

Maternal mRNAs are regulated by diverse P body–related mRNP granules during early *Caenorhabditis elegans* development

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Processing bodies (P bodies) are conserved mRNA–protein (mRNP) granules that are thought to be cytoplasmic centers for mRNA repression and degradation. However, their specific functions in vivo remain poorly understood. We find that repressed maternal mRNAs and their regulators localize to P body–like mRNP granules in the *Caenorhabditis elegans* germ line. Surprisingly, several distinct types of regulated granules form during oocyte and embryo development. 3′ untranslated region elements direct mRNA targeting to one of these granule classes. The P body factor CAR-1/Rap55 pro-

motes association of repressed mRNA with granules and contributes to repression of *Notch/glp-1* mRNA. However, CAR-1 controls *Notch/glp-1* only during late oogenesis, where it functions with the RNA-binding regulators PUF-5, PUF-6, and PUF-7. The P body protein CGH-1/Rck/Dhh1 differs from CAR-1 in control of granule morphology and promotes mRNP stability in arrested oocytes. Therefore, a system of diverse and regulated RNP granules elicits stage-specific functions that ensure proper mRNA control during early development.

Introduction

mRNAs reside in mRNA–protein (mRNP) complexes from the time they are made in the nucleus to when they are degraded in the cytoplasm. In the cytoplasm, mRNP composition controls mRNA translation, localization, and stability, processes that are critical to animal development (Alvarez-Garcia and Miska, 2005; de Moor et al., 2005; Kimble and Crittenden, 2007). In many species, mRNPs often accumulate in large cytoplasmic particles, which include processing bodies (P bodies), stress granules, germ granules, and neuronal granules (Anderson and Kedersha, 2006; Parker and Sheth, 2007). These various mRNP granules all share several conserved components and have been proposed as cytoplasmic centers for translational repression or degradation of mRNAs.

Many uncertainties remain concerning P bodies and related mRNP particles. It is not clear if each cell system contains a single type of P body. Mammalian cells exposed to stress generate at least two mRNP particles, stress granules and P bodies, which are

closely related but have differences in composition and, presumably, function (Kedersha et al., 2005; Anderson and Kedersha, 2006). In other systems, the extent and nature of mRNP granule diversity is largely unknown. Moreover, the roles of mRNP granules and their components in translational repression remain uncertain. Although repressed mRNAs can be targeted to P bodies, disruption of P body structure does not prevent mRNA repression in at least some contexts (Decker et al., 2007; Eulalio et al., 2007). It remains possible, however, that mRNP granules are important for translational control under specific conditions in vivo, especially in early development, where specific patterns of mRNA regulation require proteins that bind specific 5′ or 3′ untranslated region (UTR) elements (de Moor et al., 2005; Evans and Hunter, 2005; Wilhelm and Smibert, 2005). The functional relationship of mRNA-specific control factors with mRNP granules and their components remains poorly understood.

In the nematode *Caenorhabditis elegans*, mRNP regulation is critical to germ cell development and embryogenesis (Evans and Hunter, 2005; Lee and Schedl, 2006). Germ cells

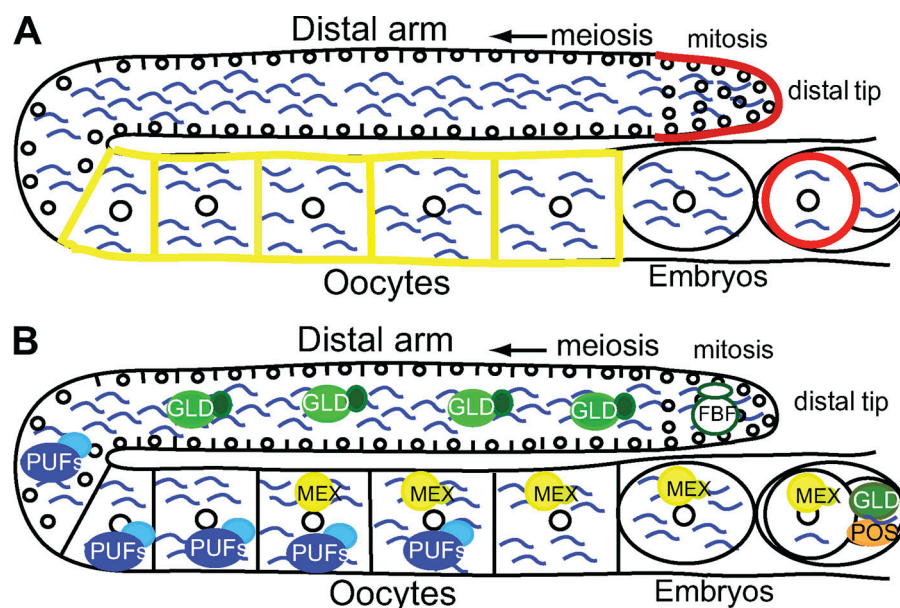
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Abbreviations used in this paper: β gal, β galactosidase; dcP bodies, DCAP-2–enriched granules related to P bodies; grP bodies, germ line RNP granules related to P bodies; IF, immunofluorescence; mRNP, messenger RNA–protein; ORF, open reading frame; P bodies, processing bodies; SCR, spatial control region; TCR, temporal control region; UTR, untranslated region.

The online version of this paper contains supplemental material.

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Figure 1. Maternal mRNAs have specific translation patterns during *C. elegans* development. One half of a hermaphrodite gonad: At the distal tip, stem cells produce germ nuclei that enter meiosis in the distal arm and differentiate into oocytes near the proximal end. Distal nuclei reside around a syncytium with a common cytoplasmic core; membranes form around oocytes. Embryos begin development in the uterus. (A) Maternal mRNAs (blue lines) like *glp-1* and *rme-2* are made by distal nuclei and distribute throughout the gonad. GLP-1 protein (red) is only made at the distal tip and in anterior embryonic cells, whereas RME-2 protein (yellow) is only made in oocytes. (B) Different RNA-binding proteins control translation at specific stages. GLD-1 (green circles) represses both *glp-1* and *rme-2* mRNAs. PUF-5, PUF-6, and PUF-7 (blue circles) are required for *glp-1* but not *rme-2* repression in late oogenesis. PUF-5 disappears upon oocyte differentiation, and new factors like MEX-3 (yellow circles) and POS-1 (orange circle) arise at specific stages.



undergo an organized program of oogenesis and fertilization within the tube-shaped hermaphrodite gonad (Fig. 1, A and B; Hubbard and Greenstein, 2000). Most maternal mRNAs are transcribed by early-stage germ nuclei and distribute throughout the gonad but are translated in very specific temporal and spatial patterns (Fig. 1 A). For example, mRNA for the Notch receptor GLP-1 is translated only in the distal gonad tip and anterior cells of the embryo, whereas mRNA for the lipoprotein receptor RME-2 is translated specifically in late-stage oocytes (Crittenden et al., 1994; Evans et al., 1994; Grant and Hirsh, 1999; Lee and Schedl, 2001). Precise control of these and many other maternal mRNAs is essential for both germ cell development in the gonad and patterning of cell fates in the embryo.

The diverse translation patterns in the *C. elegans* gonad and embryo are mediated by different sets of RNA-binding proteins that are expressed at distinct developmental stages (Fig. 1 B; Lee and Schedl, 2006). At least some of these RNA-binding proteins bind to specific UTR elements in mRNAs. The KH protein GLD-1 is expressed in early meiosis and binds directly to elements in *glp-1*, *rme-2*, and numerous other mRNAs to repress their translation (Lee and Schedl, 2001; Marin and Evans, 2003; Lee and Schedl, 2006). As germ cell nuclei enter late stages of oogenesis, GLD-1 disappears and the PUF domain protein PUF-5 is made; PUF-5 and its homologues PUF-6 and PUF-7 promote repression of *Notch/glp-1* and other mRNAs specifically in late oogenesis (Lublin and Evans, 2007). Additional RNA-binding proteins, such as MEX-3 and MEX-5/6, accumulate during late oogenesis and likely regulate distinct sets of mRNAs (Draper et al., 1996; Schubert et al., 2000; Mootz et al., 2004). In the early embryo, new combinations of RNA-binding proteins arise in specific cells to promote localized translation of mRNAs (Evans and Hunter, 2005).

The relationships of these maternal mRNA control systems to mRNP granules are not known. *C. elegans* hermaphrodite gonads have at least two distinct types of P body-like granules. Small cytoplasmic RNP particles reside in the gonad and embryo; like P bodies in other organisms, these small particles contain

the RNA helicase CGH-1/RCK/Dhh1/Me31B and the Sm domain protein CAR-1/Rap55/Sdc6/Trailer Hitch (Navarro et al., 2001; Audhya et al., 2005; Boag et al., 2005; Squirrell et al., 2006). In embryos, the decapping enzyme subunits DCAP-1 and DCAP-2 colocalize with these other P body factors in P body-like particles (Lall et al., 2005; Squirrell et al., 2006). Germ granules (P granules) represent a distinct type of mRNP granule in *C. elegans* (Strome, 2005). Unlike the small cytoplasmic RNP particles, P granules are germ-cell specific, nuclear-associated at most stages, and contain unique proteins. However, P granules also contain the P body factors CGH-1, CAR-1, and DCAP-2, which suggests some sort of link between the granule types (Navarro et al., 2001; Boag et al., 2005; Lall et al., 2005; Squirrell et al., 2006). The RNA components of these granules are not fully understood, although several maternal mRNAs associate with P granules (Subramaniam and Seydoux, 1999; Schisa et al., 2001). Collectively, these observations suggest that P body-related granules participate in the regulation of maternal mRNAs. However, their functions and relationships with specific mRNA control systems remain unclear.

In this paper, we show that translationally repressed mRNAs and their specific RNA-binding regulators are targeted to P body-related particles in the *C. elegans* gonad during oocyte arrest. 3' UTR elements that repress translation also induce mRNA localization to these granules. We also show that the P body factor CAR-1/Rap55/Trailerhitch is required for formation or maintenance of mRNP granules and for the localization of repressed mRNA to granules. In addition, CAR-1 functions with PUF-5, PUF-6, and PUF-7 to control *Notch/glp-1* and likely other mRNAs. During early meiosis, however, neither CAR-1 nor the P body factor CGH-1 are required for repression of at least two GLD-1-regulated mRNAs. Moreover, RNP granule structure undergoes dramatic changes during oocyte development, and at least four different types of granules arise at distinct stages. Collectively, the results suggest that diverse mRNP granules are dynamically regulated to elicit stage-specific functions that ensure proper mRNA control during early development.

Results

RNA-binding proteins localize to P body-like granules upon oocyte arrest

To gain insight into the function and regulation of the mRNA-specific regulator PUF-5, we examined PUF-5 localization in fixed gonads. Previous results suggested that PUF-5 accumulation in late-stage oocytes might be controlled by sperm-derived signals (Lublin and Evans, 2007). To test this, we compared PUF-5 expression in wild-type hermaphrodite gonads to gonads from *fog-2(q71)* females; *fog-2* mutations prevent sperm formation in otherwise normal XX animals (Schedl and Kimble, 1988). Oocytes arrest in meiosis, and ovulation is inhibited when sperm are absent (McCarter et al., 1999). In wild-type oocytes with active oogenesis, PUF-5 staining was diffuse and present in small particles in the cytoplasm of developing oocytes (Fig. 2 B). In arrested *fog-2(q71)* oocytes, the developmental expression pattern was not altered, but PUF-5 became tightly localized to large cytoplasmic foci (Fig. 2 C). Wild-type hermaphrodites depleted of sperm formed similar large particles, whereas *fog-2(q71)* females mated by *fog-2(q71)* males had small particles like wild-type gonads (data not shown). These observations show that PUF-5 is targeted to cytoplasmic particles that form or enlarge in the absence of sperm. These granules resemble large RNP aggregates seen previously in female and old hermaphrodite gonads (Schisa et al., 2001; Jud et al., 2007). Moreover, these particles resemble P bodies and stress granules in other organisms.

To examine the relationship of PUF-5 granules to mRNP granules in other systems, we determined if PUF-5 colocalized with the P body factors CAR-1 and CGH-1. Indeed, PUF-5 tightly colocalized with both CAR-1 and CGH-1 in large granules of arrested female oocytes (Fig. 2, C–E; and not depicted). RNAi depletion experiments showed that granule staining with antibodies to each of these proteins was specific (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200802128/DC1>). Furthermore, we also observed large aggregates that contained both CGH-1 and CAR-1 in the distal arm of female gonads, where PUF-5 is not expressed (Fig. 2, H and J; and not depicted). In *C. elegans*, the distal gonad is a syncytium in which early meiotic germ nuclei surround a shared cytoplasmic core (Figs. 1 and 2 A). CAR-1 and CGH-1 aggregates of varying size and rounded shapes accumulated in the center of the distal core of *fog-2(q71)* female gonads (Fig. 2, H and J). These CAR-1/CGH-1 particles became smaller as germ cells exited pachytene near the gonad bend before forming large spherical particles in differentiating oocytes (Fig. 2 D). Similar observations were recently made in an independent study (Jud et al., 2008). Therefore, granules related to P bodies and stress granules arise in the *C. elegans* female gonad. These granules enlarge or aggregate in the absence of sperm signaling and undergo morphological transformations from early meiosis through oocyte differentiation.

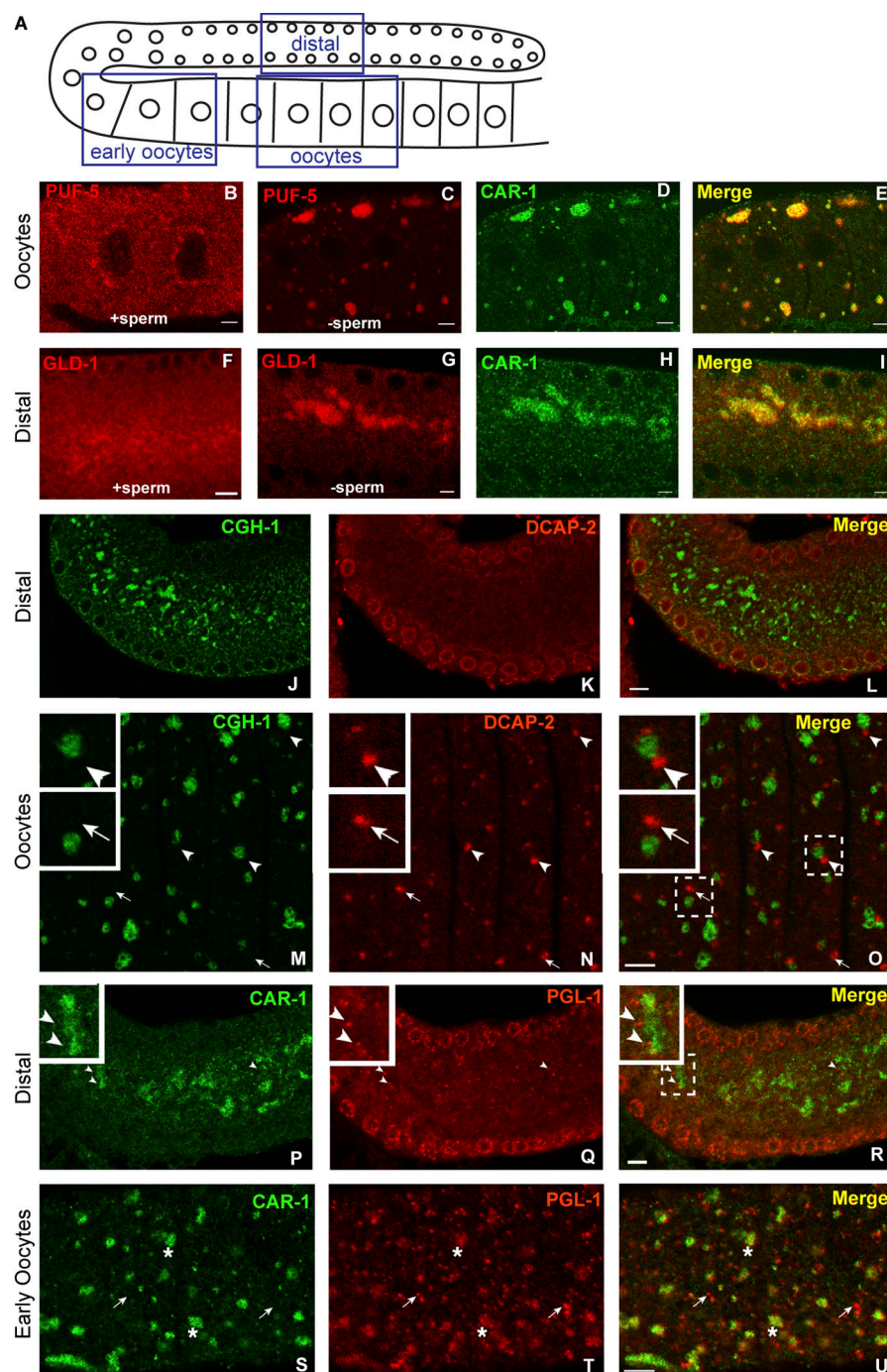
Because the mRNA regulator PUF-5 localized to P body-like granules in proximal oocytes, we asked if other mRNA-specific regulators resided in granules at other stages. In early meiotic cells of the distal arm, the KH protein GLD-1 binds specific UTR elements in several mRNAs to repress translation (Evans and Hunter, 2005; Lee and Schedl, 2006). GLD-1 in-

deed localized to distal CAR-1/CGH-1 aggregates in female gonads, although diffuse cytoplasmic GLD-1 staining was also observed (Fig. 2, G–I). In addition, we found that the KH protein MEX-3 and the CCH protein MEX-5 localized to CAR-1 and CGH-1 granules in late stage oocytes, where these putative mRNA-binding proteins are expressed (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200802128/DC1>). Other studies also observed MEX-3 localization to likely identical large aggregates in arrested oocytes (Iwasaki et al., 1996; Schisa et al., 2001; Jud et al., 2007, 2008). Therefore, several distinct RNA-binding proteins, at least some of which are mRNA-specific translational repressors, are targeted to particles that resemble P bodies and stress granules. Some of these proteins (e.g., PUF-5 and MEX-5) have overlapping expression patterns, which suggests that they are targeted to the same granules even though they likely regulate different mRNA targets.

mRNP granules are diverse and regulated during development

To further determine the similarity of *C. elegans* RNP granules to P bodies and stress granules, we examined localization of DCAP-2, the catalytic subunit of the decapping enzyme that removes the 5' cap of mRNAs before 5'-to-3' degradation. In yeast and vertebrate cells, DCAP-2 and its partner DCAP-1 are canonical P body markers (Parker and Sheth, 2007). In *C. elegans*, DCAP-1 and DCAP-2 were previously found within the early embryo in P body-like particles and in P granules (Lall et al., 2005; Squirrell et al., 2006). We examined the localization of DCAP-2 in nonmated *fog-2(q71)* female gonads using two previously characterized antibodies (Lall et al., 2005). Both antibodies stained P granules that are tightly associated with germ nuclei in the distal gonad (Fig. 2, K and L; and not depicted). Interestingly, however, neither DCAP-2 antibody stained CGH-1 aggregates in the distal cytoplasmic core (Fig. 2, J–L; and not depicted). More surprisingly, one DCAP-2 antibody (NY989) stained small particles within arrested oocytes, but these particles were distinct from granules containing CGH-1, CAR-1, and PUF-5 (Fig. 2, M–O; and not depicted). These “DCP particles” were typically small (0.2–0.5 μ m) and never colocalized with CGH-1, CAR-1, or PUF-5 ($n > 200$), although some were closely juxtaposed to the larger particles (Fig. 2, M–O, arrowheads). Staining of these unique particles was specific for DCAP-2 because NY989 staining was undetectable in 91% of *dcap-2(RNAi)*; *fog-2(q71)* gonads ($n = 44$), whereas all control gonads ($n = 35$) stained brightly for these particles (Fig. S1). Curiously, a different DCAP-2 antibody (NY990) did not stain these small DCP particles but did stain the largest CGH-1 granules (unpublished data). Some DCAP-2, therefore, is also associated with large CGH-1/CAR-1 granules in oocytes. In embryos, both DCAP-2 antibodies stained puncta in embryos that tightly colocalized with both CAR-1 and CGH-1 (unpublished data), as has been observed previously (Lall et al., 2005). These two antibodies may recognize distinct DCAP-2 epitopes that are differentially masked in two types of particles in oocytes but not in a third type of particle that forms in the embryo. Regardless, we conclude that at least two distinct granule populations are formed in arrested oocytes. We call these germ line RNP granules related

Figure 2. Diverse P body-like granules form after oocyte arrest. (A) A female gonad diagram; boxed regions represent locations of similarly labeled images. (B–U) In the wild type (+sperm), PUF-5 (B) and GLD-1 (F) were present in diffuse small puncta. In *fog-2(lf)* females (–sperm), PUF-5 (C) and GLD-1 (G) moved to large granules with CAR-1 (D, E, H, and I) and CGH-1 (not depicted). In female distal arms, DCAP-2 (K and L) was only detected in perinuclear P granules. In female oocytes, DCAP-2 (N and O) resided in granules that were distinct (arrows and arrowheads) but often adjacent (arrowheads) to CGH-1 granules (grP bodies; M and O). In female distal arms, PGL-1 (Q) stained perinuclear and cytoplasmic granules but not grP bodies (P–R); distal core PGL-1 granules clustered next to grP bodies (P–R, arrowheads). In early female oocytes, PGL-1 granules were either distinct (S–U, arrows) or coincident (S–U, asterisks) with CAR-1. In late oocytes, most PGL-1 colocalized with CAR-1 (not depicted). Dashed boxes indicate the enlarged areas shown in insets. Bars, 5 μ m.



to P bodies (grP bodies), and DCAP-2–enriched granules related to P bodies (dcP bodies). grP bodies contain the P body factors CAR-1, CGH-1, some DCAP-2, and several mRNA-binding proteins, whereas dcP bodies either do not contain CAR-1 or CGH-1 or have structural differences that mask antibody access. Both grP bodies and dcP bodies have similarities to P bodies and stress granules in other systems but also have some unique features. A third type of P body-like particle forms in embryos, and is more like canonical P bodies because it can bind all P body factor antibodies tested.

Several observations suggest that germ granules (P granules) represent a fourth type of P-body-related mRNP granule.

First, in the distal gonad, the P granule protein PGL-1 resided in perinuclear granules and in small distal core particles that were distinct from grP bodies, although distal core PGL-1 particles were often closely adjacent to grP bodies (Fig. 2, P–R). Second, as early stage oocytes began to form, some cytoplasmic PGL-1 particles colocalized with CAR-1, but others were clearly distinct (Fig. 2, S–U). PGL-1 associated with most grP bodies only in late stages of oocyte differentiation near the proximal end (unpublished data). Therefore, P granules are a distinct class of mRNP granule, although they too contain several P body factors and interact with other P body-like particles.

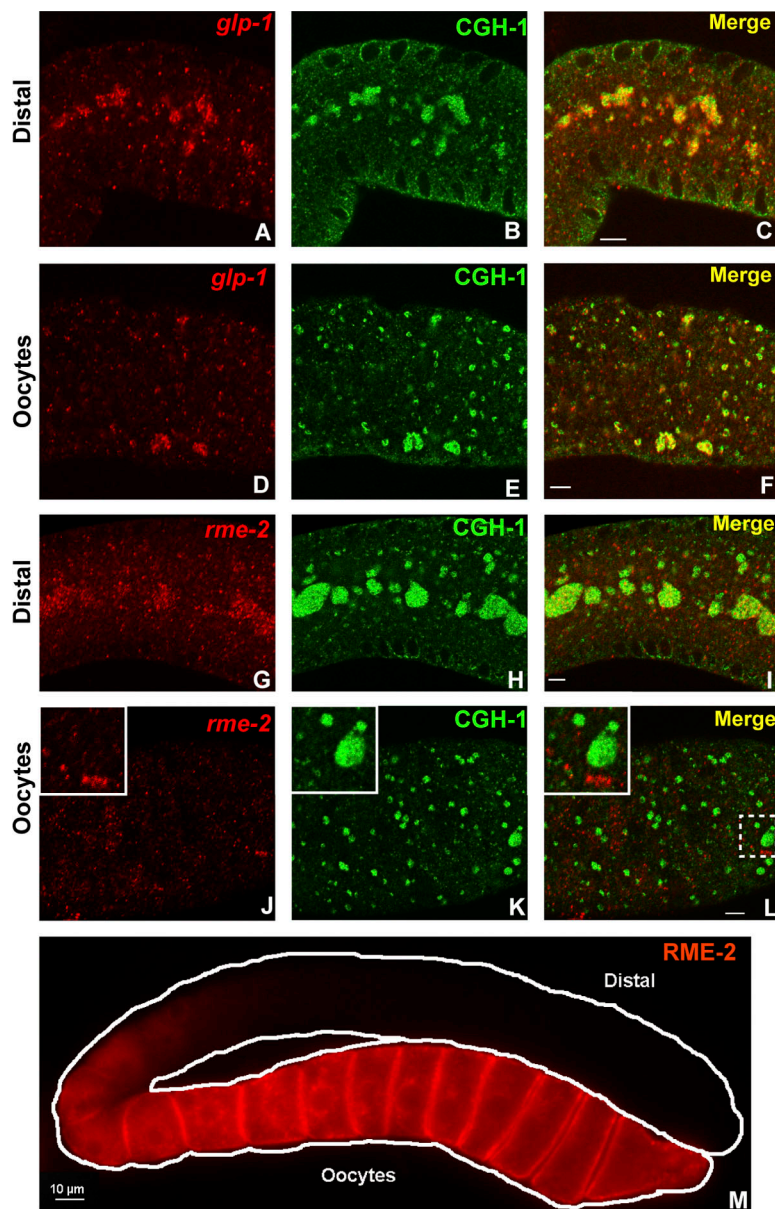


Figure 3. Repressed but not active mRNAs localize to grP bodies. (A–L) Confocal images of dissected *fog-2* female gonads stained by FISH for endogenous mRNAs (red) and by IF for CGH-1 (green). (M) A female gonad stained for RME-2 protein (red). *glp-1* mRNA colocalized with CGH-1 in grP bodies of distal arms (A–C) and oocytes (D–F), as well as distinct smaller particles. *rme-2* mRNA colocalized with CGH-1 in distal grP bodies (G–I) but not in oocytes (J–L), where *rme-2* translation is active (M). Insets in J–L are enlarged twice from the region in L indicated by the dashed box. Sense RNA probes did not stain most gonads (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200802128/DC1>). Outlines denote the gonad surface. Bars, 5 μ m.

Repressed mRNAs are specifically targeted to grP bodies

To determine if P body-like granules could be sites of repression or storage of maternal mRNAs, we investigated whether they contain specific mRNAs by FISH. FISH of both small and large particles was specific because nonspecific and sense RNA probes produced no or very weak staining (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200802128/DC1>; and not depicted). We first examined the localization of *glp-1* mRNA, which is tightly repressed throughout the gonad. We found that *glp-1* mRNA colocalized with both distal arm and proximal oocyte grP bodies (Fig. 3, A–F; also see Fig. 8, A–C; and Fig. S3). Some small *glp-1* mRNP particles did not stain for CGH-1 or CAR-1, which might reflect dynamic entry and/or exit of repressed mRNA from grP bodies, a subpopulation of distinct mRNPs, or incomplete accessibility of antibodies to mRNPs. The maternal mRNA *pos-1* also localized to grP bodies throughout the gonad (unpublished data). POS-1 expression, like GLP-1, is repressed in the

germ line, although POS-1 repression does not depend on PUF-5 and PUF-6/7 (Tabara et al., 1999; Lublin and Evans, 2007). Several other mRNAs, including translationally repressed *nos-2* (D'Agostino et al., 2006), were previously observed in RNP aggregates similar to grP bodies (Schisa et al., 2001). Therefore, translationally repressed mRNAs are targeted to grP bodies. Different mRNA-specific factors may trigger movement of different mRNAs into a common mRNP particle.

To test if only repressed mRNAs localize to grP bodies, we examined the localization of *rme-2* mRNA. *rme-2* is tightly repressed by GLD-1 in the distal gonad but is actively translated in oocytes of both hermaphrodites and females (Fig. 3 M; Grant and Hirsh, 1999; Lee and Schedl, 2001). In the distal arm of arrested female gonads, *rme-2* mRNA associated with grP bodies (Fig. 3, G–I). In contrast, *rme-2* mRNA particles were abundant but distinct from grP bodies in proximal oocytes (Fig. 3, J–L), where RME-2 protein was strongly expressed (Fig. 3 M). Actin mRNA surprisingly had the opposite

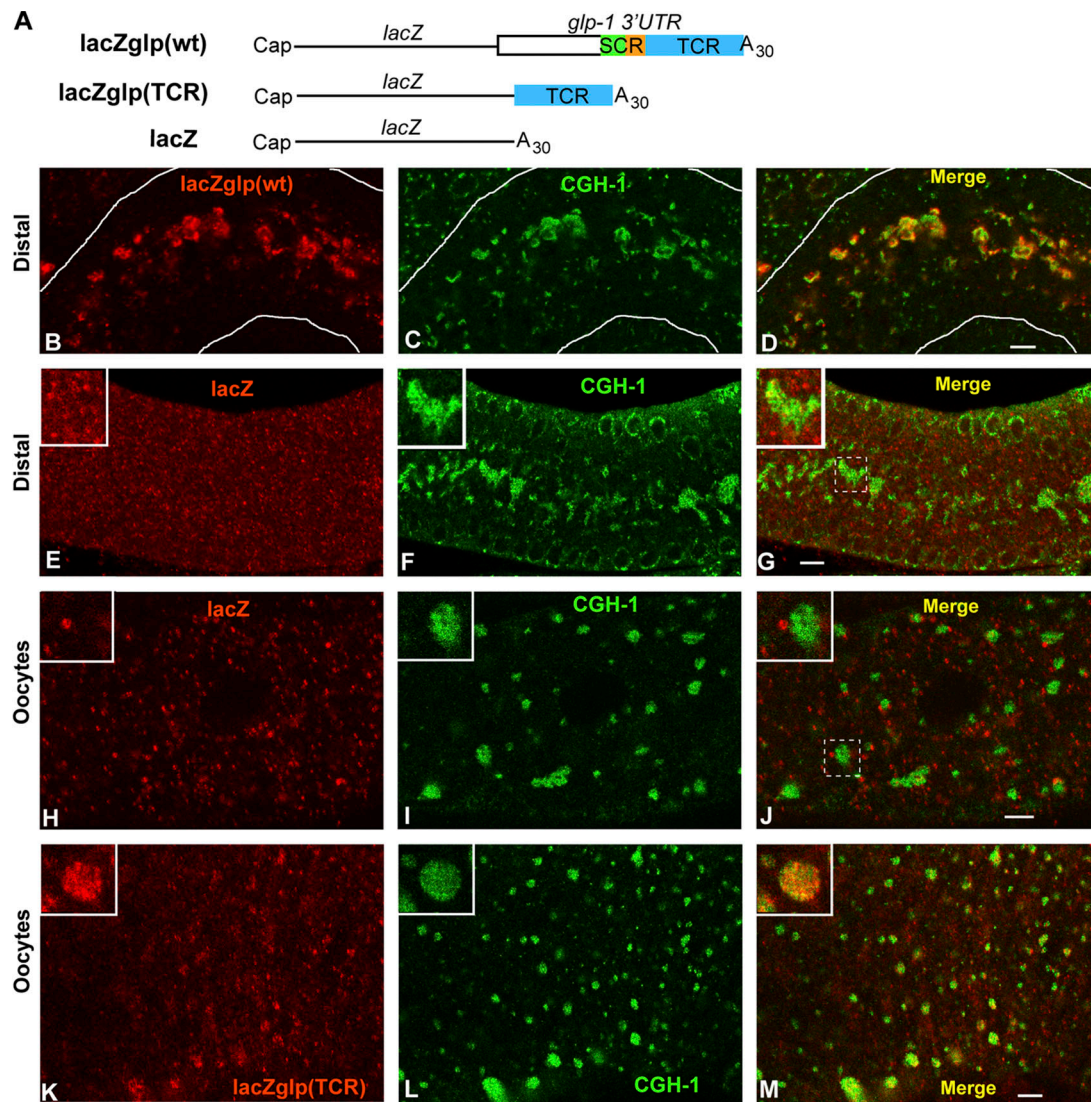


Figure 4. **Specific *glp-1* 3' UTR elements target mRNAs to grP bodies.** LacZ reporter mRNAs were injected into distal arms of *fog-2(q71)* females and localized by FISH after 17 h. (A) LacZglp(wt) carried the full length *glp-1* 3' UTR with the SCR (orange box) that contains a GLD-1-binding site (green box) and the TCR (blue box) that mediates PUF-dependent repression in oocytes. LacZglp(TCR) contained only the TCR, whereas LacZ contained no *glp-1* sequence. (B–M) Confocal images of dissected gonads stained by FISH for lacZ (red) and by IF for CGH-1 (green). Insets in E–G are 3× enlargements of the dashed box in G. Insets in H–M are 2× enlargements; the insets in H–J are from the boxed region in J and the insets in K–M are from a different focal plane of the same oocytes. The full-length *glp-1* 3' UTR targeted lacZglp(wt) to grP bodies in both distal and oocyte grP bodies (B–D and not depicted). Outlines denote the gonad surface. LacZ mRNA did not localize to grP bodies in the distal (E–G) or in proximal (H–J) gonad. The TCR region of the *glp-1* 3' UTR localized lacZglp(TCR) to grP bodies in oocytes (K–M) but not in the distal gonad (not depicted; see Results). Bars, 5 μm.

pattern; actin mRNA did not associate with grP bodies in the distal arm but did colocalize with grP bodies in proximal oocytes (Fig. S4, available at <http://www.jcb.org/cgi/content/full/jcb.200802128/DC1>). Collectively, these data suggest that mRNAs are targeted to grP bodies when repressed but are excluded from granules when actively translated. Changes in granule localization for mRNAs like *rme-2* and actin during germ cell development suggest dynamic influx and efflux of mRNAs that are dictated by mRNA-specific translational control factors.

***glp-1* 3' UTR elements are sufficient to target mRNAs to grP bodies**

If translational repression triggers movement of mRNAs into grP bodies, specific repression elements and factors should be

necessary for granule targeting. To test this, we directly injected lacZ reporter mRNAs into *fog-2(q71)* gonads and examined lacZ mRNA localization by FISH. We previously showed that lacZ mRNA carrying the *glp-1* 3' UTR (lacZglp(wt)) is strongly repressed in both the distal and proximal gonad arms, whereas a lacZ mRNA lacking elements (lacZ) is robustly translated throughout the gonad (Evans et al., 1994; Marin and Evans, 2003; Lublin and Evans, 2007). Remarkably, lacZglp(wt) mRNA localized tightly to grP bodies in all injected gonads examined ($n = 12$; Fig. 4, B–D). In contrast, lacZ mRNA formed small particles that were distinct from CGH-1-containing granules in both the distal core (Fig. 4, E–G) and oocytes (Fig. 4, H–J) in most injected gonads (95%, $n = 37$). No staining was detected in noninjected gonads (not depicted). As expected, lacZ mRNA

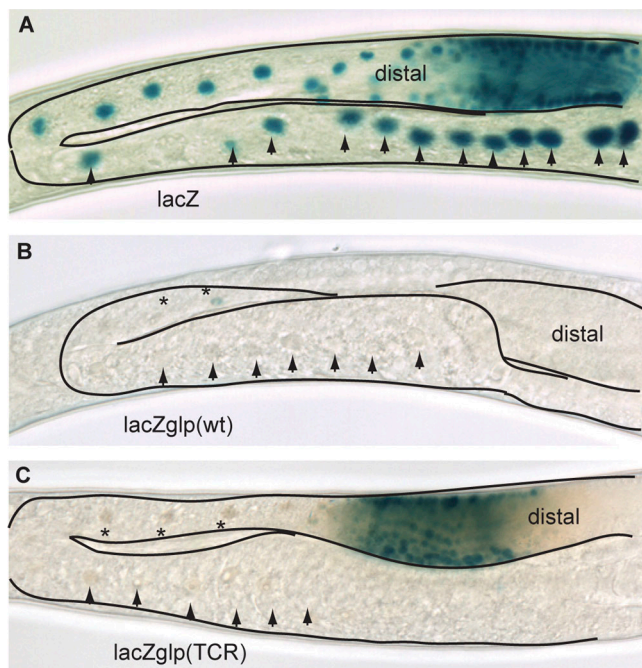


Figure 5. *Notch/glp-1* 3' UTR elements repress reporter mRNA translation in arrested female gonads. Shown are whole-mount, bright field images of *fog-2(q71)* females that were injected with *lacZ* (A), *lacZglp(wt)* (B), and *lacZglp(TCR)* (C) mRNAs and stained for β gal after 17 h. β gal contained a nuclear localization signal. Distal syncytia are labeled, whereas arrowheads mark oocyte nuclei; some nuclei reside out of the focal plane. For both *lacZglp(wt)*- and *lacZglp(TCR)*-injected gonads, some early-stage oocyte nuclei near the bend had weak β gal staining (asterisks). Table S1 (available at <http://www.jcb.org/cgi/content/full/jcb.200802128/DC1>) shows a quantitative summary of these results. Outlines denote the gonad surface.

was translated strongly in arrested female gonads, whereas *lacZglp(wt)* mRNA was strongly repressed (Fig. 5, A and B; and Table S1, available at <http://www.jcb.org/cgi/content/full/jcb.200802128/DC1>). We conclude that the *glp-1* 3' UTR both represses translation and specifically targets reporter mRNA to grP bodies. These results suggest that granule association is triggered by translational repression through *glp-1* 3' UTR elements or by specific "granule-binding" RNA elements. In rare gonads (2/37), injected *lacZ* mRNA localized to CGH-1 granules. We speculate that this represents nonspecific inactivation of mRNA, e.g., due to stress or damage, because a small percentage of *lacZ* mRNA injections produced little or no protein (Table S1). Thus, translational inactivation could be a trigger for granule targeting rather than direct 3' UTR-mediated association, although further experiments will be needed to distinguish these possibilities.

The *glp-1* 3' UTR contains two distinct regulatory regions (Fig. 4 A). The spatial control region (SCR) includes a GLD-1-binding site that represses translation primarily in the distal gonad (Marin and Evans, 2003). The temporal control region (TCR) has elements that repress translation primarily in oocytes and depend on PUF-5 and PUF-6/7 activity (Lublin and Evans, 2007). To test if functional differences in these elements influence granule targeting, we injected *lacZ* carrying only the TCR (*lacZglp(TCR)*). Strikingly, *lacZglp(TCR)* mRNA was not de-

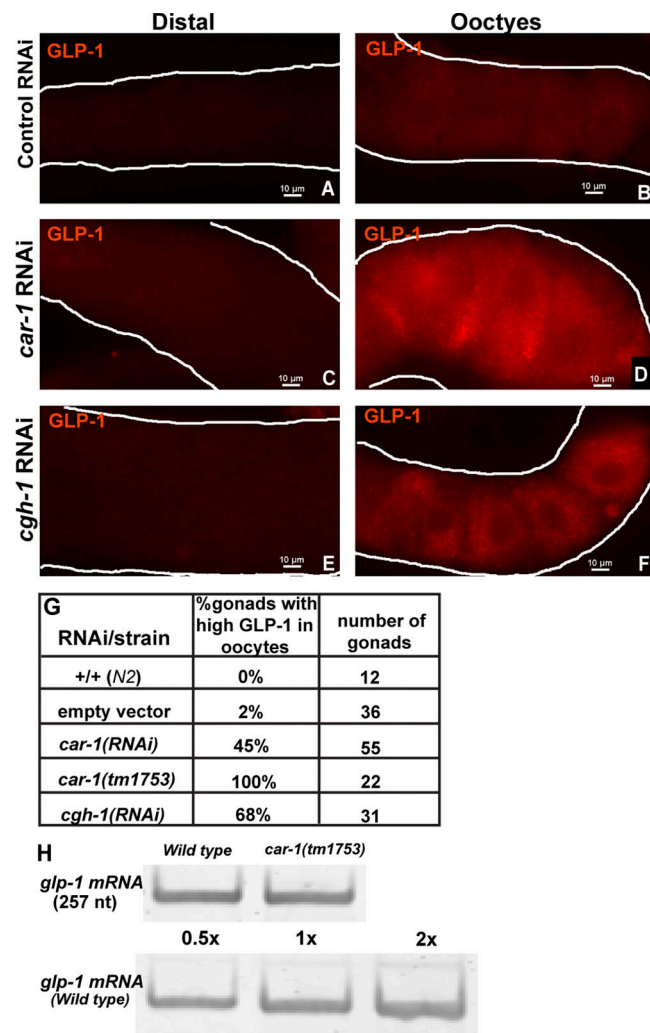


Figure 6. CAR-1 and CGH-1 control *glp-1* repression. (A–F) Identical exposures of GLP-1 protein staining after RNAi with empty vector (control RNAi; A and B), *car-1(RNAi)* (C and D), or *cgh-1(RNAi)* (E and F). In all three RNAi conditions, GLP-1 expression was high in the distal tip (not depicted) but was repressed normally in meiotic region of the distal arm (A, C, and E). In *car-1(RNAi)* (D) and *cgh-1(RNAi)* (F) oocytes, GLP-1 staining was equivalent or greater than distal tip staining on the same slide (high GLP-1 staining). Outlines denote the gonad surface. (G) The number of gonads with high GLP-1 staining in oocytes was scored blind in RNAi, *car-1(tm1753)* homozygotes and wild-type (N2) gonads. (H) *glp-1* mRNA from equal numbers of wild-type (N2) or *car-1(tm1753)* hermaphrodites was assayed by semiquantitative RT-PCR. Primers distinguished spliced (shown) from unspliced mRNA and DNA products, which were not detected (not depicted). (top) Each lane shows *glp-1* cDNA from one worm equivalent of total RNA; *actin* mRNA levels were similar in both samples (not depicted). (bottom) Changes in RT-PCR product with cDNA input amount suggest that mutant and wild-type RNA levels were equivalent or differed by less than twofold.

tected in grP bodies in 96% of injected distal gonad arms ($n = 27$) but did associate with oocyte grP bodies in 97% of stained proximal arms ($n = 29$; Fig. 4, K–M; and not depicted). Consistent with this localization pattern, *lacZglp(TCR)* translation was active in early meiotic cells but repressed in oocytes of arrested female gonads (Fig. 5 C and Table S1). These results show that the *glp-1* SCR is required for granule targeting specifically during early meiosis. The *glp-1* TCR targets mRNA to grP bodies specifically in oocytes, where it confers PUF-dependent repression

Table I. Genetic interactions between *car-1* and *puf-5*

Genotype ^a	RNAi	Small oocyte phenotype ^b	Yolk accumulation ^c	Embryo defects ^d	
				Egg shell defects	Cytokinesis defects
		%	%	%	%
+/+ (N2)	empty vector	2 (58)	0 (29)	1 (104)	0 (219)
+/ <i>hT2</i>	<i>puf-5</i>	0 (28)	0 (23)	8 (209)	8 (209)
<i>car-1</i> / <i>hT2</i>	empty vector	0 (55)	0 (48)	0 (244)	1 (357)
<i>car-1</i> / <i>hT2</i>	<i>puf-5</i>	75 (85)	96 (73)	91 (232)	98 (174)
<i>car-1</i> / <i>car-1</i>	empty vector	23 (39)	0 (37)	nd	nd
<i>car-1</i> / <i>car-1</i>	<i>puf-5</i>	86 (36)	84 (37)	90 (49)	98 (203)
+/+ (N2)	<i>puf-5</i>	3 (87)	0 (51)	4 (246)	4 (234)
+/+ (N2)	<i>car-1</i>	22 (9)	0 (9)	2 (80)	88 (80)
+/+ (N2)	<i>puf-5</i> ; <i>car-1</i>	75 (8)	100 (8)	97 (70)	97 (70)

Numbers in parentheses indicate the number of gonads,^b animals,^c or embryos^d scored. nd, not determined.

^a"*car-1*" is the *car-1(tm1753)* deletion allele; *hT2* is a balancer chromosome carrying *car-1(+)*.

^bPercentage of gonads in which >1 proximal oocytes did not span the dorsal/ventral gonad borders.

^cPercentage of animals with large yolk droplets in the pseudocoelom.

^dPercentage of embryos with undetectable egg shells or cytokinesis defects as in Fig. 7 F.

(Fig. 5 C and Table S1; Lublin and Evans, 2007). These results support the idea that distinct translational repression systems can independently trigger movement of *glp-1* mRNA into P body-like particles.

grP body components have stage-specific functions in *glp-1* repression

To test if grP body components influence maternal mRNA control, we asked if loss of CAR-1 or CGH-1 function disrupted the repression of *glp-1*. Control (mock) RNAi gonads had low or undetectable GLP-1 staining both in the distal arm and in oocytes (Fig. 6, A, B, and G). RNAi depletion of CAR-1 caused ectopic GLP-1 protein expression in oocytes of both wild-type and *fog-2(q71)* arrested gonads (Fig. 6, D and G; and not depicted). However, *car-1(RNAi)* did not disrupt *glp-1* repression in the distal arm; GLP-1 protein remained undetectable in early meiotic and pachytene regions in all *car-1(RNAi)* gonads ($n > 50$; Fig. 6 C). Gonads homozygous for the *car-1(tm1753)* deletion also exhibited ectopic GLP-1 expression only in oocytes, except with 100% penetrance (Fig. 6 G), as expected, because *car-1(tm1753)* is a strong loss-of-function or null mutation (Audhya et al., 2005). *glp-1* mRNA levels were similar in *car-1(tm1753)* mutant and wild-type animals, which supports CAR-1 control of *glp-1* translation rather than mRNA production or stability (Fig. 6 H). These results suggest that CAR-1 promotes PUF-dependent translational repression of *glp-1* in late oogenesis but does not influence GLD-1-mediated repression in early meiosis. To further explore this dichotomy, we examined control of GLD-1-regulated *rme-2* mRNA. As with *glp-1*, *rme-2* mRNA remained strongly repressed in the distal arms of all *car-1(tm1753)* gonads (unpublished data). Thus, CAR-1 function is required specifically by mRNA control systems that function in late oogenesis.

We also observed smaller but reproducible increases in GLP-1 expression in *cgh-1(RNAi)* gonads (Fig. 6, F and G). As with *car-1* loss, ectopic GLP-1 staining was restricted to proximal oocytes (Fig. 6, E and F). However, *cgh-1(RNAi)* also induced

loss of *glp-1* mRNA and CAR-1 in arrested female oocytes (described in a later section). These findings complicate conclusions on CGH-1 control of *glp-1*. However, CGH-1 may also contribute to *glp-1* mRNA repression in late stages of oogenesis.

CAR-1 functions with PUF-5 during late oogenesis

CAR-1 promotes GLP-1 repression only during late oogenesis. This suggests that it might function with PUF-5 and PUF-6/7, which control *glp-1* and other mRNAs at this time. To examine this possibility, we asked if *car-1* and these *puf* genes had related functions. Previous studies showed that *car-1* loss does not severely perturb oogenesis in most gonads (Audhya et al., 2005; Boag et al., 2005; Squirrell et al., 2006), which is unlike the potent defects in oocyte differentiation seen after depletion of PUF-5 and PUF-6/7 (Lublin and Evans, 2007). However, oogenesis defects that resemble *puf-5*; *puf-6/7(RNAi)* were seen in a low percentage of *car-1(RNAi)* animals, particularly as adults aged or if cell death was blocked in *car-1(RNAi)* animals (Table I; Boag et al., 2005). Thus, *car-1* and these *puf* genes may have some functions in common. To test this further, we looked for genetic interactions between *car-1* and *puf-5*. RNAi depletion of only PUF-5 produced normal oocytes arranged in a typical single row, as well as normal viable embryos (Fig. 7, A and D; and Table I), as expected, because PUF-5 is functionally redundant with PUF-6/7 (Lublin and Evans, 2007). Loss of only one *car-1(wt)* allele (in *car-1(tm1753)/hT2* heterozygotes) also did not perturb oogenesis or early embryo morphology (Fig. 7, B and E; and Table I). Strikingly however, when PUF-5 was depleted in *car-1(tm1753)/hT2* heterozygotes, severe defects were observed that strongly resembled loss of PUF-5 and PUF-6/7. Many *puf-5(RNAi)*; *car-1(tm1743)/hT2* gonads had small oocytes arranged in two rows, produced embryos that lacked detectable egg shells, and had severe cytokinesis defects similar to *puf-5*; *puf-6/7* embryos (Fig. 7, C and F; and Table I). The same phenotype was seen in PUF-5-depleted *car-1(tm1753)* homozygotes and when both CAR-1 and PUF-5

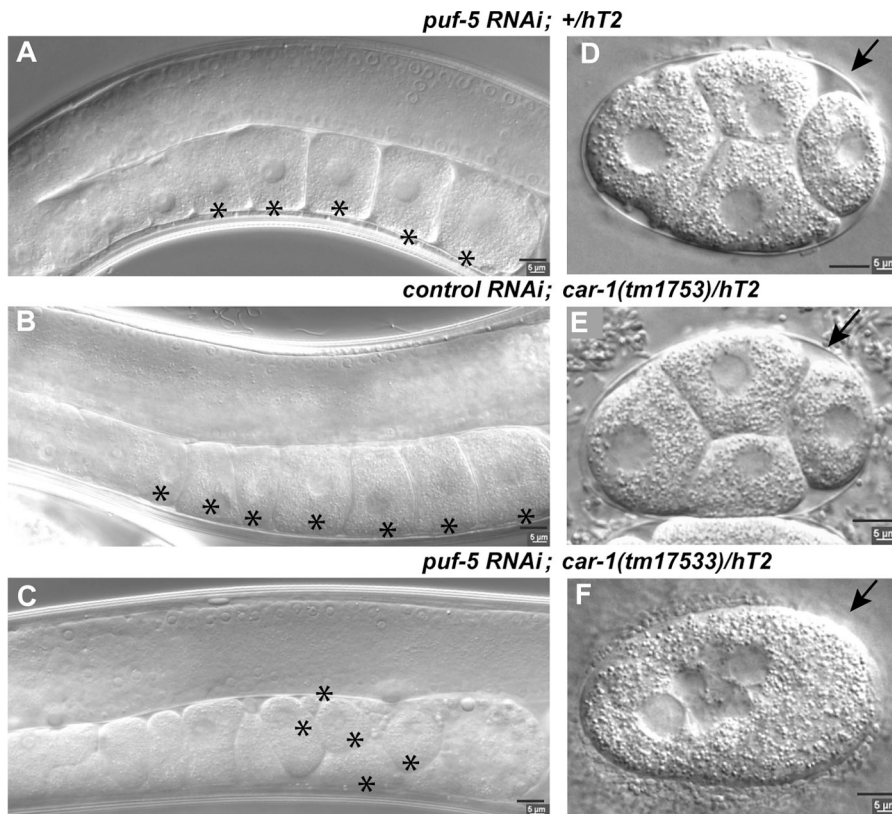


Figure 7. *car-1* and *puf-5* genetically interact to control oocyte formation and embryogenesis. Nomarski images of hermaphrodites (A–C) and embryos (D–F) of the indicated genotypes are shown. As in the wild type, most *puf-5(RNAi);+/hT2* and empty vector RNAi (control); *car-1(tm1753)/hT2* animals had a single row of oocytes (A and B, asterisks), and embryos with a clear eggshell (D and E, arrow) and normal cytokinesis. However, *puf-5* RNAi of *car-1(tm1753)/hT2* heterozygotes caused abnormal small oocytes (C, asterisks) and embryo cytokinesis defects (F); embryonic egg shells (F, arrow) were not detectable. Black bars, 10 μ m; white bars, 5 μ m.

were depleted by RNAi, which confirms that these phenotypes were induced by CAR-1 loss (Table I). Together with effects of CAR-1 on *glp-1* repression, this synergistic genetic interaction suggests that CAR-1 functions together or in parallel with the PUF-5 and PUF-6/7 regulatory system, perhaps to influence common mRNA targets. Because *car-1* loss alone gives only a weakly penetrant oogenesis phenotype, CAR-1 likely contributes but is not essential for repression of PUF-bound mRNAs.

CAR-1 controls grP body formation and mRNA targeting

CAR-1 could influence translational control by regulating granules themselves. To test this possibility, we examined protein and mRNA granule components in *car-1(RNAi)* and *car-1(tm1753)* gonads. When CAR-1 was depleted in *fog-2(q71)* female gonads, CGH-1–stained granules in oocytes became substantially smaller (Fig. 8, compare B to E); numerous oocyte granules larger than 2 μ m were present in 96% of control gonads ($n = 51$), whereas only 33% of *car-1(RNAi)* gonads ($n = 54$) contained any granules larger than 2 μ m. Strikingly, *glp-1* mRNPs were present at similar levels throughout *car-1(RNAi)* oocytes but no longer colocalized with CGH-1–stained granules in oocytes (Fig. 8, D–F). These results suggest that CAR-1 either promotes *glp-1* mRNA targeting to granules or maintains *glp-1* in granules once it has been localized. Similarly, all *car-1(tm1753);fog-2(q71)* female gonads also had abnormally small CGH-1 granules in arrested oocytes (unpublished data). In contrast, *dcap-2* RNAi did not obviously alter the abundance, size, or morphology of grP bodies (unpublished data), which is

consistent with recent studies of DCAP-2 in activated hermaphrodite gonads (Boag et al., 2008). Interestingly, *car-1(RNAi)* also disrupted grP bodies in the distal arm, with only 6% ($n = 36$) of *car-1(RNAi)* distal gonads containing granules over 2 μ m compared with 93% ($n = 46$) of control RNAi distal gonads (Fig. 8, compare G–I to J–L). Furthermore, distal *glp-1* mRNPs no longer aggregated with CGH-1 granules in *car-1(RNAi)* distal arms (Fig. 8, J–L). Therefore, CAR-1 is required for grP body formation and *glp-1* mRNA targeting both in early meiosis and late oogenesis of arrested gonads but only contributes to *glp-1* repression during late oogenesis.

Loss of CGH-1 had very different effects on mRNA granules. Previous work showed that CGH-1 loss caused CAR-1 to form abnormal enlarged structures in hermaphrodites (Audhya et al., 2005; Boag et al., 2005), which we confirmed (unpublished data). We extended this analysis to arrested gonads of *fog-2(q71)* females. In the distal gonad, depletion of CGH-1 in *fog-2(q71)* females altered granule shape; granules became large elongated rod- or sheetlike structures in *fog-2(q71)* gonads, unlike the more rounded particles in control gonads (Fig. 9, A and B). CGH-1 loss, however, did not prevent association of *glp-1* mRNA with these sheetlike structures (Fig. 9, B and C). Interestingly, these structures appeared to break down into small puncta in the bend region of the gonad and then disappear in 97% ($n = 63$) of *cgh-1(RNAi);fog-1(q71)* oocytes (Fig. 9 D). In addition, *glp-1* mRNA levels became low or undetectable in oocytes where these particles disappeared (100%, $n = 18$; Fig. 9 E). Therefore, CGH-1 functions are distinct from CAR-1. CGH-1 is required to maintain CAR-1 and *glp-1* mRNA stability during this late stage of oocyte development.

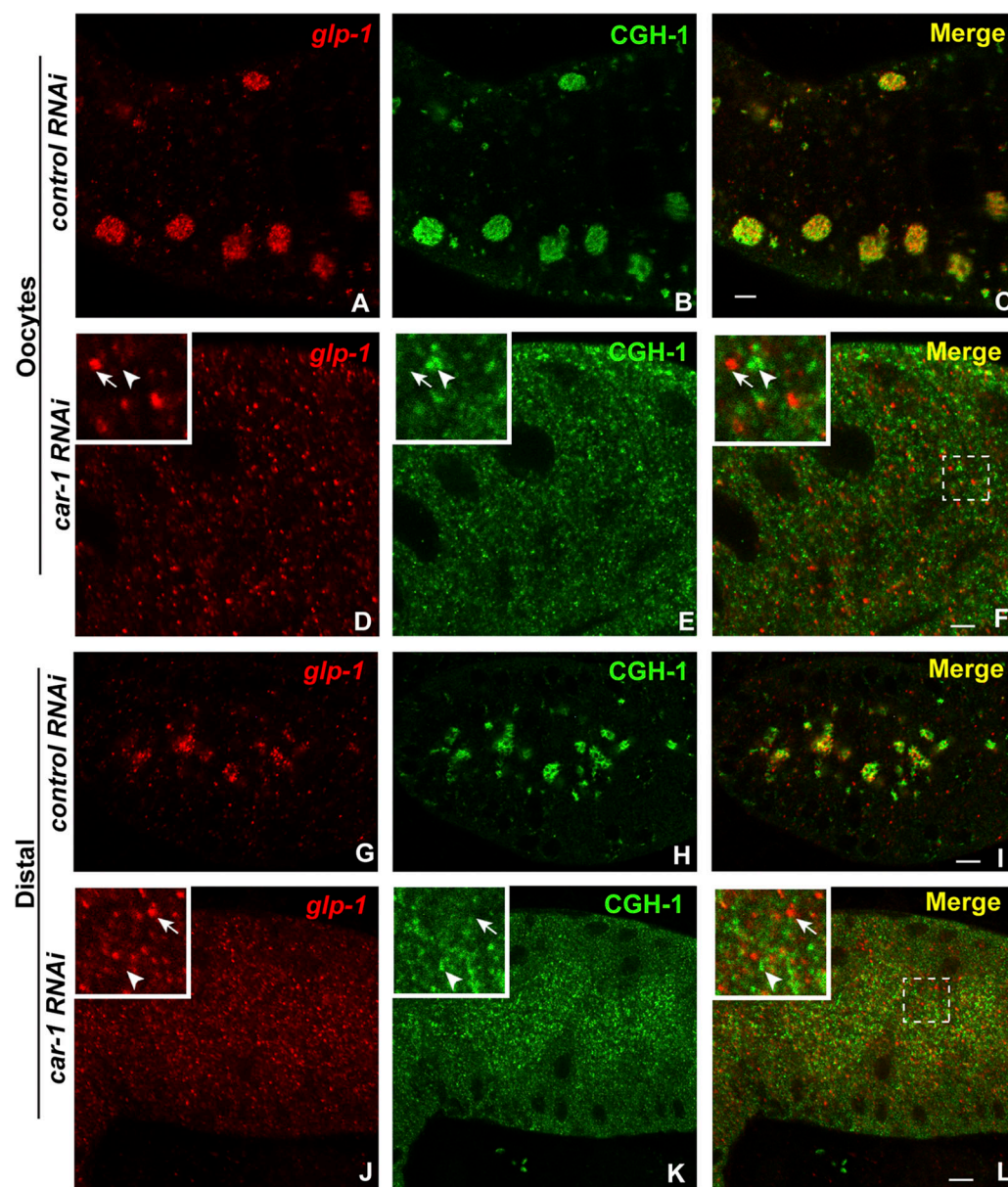


Figure 8. **CAR-1 regulates mRNP granule size and mRNA targeting.** (A–L) Confocal images of gonads stained for endogenous *glp-1* mRNA (red) and CGH-1 protein (green) after empty vector RNAi (control RNAi) or *car-1* RNAi. (A–F) Oocyte sections; (G–L) distal gonad sections. After *car-1* RNAi, CGH-1 granule size in both oocytes (E) and in distal arms (K) was reduced, and *glp-1* mRNPs (arrows in insets) were distinct from CGH-1 granules (arrowheads in insets). Insets are enlargements of the dashed boxes in F and L. Bars, 5 μ m.

Discussion

P bodies, stress granules, and related mRNP particles have been proposed as general sites for mRNA repression and degradation. Our studies suggest that within the *C. elegans* gonad, mRNP granules are diverse, have stage-specific functions in maternal mRNA control, and are dynamically regulated during development. At least three types of mRNP granules are made in the *C. elegans* germ line, and a fourth is made in the embryo. Repressed maternal mRNAs are targeted to at least one of these granule types, whereas activated mRNAs are excluded. Furthermore, the granule component CAR-1 controls this targeting and functions with mRNA-specific PUF proteins to promote differentiation during late oogenesis. These results suggest that oo-

cyte grP bodies participate in translational silencing of mRNAs that are needed for oocyte and embryo development. However, this function is stage specific, and mRNP granule formation depends on specific developmental signals. These data suggest that P body-like granules are not primary mediators of mRNA repression but have specific modulatory functions that change during development.

Different mRNP granule components have distinct functions

CAR-1 homologues are found in P body-related granules from yeast to human cells, but their functions are unclear. Previous work showed that CAR-1 proteins control germ cell apoptosis, embryonic cell division, oocyte-somatic gonad interactions, and

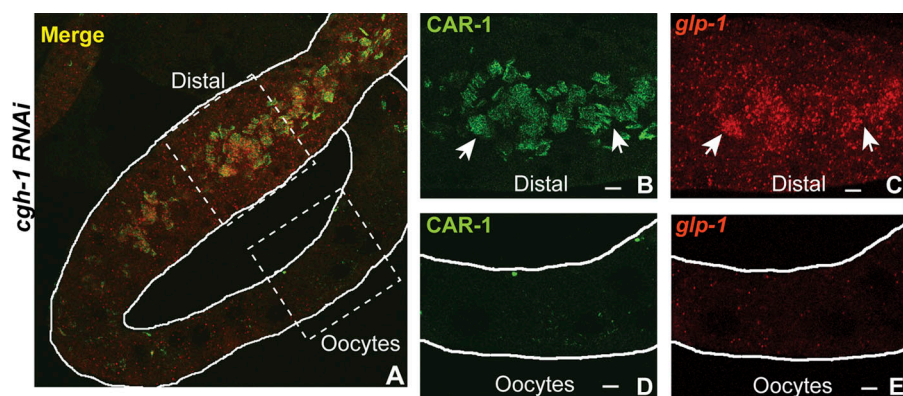


Figure 9. CGH-1 regulates mRNP granule morphology and stability. (A) A *cgh-1(RNAi);fog-2(q71)* gonad stained for both CAR-1 and *glp-1* mRNA (merged image) is shown. In the distal core, CAR-1 (B) localized with *glp-1* mRNA (C) in abnormal elongated sheetlike particles (compare with controls in Fig. 8). Arrows indicate examples of the same two particles in both images. In oocytes, both CAR-1 (D) and *glp-1* mRNA (E) levels were very low. B–E are 1.5× enlarged views of the boxed regions in A. Outlines denote the gonad surface. Bars, 5 μm.

neuronal morphogenesis (Audhya et al., 2005; Boag et al., 2005; Wilhelm et al., 2005; Barbee et al., 2006; Squirrell et al., 2006). At a cellular level, CAR-1-related proteins control granule size and endoplasmic reticulum dynamics, and can promote translational repression when synthetically tethered to a reporter mRNA (Wilhelm et al., 2005; Tanaka et al., 2006; Yang et al., 2006). Our results suggest a specific relationship of CAR-1 with mRNA-specific repressors and mRNP granules in vivo. CAR-1 contributes to endogenous *Notch/glp-1* repression specifically during late oogenesis, when *glp-1* silencing depends on three functionally redundant PUF proteins (PUF-5, PUF-6, and PUF-7). Synergistic genetic interactions between *car-1* and *puf-5* suggest that CAR-1 works with or in parallel to these PUFs to control several and perhaps all of their mRNA targets. Furthermore, CAR-1 promotes formation or maintenance of grP bodies in arrested oocytes and is required for efficient targeting of *Notch/glp-1* mRNPs to these particles throughout the germ line. Thus, CAR-1 may promote repression of PUF mRNA substrates by inducing association of mRNA targets with these mRNP granules or by maintaining granule integrity (Fig. 10). Alternatively, CAR-1 could participate in an early step of translational inactivation for many maternal mRNAs, which, in turn, stimulates mRNP aggregation. However, *car-1* deletion alone produces relatively mild germ line phenotypes, unlike loss of GLD-1 or

PUF-5 and PUF-6/7, and CAR-1 is not required for repression of at least two GLD-1-regulated mRNAs (this paper; Francis et al., 1995; Lublin and Evans, 2007). Therefore, CAR-1 control of granule structure is not essential for and can be separated from repression. This is consistent with the idea that RNA-binding factors provide translational repression and that CAR-1 subsequently promotes association of repressed mRNPs with granules. Alternatively, CAR-1 could have diverse functions that independently influence repression and mRNP aggregation. Regardless, these observations suggest that the influence of CAR-1 on translational repression is specific to particular repression factors (e.g., PUFs) or specific subsets of mRNAs.

The RNA helicase CGH-1/Dhh1/RCK also regulates mRNP granules in the gonad, but its functions are distinct from CAR-1. CGH-1 likely controls unique aspects of mRNP granule dynamics and composition, and is required to maintain at least one granule factor (CAR-1) and a maternal mRNA (*glp-1*) as oocytes differentiate and arrest. Thus, CGH-1 may promote the stability of mRNP particles when oocytes enter an arrested state. A recent study showed that CGH-1 stabilizes numerous maternal mRNAs even during active oogenesis, which suggests that mRNA protection may in fact be central to CGH-1's germ-line function (Boag et al., 2008). This finding is surprising given that CGH-1 orthologues in other systems promote mRNA decay

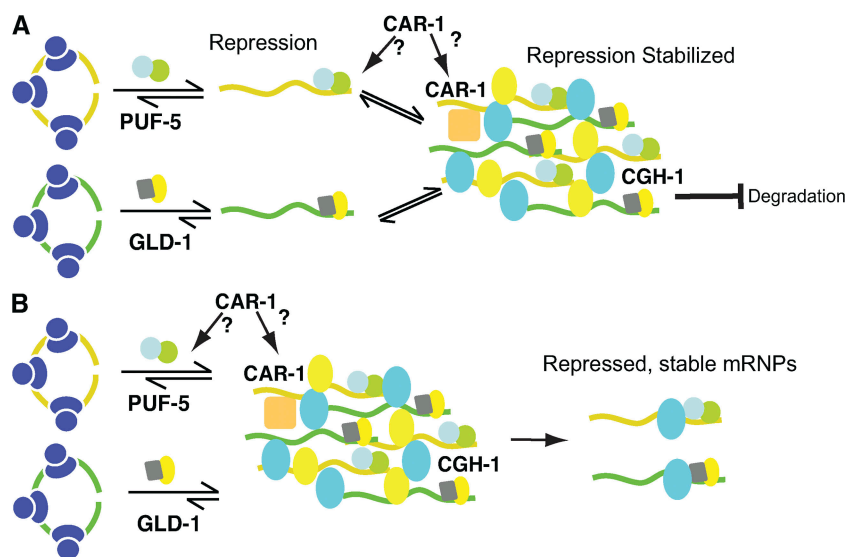


Figure 10. Models for mRNP regulation during oogenesis. (A) A two-step model; mRNA repression is triggered by specific mRNA-binding factors (GLD-1 and PUF-5). Aggregation of repressed mRNPs reinforces repression by reducing rates of reentry of mRNAs into translation complexes (circular mRNPs on the left). CAR-1 may influence translation by controlling aggregation or mRNA targeting, or it could independently regulate translation and granule formation. (B) Granules as mRNP modification centers: Specific factors induce transient association of mRNAs with mRNP modification complexes where mRNPs exit in a repressed state; aggregation of these complexes into granules could improve efficiency of mRNP modification.

(Parker and Sheth, 2007). Therefore, the function of this DEAD box helicase depends on cellular and developmental context, and differs from CAR-1 during oocyte development.

mRNP granule diversity and regulation

Our results suggest that the *C. elegans* germ line generates multiple types of mRNP granules that likely have different functions. During early meiosis, germ granules associate with germ nuclei, whereas distinct grP bodies accumulate in the cytoplasmic core. As oocytes form and arrest, a third type of particle arises in the oocyte cytoplasm; these dcP bodies are unique in that they lack detectable CGH-1 and CAR-1 but contain the decapping enzyme DCAP-2. Once oocytes are fertilized and embryonic divisions begin, new particles form in somatic blastomeres, in which DCAP-1, DCAP-2, CAR-1, and CGH-1 all tightly colocalize together (this paper; Audhya et al., 2005; Boag et al., 2005; Lall et al., 2005; Squirrell et al., 2006). Therefore, a diverse collection of particles that defies a simple nomenclature forms in *C. elegans* germ cells and embryos. All share some features with P bodies and stress granules in other organisms. These findings raise the possibility that mRNP granules are more diverse than previously appreciated in other metazoans. Different *C. elegans* granules appear to dynamically interact with each other (see Fig. 2), which may reflect mRNP movements from one granule class to another. These interactions are reminiscent of the docking interactions between P bodies and stress granules in mammalian cells (Kedersha et al., 2005). Thus, direct interactions between different granule types may be common.

The various RNP granules in the *C. elegans* gonad are regulated by developmental transitions and cues. Both grP bodies and dcP bodies form or enlarge when sperm are absent. In addition, dcP bodies arise only during late oogenesis, and embryonic P bodies are only detected after cell division begins in the embryo. P granules also follow unique dynamic transitions during early development (this paper; Strome, 2005). Therefore, mRNP granule diversity, structure, and localization are tightly controlled during development, which suggests that each granule type has unique functions that change with different developmental states.

The roles of P body-like particles in mRNA control

A key question is how the various mRNP granules influence mRNA regulation. One function for oocyte grP bodies may be to protect repressed maternal mRNAs from degradation. The importance of CGH-1 to maternal mRNA stability supports this idea (this paper; Boag et al., 2008). This hypothesis is attractive because maternal mRNAs are not transcribed in arrested oocytes and many are needed for embryonic development. Perhaps dcP bodies arise specifically in late-stage arrested oocytes to help segregate or inhibit decapping and degradation activities.

Several results suggest that *C. elegans* grP bodies are tied to translational repression. Repressed mRNAs localize to grP bodies, whereas translationally active mRNAs are excluded. 3' UTR regions that trigger repression promote mRNA localization to these particles. Furthermore, CAR-1 loss disrupts both granules and repression. However, three findings argue that mRNP aggregation

into enlarged granules is not essential for translational silencing. First, grP body disruption in early meiosis did not perturb repression of at least some GLD-1-regulated mRNAs. Second, *car-1* loss strongly disrupted grP body aggregation but only causes relatively weak phenotypes, unlike loss of mRNA-specific repressors. Third, *Notch/glp-1* mRNA is strongly repressed when oogenesis is activated by sperm and mRNPs are very small. Thus, mRNA repression does not strictly require sequestration into large grP bodies. A similar conclusion was drawn from yeast and fly cells, where disruption of visible P bodies did not impair repression or mRNA decay (Decker et al., 2007; Eulalio et al., 2007). One simple idea is that mRNP granules are not central to repression but reinforce mRNA controls imposed by specific regulatory factors (Fig. 10). In one model, mRNA-specific RNA-binding proteins trigger translational repression, then subsequent localization of repressed mRNPs to grP bodies ensures sustained tight repression by limiting access of repressed mRNAs to translation machinery (Fig. 10 A). Different repressors likely trigger mRNA targeting to a common granule, but this reinforcement system may only influence repression systems that are relatively inefficient; in the *C. elegans* gonad, GLD-1 may tightly repress mRNAs such that grP body localization is dispensable, whereas PUF-5 and PUF-6/7 may require reinforcement to maintain the repressed state. An alternative model is that granules represent mRNP modification centers that interact transiently with mRNAs (Fig. 10 B); enlarged granule size could reflect aggregation of these complexes or increased duration of mRNP residence. In this scenario, aggregation of modification complexes (granule formation) could improve mRNP assembly rates, which might only significantly impact some regulatory systems under specific physiological conditions. Future studies of mRNP composition and dynamics will be needed to explore these ideas.

Materials and methods

Plasmids and strains

Strains used were N2 (Bristol wild-type strain), *fog-2(q71)*, *car-1(tm1753)*, *car-1(tm1753)/ht2*, *+/ht2*, and *car-1(tm1753)/ht2;fog-2(q71)* maintained at 20°C using standard methods (Brenner, 1974). The *car-1(tm1753)* mutant was provided by S. Mitani (National BioResource Project, Tokyo, Japan) and *car-1(tm1753)/ht2* was provided by J. Squirrell and J. White (University of Wisconsin, Madison, WI; Squirrell et al., 2006). For RNAi feeding vectors, cDNA fragments for *puf-5* [full open reading frame (ORF)], *car-1* [full ORF], and *cgh-1* (267–1284) were made by RT-PCR and cloned into L4440 to make pTE7.3, pTE7.4, and pTE7.5, respectively. The *dcap-2* L4440 feeding vector was obtained from the Ahinger library (Kamath and Ahinger, 2003). Reporter mRNA plasmids pJK370 (lacZ), pTE4.0 (lacZglp[wt]), and pTE13.0 (lacZglp[TCR]) were as described previously (Marin and Evans, 2003; Lublin and Evans, 2007). pTE2 was as described previously (Barbee and Evans, 2006). All DNAs were sequenced by the University of Colorado Cancer Center DNA Sequencing and Analysis Center.

Immunofluorescence

Animals were dissected, fixed, and stained with antibodies as described previously (Barbee et al., 2002). Antibodies used for immunofluorescence (IF) include rabbit anti-CGH-1 (Navarro et al., 2001), chicken anti-CAR-1 (gifts from K. Blackwell, Joslin Diabetes Center, Harvard Medical School, Boston, MA; Boag et al., 2005), rabbit anti-PUF-5 (Lublin and Evans, 2007), two rat anti-DCAP-2 (NY989 and NY990; gifts from R. Davis, University of Colorado at Denver and Health Sciences Center, Denver, CO; Lall et al., 2005), rabbit anti-GLD-1 (gift from T. Schedl, Washington University School of Medicine at St. Louis, St. Louis, MO; Jones et al., 1996), rabbit anti-MEX-3 (gift from C. Hunter, Harvard University, Cambridge, MA; Huang et al., 2002) and monoclonal anti-MEX-5 (gift from J. Priess, Fred Hutchinson

Cancer Research Center, Seattle, WA; Schubert et al., 2000), rabbit anti-GLP-1 (gift from J. Kimble, University of Wisconsin, Madison, WI; Evans et al., 1994), rabbit anti-PGL-1 (gift from S. Strome, University of California, Santa Cruz, Santa Cruz, CA; Kawasaki et al., 1998), rabbit anti-RME-2 (gift of B. Grant, Rutgers University, Piscataway, NJ; Grant and Hirsh, 1999) and K76 monoclonal anti-PGL-1 (K76 was developed by S. Strome and provided by the Developmental Studies Hybridoma Bank, which is maintained by grant N01-HD-7-3263 of the National Institute of Child Health and Human Development). Antibody staining was shown to be specific by RNAi depletion (Fig. S1, also see Results) or by the expected specific staining pattern of previously characterized antibodies (Evans et al., 1994; Jones et al., 1996; Grant and Hirsh, 1999; Schubert et al., 2000; Huang et al., 2002). Secondary antibodies were conjugated to Alexa fluor 488, 546, or 594 (Invitrogen), or Cy2 or Cy3 (Jackson ImmunoResearch Laboratories). Images were acquired at room temperature from fixed tissue in 50% glycerol by a laser scanning confocal microscope (LSM 510; Carl Zeiss, Inc.) with a META detector and LSM 510 4.0 software, using a 63x 1.4 NA Plan Apochromat objective (for Figs. 2–4 and 8; Carl Zeiss, Inc.). Images were also acquired with an Axioskop microscope (Carl Zeiss, Inc.) with epifluorescence using a 63X 1.4 NA Plan Apochromat objective and imaged with AxioCam (HRC) and Axiovision 4.6 software (both from Carl Zeiss, Inc.). Images were imported into Photoshop CS2 (Adobe); no adjustments were made for images in Fig. 6; identical enhanced per channel contrast adjustments were made for all fluorescence images in Figs. 2–4, 8, and 9. For all paired test and control specimens, identical exposures and adjustments were used (e.g., RNAi experiments in Figs. 6, 8, and S1).

FISH

Antisense or sense digoxigenin-labeled (dig) RNA probes were synthesized by Megascript in vitro transcription (Ambion) as described previously (Barbee and Evans, 2006). Templates were linearized pTE2 (for *glp-1*), or PCR templates were made from cDNA (ORFs) for *lacZ* (256–3200), *rme-2* (632–2676), *act-1* (8–1125), and *pos-1* (full length), as described previously (Barbee and Evans, 2006). Adult animals were dissected, freeze-cracked, and fixed in methanol on slides as described previously (Barbee et al., 2002); rehydrated progressively in 90%, 70%, and 50% methanol; washed with PBS + 0.5% BSA (PBS/BSA); and then fixed with 4% formaldehyde. Slides were washed with PBS + 2 mg/ml glycine and preincubated in hybridization buffer (50% formamide, 5x SSC, 100 µg/ml salmon sperm DNA, and 0.01% Tween 20) at 50°C for 1 h. Dig-labeled RNA probes in hybridization buffer were hybridized at 50°C overnight. Slides were then washed at 50°C over 5 h in wash buffer (50% formamide, 5x SSC, and 0.01% Tween 20), 2x SSC at room temperature, and finally washed in PBS/BSA. Probes were detected with Fluorescent Antibody Enhancer Set for DIG Detection (Roche) using Cy3-labeled mouse anti-digoxigenin (Cy3-anti-dig) antibody (Jackson ImmunoResearch Laboratories). For simultaneous IF and FISH, primary antibodies were included in the first anti-dig incubation, and fluor-tagged secondary antibodies were included with Cy3-anti-dig. Images were acquired as described in the previous section. Sense or non-specific RNA probes produced very low staining (Fig. S2), which indicates that the signal seen by probes against endogenous mRNAs is specific.

RNAi

RNAi was performed by feeding nematodes HT115 bacteria that were induced to express double-stranded RNAs from L4440-based plasmids as described previously (Timmons et al., 2001; Barbee and Evans, 2006). For each experiment, L4440 with no insert (empty vector) was used as a control. For *car-1*(RNAi) and *cgh-1*(RNAi), L4 worms were grown on test or control feeding plates at 20°C for 38–42 h. For antibody specificity tests (Fig. S1), some test and control RNAi plates were incubated for 48 h. RNAi-treated worms were examined by Nomarski optics or by IF and FISH. Each experiment was repeated 2–5 times.

Reporter mRNA localization and translation assays

Capped and polyadenylated *lacZ* reporter mRNAs were made in vitro and microinjected as described previously (Evans et al., 1994; Marin and Evans, 2003). Reporter mRNAs (80 nM) were injected into young virgin *fog-2(q71)* females, and worms were incubated on OP-50-seeded plates for 17–18 h at 20°C. Gonads were then fixed and stained by FISH using an antisense *lacZ* probe and costained for CGH-1. FISH of noninjected *fog-2(q71)* gonads revealed that background staining by the *lacZ* probe was negligible, similar to sense RNA probes (Fig. S3). For β galactosidase (β gal) expression assays, *fog-2(q71)* females were injected and incubated exactly as for FISH but were instead fixed and stained with Xgal for 5 h at 37°C as described previously (Evans et al., 1994). Xgal-stained whole mount animals were examined under bright-field illumination with an Axio-

phot microscope (Carl Zeiss, Inc.) using 10x 0.3 NA and 40x 1.3 NA oil Plan Apochromat objectives (Carl Zeiss, Inc.), and scored for β gal expression as described in Table S1. Images were acquired with an AxioCam (HRC; Carl Zeiss, Inc.) as described in the “Immunofluorescence” section, using identical exposures and no adjustments for all images.

RNA extraction and RT-PCR

100 N2 or homozygous *car-1(tm1753)* worms were picked to tubes, washed once with PBS, once with cold PBS, once with cold double distilled H₂O, and frozen in dry ice/ethanol. RNA was isolated directly using TRIzol (Invitrogen). cDNA was made using Superscript III (Invitrogen) with random hexamer priming. Specific cDNAs were detected by PCR using primers that flanked either side of the last intron of *glp-1* or *act-1* genes; PCR products from spliced mRNA were easily resolved from DNA and nonspliced RNA products on 5% PAGE gels. For *glp-1*, we used primers 5'-CATCGACACCGAATCGAATGG-3' and 5'-AGTTAGGAGATATGTTGGGAGG-3'. The PCR products were run on a gel, stained with Sybr green nucleic acid stain, and analyzed on a Typhoon 940 scanner with ImageQuant 5.4 (MDS Analytical Technologies). No adjustments were made to scanned gel images in Fig. 5 H. PCR amplification conditions were optimized to obtain a semi-quantitative range (Fig. 5 H).

Online supplemental material

Fig. S1 shows specificity of antibodies to PUF-5, CAR-1, CGH-1, and DCAP2 for staining of RNP granules in arrested oocytes. Fig. S2 shows association of MEX-3 and MEX-5 with oocyte grP bodies. Fig. S3 shows that staining of mRNAs by FISH is specific. Fig. S4 shows that actin mRNA has a unique pattern of localization to grP bodies that is the opposite of *rme-2*. Table S1 presents summed data for β gal expression from reporter mRNAs injected into arrested female gonads. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200802128/DC1>.

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