schetter, A. J., Morton, D. G., Gunsalus, K. C., Reinke, V., Kim, S. K. and Kemphues, K. J. (2002). Gene clustering based on RNAi phenotypes of ovary-enriched genes in *C. elegans*. *Curr. Biol.* **12**, 1959-1964.

Pitt, J. N., Schisa, J. A. and Priess, J. R. (2000). P granules in the germ cells of *Caenorhabditis elegans* adults are associated with clusters of nuclear pores and contain RNA. *Dev. Biol.* **219**, 315-333.

Richter, J. D. and Smith, L. D. (1984). Reversible inhibition of translation by *Xenopus* oocyte-specific proteins. *Nature* **309**, 378-380.

Riddle, D. L., Blumenthal, T., Meyer, B. J. and Priess, J. R. (1997). *C. elegans* II. New York: Cold Spring Harbor Press.

Rocak, S. and Linder, P. (2004). DEAD-box proteins: the driving forces behind RNA metabolism. *Nat. Rev. Mol. Cell. Biol.* **5**, 232-241.

Rongo, C., Broihier, H. T., Moore, L., Van Doren, M., Forbes, A. and Lehmann, R. (1997). Germ plasm assembly and germ cell migration in Drosophila. *Cold Spring Harb. Symp. Quant. Biol.* **62**, 1-11.

Saffman, E. E. and Lasko, P. (1999). Germline development in vertebrates and invertebrates. *Cell Mol. Life Sci.* **55**, 1141-1163.

Salgado-Garrido, J., Bragado-Nilsson, E., Kandels-Lewis, S. and Seraphin, B. (1999). Sm and Sm-like proteins assemble in two related complexes of deep evolutionary origin. *EMBO J.* **18**, 3451-3462.

Schedl, T. (1997). Developmental genetics of the germ line. In *C. elegans II*, (ed. D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess), pp. 241-270. New York: Cold Spring Harbor Press.

Schisa, J. A., Pitt, J. N. and Priess, J. R. (2001). Analysis of RNA associated with P granules in germ cells of *C. elegans* adults. *Development* **128**, 1287-1298.

Schumacher, B., Schertel, C., Wittenburg, N., Tuck, S., Mitani, S., Gartner, A., Conradt, B. and Shaham, S. (2005). *C. elegans ced-13* can promote apoptosis and is induced in response to DNA damage. *Cell Death Differ.* **12**, 153-161.

Seraphin, B. (1995). Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. *EMBO J.* **14**, 2089-2098.

Seydoux, G. and Fire, A. (1994). Soma-germline asymmetry in the

distributions of embryonic RNAs in *Caenorhabditis elegans*. *Development* **120**, 2823-2834.
Sheth, U. and Parker, R. (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science* **300**, 805-808.
Smillie, D. A. and Sommerville, J. (2002). RNA helicase p54 (DDX6) is a shuttling protein involved in nuclear assembly of stored mRNP particles. *J. Cell Sci.* **115**, 395-407.
Sommerville, J. (1999). Activities of cold-shock domain proteins in translation control. *BioEssays* **21**, 319-325.
Stebbins-Boaz, B., Cao, Q., de Moor, C. H., Mendez, R. and Richter, J. D. (1999). Maskin is a CPEB-associated factor that transiently interacts with eIF-4E. *Mol. Cell* **4**, 1017-1027.
Strome, S. and Wood, W. B. (1982). Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **79**, 1558-1562.
Tay, J. and Richter, J. D. (2001). Germ cell differentiation and synaptonemal complex formation are disrupted in CPEB knockout mice. *Dev. Cell* **1**, 201-213.
Teixeira, D., Sheth, U., Valencia-Sanchez, M. A., Brengues, M. and Parker, R. (2005). Processing bodies require RNA for assembly and contain nontranslating mRNAs. *RNA* **11**, 371-382.
Tharun, S., He, W., Mayes, A. E., Lennertz, P., Beggs, J. D. and Parker, R. (2000). Yeast Sm-like proteins function in mRNA decapping and decay. *Nature* **404**, 515-518.
Villeneuve, A. M. (1994). A cis-acting locus that promotes crossing over between X chromosomes in *Caenorhabditis elegans*. *Genetics* **136**, 887-902.
Wilczynska, A., Aigueperse, C., Kress, M., Dautry, F. and Weil, D. (2005). The translational regulator CPEB1 provides a link between dcp1 bodies and stress granules. *J. Cell Sci.* **118**, 981-992.
Wilhelm, J. E., Mansfield, J., Hom-Booher, N., Wang, S., Turck, C. W., Hazelrigg, T. and Vale, R. D. (2000). Isolation of a ribonucleoprotein complex involved in mRNA localization in Drosophila oocytes. *J. Cell Biol.* **148**, 427-440.
Wilhelm, J. E., Hilton, M., Amos, Q. and Henzel, W. J. (2003). Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. *J. Cell Biol.* **163**, 1197-1204.
Wylie, C. (2000). Germ cells. *Curr. Opin. Genet. Dev.* **10**, 410-413.