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Protection of specific maternal messenger RNAs by the P body protein CGH-1 (Dhh1/RCK) during Caenorhabditis elegans oogenesis

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During oogenesis, numerous messenger RNAs (mRNAs) are maintained in a translationally silenced state. In eukaryotic cells, various translation inhibition and mRNA degradation mechanisms congregate in cytoplasmic processing bodies (P bodies). The P body protein Dhh1 inhibits translation and promotes decapping-mediated mRNA decay together with Pat1 in yeast, and has been implicated in mRNA storage in metazoan oocytes. Here, we have investigated in Caenorhabditis elegans whether Dhh1 and Pat1 generally function together, and how they influence mRNA sequestration during oogenesis. We show that in somatic tissues, the Dhh1 orthologue (CGH-1) forms Pat1 (patr-1)-dependent P bodies that are involved in mRNA decapping. In contrast, during oogenesis, CGH-1 forms patr-1-independent mRNA storage bodies. CGH-1 then associates with translational regulators and a specific set of maternal mRNAs, and prevents those mRNAs from being degraded. Our results identify somatic and germ cell CGH-1 functions that are distinguished by the involvement of PATR-1, and reveal that during oogenesis, numerous translationally regulated mRNAs are specifically protected by a CGH-1–dependent mechanism.

Introduction

Accurate temporal and spatial regulation of mRNA translation and degradation is critical for control of gene expression. In a dramatic example of this regulation, developing oocytes produce thousands of different mRNAs that must be maintained in a translationally silent state in order to prevent premature differentiation (Johnstone and Lasko, 2001; Ciosk et al., 2006; Richter, 2007). Many of these mRNAs are provided to the embryo maternally and then destroyed during the transition to zygotic gene expression (Schier, 2007; Stützel and Seydoux, 2007). Much has been learned about how specific mRNA-binding proteins control translation of individual maternal mRNAs in oocytes and early embryos (Lipshitz and Smibert, 2000; Wickens et al., 2002; Richter, 2007). However, less is understood about how these mRNAs are influenced by mechanisms that affect mRNA translation or turnover on a broader scale.

Various mechanisms that repress translation or degrade mRNAs are concentrated in cytoplasmic structures known as processing bodies (P bodies; also referred to as GW or Dcp bodies; Anderson and Kedersha, 2006; Eulalio et al., 2007a; Parker and Sheth, 2007). These mechanisms include decapping- and nonsense-mediated mRNA degradation, as well as translational silencing and mRNA degradation directed by small RNAs. P bodies are defined by a set of “core” proteins that are involved in decapping-dependent decay, a process in which the protective 5′ cap is removed from a deadenylated mRNA, thereby exposing it to 5′-to-3′ degradation (Parker and Sheth, 2007). These core P body proteins, as described in yeast, include the decapping enzyme subunits Dcp1 and Dcp2 and a set of accessory proteins that form a decapping complex on mRNAs.
and other metazoans, the Dhh1 helicase is found in a germ line RNP complex that includes CAR-1 and Y-box proteins (Table I; Wilhelm et al., 2000; Nakamura et al., 2001; Minshall and Standart, 2004; Weston and Sommerville, 2006). In early biochemical studies of *Xenopus laevis* oocytes, it was proposed that this set of proteins "masks" mRNAs to prevent their translation (Richter and Smith, 1984; Weston and Sommerville, 2006). It is still unknown whether the Dhh1 helicase associates with metazoan maternal mRNAs broadly or specifically, and how in general it influences their regulation in vivo. It has also not been determined whether the putative oocyte mRNA storage particles that are defined by this helicase correspond simply to a form of P body in which mRNA degradation has been blocked, or a different type of structure.

In *C. elegans*, maternally provided CGH-1 and CAR-1 perdure in the embryo. They then form particles that begin to disappear around the four-cell stage in somatic precursors but persist until around the 200-cell stage in the germ lineage (Navarro et al., 2001; Audhya et al., 2005; Boag et al., 2005). During *Caenorhabditis elegans* oogenesis (Fig. 1), Dhh1 (CGH-1) colocalizes with the P body protein CAR-1 in RNP particles that accumulate in the syncytial gonad cytoplasm in parallel to newly synthesized mRNAs (Navarro et al., 2001; Boag et al., 2005; Lall et al., 2005; Jud et al., 2008). In *D. melanogaster*, translational silencing of some oocyte mRNAs depends upon the Dhh1 orthologue Me31B (Nakamura et al., 2001). In these and other metazoans, the Dhh1 helicase is found in a germ line RNP complex that includes CAR-1 and Y-box proteins (Table I; Wilhelm et al., 2000; Nakamura et al., 2001; Minshall and Standart, 2004; Weston and Sommerville, 2006). In early biochemical studies of *Xenopus laevis* oocytes, it was proposed that this set of proteins "masks" mRNAs to prevent their translation (Richter and Smith, 1984; Weston and Sommerville, 2006). It is still unknown whether the Dhh1 helicase associates with metazoan maternal mRNAs broadly or specifically, and how in general it influences their regulation in vivo. It has also not been determined whether the putative oocyte mRNA storage particles that are defined by this helicase correspond simply to a form of P body in which mRNA degradation has been blocked, or a different type of structure.

Among these decapping accessory proteins are the Pat1 protein and the DEAD box RNA helicase Dhh1, which inhibit translation competitively and promote decapping complex assembly and P body formation (Coller and Parker, 2005; Pilkington and Parker, 2007). If decapping is prevented, this mechanism can maintain mRNAs in a translationally arrested state, providing a model for how silenced mRNAs might be sequestered in potentially any cell type. Evidence from human and *Drosophila melanogaster* cell lines indicates that the Dhh1 orthologue is important for microRNA (miRNA)-induced translational silencing (Chu and Rana, 2006; Eulalio et al., 2007c), which suggests that this inhibitory mechanism might be involved in posttranscriptional regulation in many contexts.

In developing oocytes, early embryos, and neurons, the Dhh1 orthologue and other core P body proteins are found in RNA–protein (RNP) granules that are proposed to correspond to mRNA storage particles (Navarro et al., 2001; Coller and Parker, 2004; Eulalio et al., 2007; Parker and Sheth, 2007). During *Caenorhabditis elegans* oogenesis (Fig. 1), Dhh1 (CGH-1) colocalizes with the P body protein CAR-1 in RNP particles that accumulate in the syncytial gonad cytoplasm in parallel to newly synthesized mRNAs (Navarro et al., 2001; Boag et al., 2005; Lall et al., 2005; Jud et al., 2008). In *D. melanogaster*, translational silencing of some oocyte mRNAs depends upon the Dhh1 orthologue Me31B (Nakamura et al., 2001). In these

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**Figure 1. A *C. elegans* hermaphrodite gonad, illustrating models discussed in the text.** Germ cells develop in an "assembly line" fashion from stem cells that are regulated by the somatic distal tip cell (Hubbard and Greenstein, 2005). Each of two gonad arms produces sperm during the fourth larval stage, then oocytes during adulthood. Germ cells initially share a common cytoplasm, which flows as shown by arrows (Wolke et al., 2007). Transcription increases sharply upon progression through the transition zone and entry into meiosis I. As new mRNAs move into the syncytial gonad core, some appear to pass through germ (P) granules (inset, green), which are located just outside of most nuclear pores (Pitt et al., 2000; Schisa et al., 2001). CGH-1 associates with and protects particular translationally regulated maternal mRNAs in the context of storage bodies, which may be assembled on germ (P) granules (inset). Oocytes enlarge and become cellularized in the proximal region, and fertilization occurs in the spermatheca. Transcription ceases as oocytes enter diakinesis (Walker et al., 2007). Further development depends upon translationally regulated maternal gene products until embryonic transcription begins at the four-cell stage (Seydoux and Dunn, 1997). CGH-1 then associates with PATR-1 in *patr-1*–dependent somatic P bodies (red dots).
Results

Somatic but not germ line CGH-1 particles correspond to decapping-associated P bodies

We first investigated whether the CGH-1 particles that are present during oogenesis are assembled by the same mechanism as "typical" P bodies, and whether such structures are present in C. elegans tissues. P bodies are dynamic structures that accumulate in proportion to their steady-state supply of mRNA (Parker and Sheth, 2007). If Pat1 or Dhh1 (CGH-1) is lacking, entry of mRNA into the decapping pathway is inefficient and P bodies are extremely small (Sheth and Parker, 2003; Eulalio et al., 2007b). In contrast, P bodies accumulate mRNA and become large if mRNA decapping is prevented. This paradigm is now well established in yeast and mammalian cells. As CGH-1 is a core P body component (Parker and Sheth, 2007), it is possible to detect C. elegans P bodies that are involved in decapping-dependent decay by identifying CGH-1 particles that are smaller when PATR-1 (Pat1) is lacking, and larger when decapping-dependent degradation is blocked.
We performed these analyses using RNAi against patr-1 and decapping-associated genes, and by examining previously uncharacterized predicted null mutations in patr-1 and dcap-2, the C. elegans orthologue of Dcp2 (decapping enzyme catalytic subunit; Fig. S1, A–C, and Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200801183/DC1). dcap-2(tm2470) homozygotes produce fertile offspring, though in reduced numbers, which makes it possible to examine the consequences of an absence of mRNA decapping in animals of any stage. In contrast, patr-1(tm2402) homozygotes produce offspring that arrest development during late embryonic or early larval stages.

To examine how lack of dcap-2 or patr-1 affects the distribution of CGH-1 during oogenesis, we used antibody staining to visualize endogenous CGH-1 in gonads. In wild-type (WT) hermaphrodites, in the mitotic precursors at the distal gonad end, CGH-1 and CAR-1 are apparent on germ (P) granules around the nuclear periphery (Fig. 1; Navarro et al., 2001; Boag et al., 2005). As germ cells enter meiosis, CGH-1 and CAR-1 together form structures that accumulate within the syncytial gonad core and lack characteristic germ (P) granule proteins (Figs. 1 and S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200801183/DC1; Navarro et al., 2001; Boag et al., 2005). Strikingly, this distribution of CGH-1 and CAR-1 was not detectably altered by lack of either PATR-1 or DCAP-2 (Fig. S2 A), which suggests that these structures are formed differently from “standard” P bodies. We will refer to the CGH-1/CAR-1 particles that are seen in the gonad core as storage bodies because of this distinction, and because we have found that CGH-1 protects many oocyte mRNAs from degradation (Fig. 1; see subsequent paragraphs).

Our failure to identify PATR-1-dependent CGH-1 particles in the germ line made it important to investigate whether such structures might be present in C. elegans somatic tissues. In the embryo, CGH-1 staining overlaps with CAR-1, DCAP-2, and its cofactor DCAP-1, as predicted for P bodies (unpublished data; Audhya et al., 2005; Boag et al., 2005; Squirrell et al., 2006). In somatic precursors, CGH-1 particles become increasingly less prominent after the four-cell stage in the WT but were far more prevalent at later stages after RNAi of dcap-1 and dcap-2 (dcap-1/-2[RNAi]; Fig. 2). This was also true in dcap-2(tm2470) embryos and after RNAi of the decapping-associated 5′-to-3′ exonuclease XRN-1 (unpublished data), which indicates that the decapping pathway is required for the normal disappearance of CGH-1 particles from the soma. In striking contrast, after the four-cell stage, CGH-1 foci were far less prevalent in patr-1[RNAi] and patr-1(tm2402) embryos than in WT so that by the 100-cell stage, patr-1[RNAi] embryos contained 12-fold fewer CGH-1 foci (Fig. 2, B–D). In somatic embryonic cells, CAR-1 antibody staining was affected similarly by dcap-1/-2 and patr-1[RNAi] (unpublished data), as was a rescuing transgenic fusion protein in which CGH-1 is linked to GFP (Fig. 2 E).

During postembryonic stages in WT animals, antibody staining detects CGH-1 and CAR-1 only in germ cells (Navarro et al., 2001; Boag et al., 2005). In contrast, in dcap-2(tm2470) larvae and adults, CGH-1 particles were readily apparent in many somatic tissues (Figs. 3 A and S1 F). Western blotting indicated that total CGH-1 levels were not dramatically affected in either patr-1(tm2402) or dcap-2(tm2470) adults (Fig. 3 C), which suggests that these postembryonic CGH-1 foci may be formed mainly through aggregation, not new CGH-1 synthesis. We conclude that these somatic CGH-1 foci, which we will refer to as somatic P bodies, may represent sites of decapping-mediated mRNA turnover.

Association of PATR-1 with somatic P bodies

We next investigated whether PATR-1 is present in these various types of CGH-1 particles (somatic P bodies, germ [P] granules, and storage bodies). Western blotting with an antiserum against a PATR-1 N-terminal epitope (PATR-1[N]) revealed that PATR-1 is expressed primarily in somatic tissues (Figs. 3 D and Fig. S1 D), and we did not detect PATR-1(N) immunostaining in dissected gonads (not depicted). In the embryo, PATR-1(N) staining was diffuse and barely detectible through the two-cell stage. Robust PATR-1(N) staining then appeared specifically in somatic P bodies at the four-cell stage (Fig. 3 B and not depicted), just before these structures became patr-1 dependent (Fig. 2 B). Subsequently, PATR-1(N) staining appeared in the somatic daughter of the germ line precursor shortly after each successive division and persisted in somatic cells until late embryonic stages (Fig. 3 B and not depicted). In dcap-2(tm2470) mutants but not the WT, PATR-1(N) staining that colocalized with CGH-1 was readily detectable during larval and adult stages (Figs. 3 A and S1 F). It is possible that in some cells, PATR-1 levels might be too low for detection, or the PATR-1(N) epitope might not be accessible. However, our finding that PATR-1(N) staining is detectable in CGH-1 foci only in somatic cells suggests that it represents a specific marker of degradative somatic P bodies.

Interactions between CGH-1 and translational regulators during oogenesis

Having observed that germ line CGH-1 particles seem to be formed differently from these somatic P bodies, we examined the germ line CGH-1 RNP complex further. In WT adults, the bulk of CGH-1 protein is found in germ cells (Boag et al., 2005). When we previously immunoprecipitated endogenous CGH-1 from adult extracts, we detected interactions between CGH-1 and CAR-1, Y-box proteins, and two prominent proteins of ~75 and ~70 kD (Boag et al., 2005). Through mass spectroscopy, we have now identified those last two proteins as ATX-2 (ataxin 2) and PAB-1 (poly–A–binding protein [PABBP]), respectively. ATX-2 and PAB-1 are RNA-binding proteins that are involved in translational regulation, and PABP protects mRNAs from deadenylation and degradation (Mangus et al., 2003; Ciosk et al., 2004). These two proteins interact with CGH-1 orthologues and each other in multiple species, but neither is typically found in P bodies (Table I; Mangus et al., 2003; Ciosk et al., 2004; Ralser et al., 2005; Wilhelm et al., 2005; Nonhoff et al., 2007; Anderson and Kedersha, 2008). We could detect PATR-1 in CGH-1 immunoprecipitates (IPs) by Western blotting (Fig. 3 E), which is consistent with evidence that this helicase interacts with Pat1 in other species (Ho et al., 2002; Coller and Parker, 2005; Krogan et al., 2006; Eulalio et al., 2007b). Neither actin nor the P body
Association of CGH-1 with specific maternal mRNAs during oogenesis

Interactions between CGH-1 and these proteins were abolished by RNase treatment, which suggests that mRNA might be present in this germ line CGH-1 complex (Boag et al., 2005). To test this idea, we performed four experiments in which we used RNA IP and microarray analysis (RIP-Chip) to detect mRNAs that coIP with CGH-1 (Fig. 4 A). We performed these IPs using extracts that were prepared from intact 1-d-old adults in a way...
Figure 3. **Association of CGH-1 and PATR-1 in somatic P bodies.** (A) Somatic P bodies in *dcap-2(tm2470)* larvae. Single-plane confocal images reveal colocalizing CGH-1 (green) and PATR-1(N)-staining (red) particles in many somatic cells in *dcap-2(tm2470)* but not N2 (WT) L1-stage larvae (outlined by broken lines). Regions outlined by dashed boxes are enlarged on the far right. *n > 100* for each set. Bars: 10 μm; [far right] 2 μm. [B] PATR-1(N) staining marks somatic P bodies in the embryo. WT embryos were analyzed by antibody staining and confocal microscopy (z series projections are shown). Germ cell precursors are outlined by broken lines, and in eight-cell embryos, the somatic sister of P3 (C blastomere) is similarly delineated. Robust PATR-1(N) staining is first detected in four-cell embryos, in which it colocalizes with CGH-1 in somatic blastomeres. After each successive division of the germ cell precursor PATR-1(N), staining is initially low, then increases. These low levels are apparent in the EMS blastomere (detail of the boxed area shown on the right), and also in the C blastomere (sister of P3) at the eight-cell stage. More than 50 embryos in each set were examined in each of at least two experiments. Bars: 10 μm; [far right] 2 μm. (C) CGH-1 and PATR-1 levels in N2, *dcap-2(tm2470)*, and *patr-1(2402)* adult hermaphrodites. PATR-1 was detected with anti-PATR-1(N). (D) PATR-1 expression in N2 and *glp-4(bn2)* adults, which essentially lack germ cells when grown at the restrictive temperature of 25°C. Densitometry indicated that *glp-4(bn2)* adult PATR-1 levels were ~68% of WT. (E) PATR-1 coIPs with CGH-1. CGH-1 IPs from 2.5 mg of adult (A) and embryo (Em) protein extracts were Western blotted for PATR-1.
that excluded embryos so that they were enriched for CGH-1 that is expressed in adult germ cells. We then identified mRNAs that were on average at least threefold overrepresented in CGH-1 IPs relative to IgG controls because we expected that this arbitrarily defined level would identify specific interactions. Surprisingly, only 392 mRNAs met this criterion (Fig. 4 B, and Tables II and S2, available at http://www.jcb.org/cgi/content/full/jcb.200801183/DC1).

If CGH-1 associates with C. elegans mRNA nonspecifically, our IPs should have isolated a random distribution of the most abundant somatic and germ line mRNAs because they were performed from whole animal extracts. Significantly, however, nearly all of the mRNAs that were enriched in our CGH-1 IPs are known to be expressed primarily in germ cells and to be provided to embryos as maternally supplied mRNA. Of the 305 CGH-1–associated mRNAs for which expression patterns have been characterized (Reinke et al., 2004), 92% (282) are expressed mainly in the hermaphroditic gonad and not during spermatogenesis, which indicates that they are produced during oogenesis. In addition, most CGH-1–associated mRNAs (85%) have been classified as maternal mRNAs (present in embryos before the onset of zygotic transcription), and 52% have expression profiles consistent with class II maternal mRNAs (Baugh et al., 2003). These CGH-1–associated mRNAs correspond to a significant but restricted fraction of the ~6,000 genes that are expressed in the hermaphrodite gonad (Baugh et al., 2003; Reinke et al., 2004).

Taken together, our findings suggest that CGH-1 may associate with a limited set of maternal mRNAs. To test this idea further, we examined the relative abundance of CGH-1–associated mRNAs by interrogating C. elegans serial analysis of gene expression (SAGE) databases (McKay et al., 2003; Khattra et al., 2007). The number of SAGE tags recovered for each mRNA is indicative of its relative abundance. If CGH-1 simply associates randomly with mRNAs that are present in developing germ cells, the most abundant germ line mRNAs should have been overrepresented in our CGH-1 IPs. This was not the case, however. None of the 50 most abundant gonad mRNAs were enriched at least threefold in our CGH-1 IPs (unpublished data). In addition, although the 50 most highly enriched CGH-1–associated mRNAs were represented by a mean of 9.8 nonambiguous SAGE tags (per 100,000) in a gonad mRNA library, the mean number of SAGE tags for the 50 most abundant gonad mRNAs was >38-fold higher (Fig. 4 C). This indicates that our CGH-1 IPs did not isolate the most abundant germ line mRNAs. A similar difference was seen when these CGH-1–associated mRNAs were compared with the 50 most abundant of all adult C. elegans mRNAs (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200801183/DC1). We conclude that during oogenesis, CGH-1 associates specifically with a discrete subset of germ line mRNAs, nearly all of which are expressed during oogenesis and provided to the embryo maternally.

The proteins encoded by these CGH-1–associated mRNAs are involved in various processes, including RNA binding, ubiquitylation, and gene regulation (Fig. S3 B). These functional categories are also highly represented among mRNAs that are present in mammalian oocytes (Evsikov et al., 2006; Kocabas et al., 2006).

Many CGH-1–associated mRNAs are involved in embryonic fate specification or cell cycle control (i.e., nos-2, pos-1, cdk-1, and cdk-5; Tables II and S2). In addition, many of these mRNAs are known to be translationally regulated during oogenesis, in that their protein products are not detected until late oogenesis or early embryonic stages (i.e., nos-2, pos-1, and pie-1; Tenenhaus et al., 1998; Subramaniam and Seydoux, 1999; Tabara et al., 1999). Together, the data suggest that CGH-1 associates with a particular subset of maternal mRNAs, many of which are subject to posttranscriptional regulation during oogenesis.

This interaction between CGH-1 and maternal mRNAs predicts that in oocytes, mRNA should be present in storage bodies along with CGH-1. To test this idea, we performed CGH-1 antibody staining simultaneously with in situ hybridization with a probe for SL1, a trans-spliced leader sequence that is found on
Table II. mRNAs that are most highly enriched in CGH-1 IPs

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<th>Reinke germ line expression</th>
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The CGH-1/IgG ratio indicates fold enrichment.

aMicroarray expression data from Baugh et al. (2001). MD, maternal degradation class (genes that are the subset of maternal genes that decrease without first increasing in abundance; ME, maternal-embryonic class [mRNAs with similar expression levels in pre- and postzygotic embryos]; SMD, strictly maternal degradation class (genes are the subset of maternal degradation genes that are not also classified as embryonic).

bMicroarray expression data from (Reinke et al. (2004). Intrinsic, similar expression in gonads that produce either oocytes or sperm; Oogenesis-enriched, mRNAs that were more abundant during oogenesis than spermatogenesis.

cEukaryotic clusters of orthologous groups of proteins database classification. A complete list of CGH-1 associated mRNA is available in Table S2 (available at http://www.jcb.org/cgi/content/full/jcb.200801183/DC1).

~60% of C. elegans mRNAs (Zorio et al., 1994). When C. elegans oogenesis is arrested, mRNA accumulates in large cytoplasmic foci, greatly enhancing its detection (Schisa et al., 2001). We therefore performed our experiments in the feminized strain fog-2(q71), which ovulates infrequently because it does not produce sperm (Schedl and Kimble, 1988). In fog-2(q71) oocytes, CGH-1 formed large particles that colocalized with the SL1 signal, which was also detected in smaller particles that lacked CGH-1 staining (Fig. 5, A–C). In fog-2(q71), this CGH-1 staining has been reported to colocalize with large CAR-1 foci, only some of which overlap with germ (P) granules (Jud et al., 2008). Taken together, this suggests that some but not all SL1-containing mRNA colocalizes with CGH-1 and CAR-1 in storage bodies and germ (P) granules.

Mislocalization and specific destabilization of germ line mRNAs in the absence of CGH-1

Our results raise the important question of how CGH-1 influences the localization, stability, or translational regulation of maternal mRNAs during oogenesis. Null cgh-1(ok492) and cgh-1(RNAi) hermaphrodites are sterile but produce oocytes in which gross abnormalities in gene expression have not been detected (Navarro et al., 2001; Boag et al., 2005). This makes it very unlikely that the lack of CGH-1 results simply in a widespread release of germ line translational suppression, which would lead to somatic differentiation in the gonad (Ciosk et al., 2006). CGH-1 is required for appropriate localization of CAR-1, however. If CGH-1 is absent, CAR-1 forms aberrant sheetlike structures within the syncytial gonad core (Fig. S2 B; Audhya et al., 2005; Boag et al., 2005). This appears to involve a defect in storage body formation because germ (P) granules remain largely intact during oogenesis in cgh-1 null mutants (Fig. S2 B; Boag et al., 2005). We examined how lack of CGH-1 affects the distribution of mRNAs during oogenesis by hybridizing with the SL1 probe, which in WT animals detects small foci throughout the syncytial gonad core (Fig. 5 D). Remarkably, in cgh-1(ok492) animals, some SL1-containing mRNA was similarly found in small puncta, but a significant proportion of the SL1 signal colocalized with large CAR-1 sheets (Fig. 5, F–I). We conclude that during oogenesis, CGH-1 is required for correct localization of a substantial proportion of SL1-containing mRNAs.

We next investigated whether a lack of CGH-1 differentially affects the localization or stability of representative mRNAs that our RIP-Chip experiments identified as being associated with CGH-1. For controls, we examined germ line enriched mRNAs (Reinke et al., 2004; Reinke et al., 2000) that were indicated by SAGE databases to be of comparable abundance but had not been detected as CGH-1–associated in our IPs. We first performed in situ hybridization of dissected gonads
dissected gonads of WT and cgh-1(ok492) adult hermaphrodites, and similar results were obtained (unpublished data). Within a population of cgh-1(ok492) animals, each of the CGH-1–associated mRNAs that we tested was present at dramatically lower levels, but a reduction was seen for only one of the control mRNAs (gld-1; Fig. 6 B).

Our findings suggest that CGH-1–associated mRNAs become differentially destabilized if CGH-1 is absent. Alternatively, it might be possible that cgh-1(ok492) animals have lower levels of these maternal mRNAs because they do not ovulate and therefore produce fewer oocytes, or if they have a germ cell differentiation block. cgh-1(ok492) and cgh-1(RNAi) animals switch from sperm to oocyte production at the appropriate stage, however, and

Figure 5. CGH-1 affects mRNA localization during oogenesis. (A–C) CGH-1 and SL1 leader sequence localization in proximal oocytes in fog-2(q71) females. Extruded gonads from 1-d-old adults were stained for CGH-1 and analyzed by in situ hybridization to an Alexa 488–linked SL1 antisense probe. SL1–containing mRNA accumulates in large foci that colocalize with CGH-1 and in separate small puncta. In parallel, an SL1 sense probe showed little or no diffuse signal (not depicted). Oocytes are outlined by broken lines. n > 60. (D and E) SL1 localization in WT hermaphrodite gonads analyzed by in situ hybridization. (F–I) Abnormal mRNA localization during oogenesis in cgh-1(ok492). Extruded gonad of a typical gonad are shown, with distal toward the top right. CAR-1 and some SL1-containing mRNAs colocalize in sheetlike structures. The bulbous shape of this gonad is characteristic of many animals that lack CGH-1 (Navarro et al., 2001). Confocal z series projections are shown. For D–I, n > 60 for WT and cgh-1(ok492). Bars, 10 μm.
Figure 6. CGH-1 binds and stabilizes particular mRNAs during oogenesis. (A) Analysis of mRNA abundance and distribution by in situ hybridization. Probes were hybridized to dissected gonads from 1-d-old N2 and cgh-1(ok492) animals. For each probe and genotype tested, at least 20–25 gonads were examined in each of two or more experiments. Six of seven mRNAs that were not associated with CGH-1 did not differ detectably between N2 and cgh-1 mutant animals (Fig. S3, C and D, available at http://www.jcb.org/cgi/content/full/jcb.200801183/DC1). In contrast, all six CGH-1–associated mRNAs analyzed were present at markedly lower levels in cgh-1(ok492) gonads. Distal is shown to the right (asterisks). Each in situ hybridization experiment gave consistent results among gonads examined, for which representative images are shown. Bar, 50 μm. (B) Relative abundance of CGH-1–associated and unassociated mRNAs in cgh-1(ok492) adults compared with WT, assayed by RT-qPCR. Each mRNA value was normalized to actin (act-1), and WT levels were converted to 1. The mean of three biological experiments is graphed. (C) Relative abundance of CGH-1–associated and unassociated mRNAs in fog-2(q71) adults compared with WT. Data are from two independent experiments. Error bars indicate SEM. (D) A model for germ line CGH-1 functions. Many maternal mRNAs rely on the CGH-1 complex to protect them from degradation and possibly for translational silencing. Some CGH-1–associated mRNAs are known to be translationally regulated through binding of sequence-specific proteins to their 3′ UTRs (see text). Such proteins might be present on individual mRNAs in conjunction with the CGH-1 complex or could assume control of these mRNAs after they are released by this complex.
as adults, produce only oocytes (Navarro et al., 2001; Boag et al., 2005). Furthermore, cgh-1 mutants express typical oocyte-specific genes. For example, three of the CGH-1–unassociated mRNAs that we tested (gld-1, air-1, and zip-4) are expressed primarily during oogenesis and not in larvae (Reinke et al., 2004), and only one of these (gld-1) is present at modestly lower levels in cgh-1(ok492) hermaphrodites (Figs. 6 B and S3 D). Finally, mRNAs that are associated with (and dependent upon) CGH-1 are present at normal or increased levels in fog(q71) females, which also ovulate infrequently (Fig. 6 C). We conclude that during oogenesis, CGH-1 is needed to protect the specific group of mRNAs with which it normally associates but not most other mRNAs, in striking contrast to its previously described role in promoting mRNA decay in P bodies (Coller and Parker, 2004; Eulalio et al., 2007a; Parker and Sheth, 2007).

Discussion

Studies in yeast, cultured cells, and in vitro have shown that Dhh1(CGH-1) and Pat1 act in concert to recruit mRNAs to P bodies, inhibit their translation, and promote decapping (see Introduction). We now find that in C. elegans, the oogenesis functions of CGH-1 are distinguishable genetically from its role in “typical” P bodies. In somatic tissues, CGH-1 is found in patr-1–dependent decapping-associated P bodies, but during oogenesis, CGH-1 forms storage bodies independently of patr-1, interacts primarily with a set of translational regulators, and protects a specific group of maternal mRNAs from degradation.

PATR-1–dependent somatic CGH-1 particles have properties of decapping-associated P bodies

When we investigated whether germ line or embryonic CGH-1 particles have characteristic properties of P bodies (Parker and Sheth, 2007), we found that this was the case only in somatic cells. The CGH-1 particles that are present in somatic cells after the four-cell stage (somatic P bodies) were stabilized if decapping-associated factors were lacking, and were essentially undetectable in the absence of PATR-1 (Fig. 2, B–E). PATR-1(N) staining was seen almost exclusively in somatic P bodies, and in dcap-2(tm2470) animals, somatic P bodies that contained both PATR-1 and CGH-1 were prominent into adulthood (Figs. 3 A and S1 F). We conclude that PATR-1 dependence and PATR-1(N) staining are specific characteristics of somatic P bodies, and that in metazoan, Pat1 may represent a functional marker of degradative P bodies.

The somatic P bodies we identified are likely to correspond to previously described embryonic particles containing DCAP-2 and the miRNA–associated proteins ALG-1 (argonauta) and AIN-1 (GW182; Ding et al., 2005). Cell culture experiments suggest that mammalian CGH-1 (RCK) is required for gene silencing by miRNAs (Chu and Rana, 2006), but cgh-1(ok492) homozygotes do not show either the devastating germ line defects caused by lack of the nuclease dicer (dcr-1) or the heterochronic phenotypes associated with some miRNAs (Pasquinelli et al., 2000; Knight and Bass, 2001). Although this suggests that CGH-1 is not generally essential for miRNA function, it will be important to investigate whether CGH-1 might have redundant or more specific functions in miRNA-directed silencing.

Somatic CGH-1 particles became patr-1 dependent approximately as decay of class II maternal mRNAs begins (see Introduction), which suggests that class II mRNAs might be degraded through decapping. However, class II mRNAs that we have analyzed by in situ hybridization and RT-qPCR (pos-1 and nos-2) were not detectably stabilized in dcap-2(tm2470) embryos (unpublished data). Perhaps the decapping pathway degrades only some class II mRNAs or acts redundantly with other mechanisms. Consistent with the latter idea, dcap-2(tm2470) mutants develop to adulthood and are fertile (Table S1), which suggests that the decapping pathway is unlikely to be the most important mRNA decay mechanism in C. elegans.

CGH-1 binds and stabilizes specific maternal mRNAs

In contrast to somatic P bodies, the CGH-1–containing storage bodies found in germ cells were not detectably influenced by lack of dcap-2 or patr-1, and they lacked PATR-1(N) staining (Fig. S2 and not depicted). In addition, neither the decapping proteins nor PATR-1 were among the most prominent CGH-1 interactors in C. elegans, in which the bulk of CGH-1 expression derives from developing oocytes (Table I; Boag et al., 2005). During oogenesis, CGH-1 therefore appears to act in a manner that is distinct from its P body functions. Accordingly, we identified two prominent CGH-1 interactors to be ATX-2 and PAB-1, which in mammalian cells are not found not in P bodies but are characteristic of stress granules, structures in which some mRNAs are transiently sequestered during stress (Anderson and Kedersha, 2008). We speculate that CGH-1 and the translational regulators with which it forms a germ line RNP (Table I; Boag et al., 2005) may assemble on an mRNA to form the functional unit of the storage body, much as the decapping complex is the “building block” of the P body (Parker and Sheth, 2007). Although germ line storage bodies are distinct from P bodies, it should be remembered that core P body mechanisms have important functions during oogenesis: dcap-2 and patr-1 mutants show minimal germ line abnormalities (Fig. S1 C and Table S1), and in D. melanogaster, Dcp1 is required for localization of some maternal mRNAs (Lin et al., 2006).

We investigated the specificity with which CGH-1 acts on mRNA during oogenesis by performing a systems analysis of its interactions with mRNA. In RIP-Chip experiments, we obtained the unexpected finding that CGH-1 interacts specifically with a particular set of oocyte and maternal mRNAs (Fig. 4 and Table S2). Our stringent criteria for CGH-1 association might have underestimated the number of mRNAs that interact specifically with CGH-1 in vivo, but we established that these CGH-1–associated mRNAs are functionally linked to CGH-1, as we obtained the surprising finding that they were differentially destabilized in cgh-1(ok492) animals (Fig. 6 and Fig. S3, C and D). Our findings indicate that CGH-1, and by extension the conserved CGH-1 germ line complex, defines a large but discrete maternal mRNA storage pathway.
What is the main function of the CGH-1 complex in regulating these mRNAs? In cgh-1(ok492) animals, we did not detect premature translation of the CGH-1–associated mRNA pos-1 by antibody staining (not depicted), although the destabilization of this mRNA (Fig. 6, A and B) might have masked any translational derepression. It is intriguing that CGH-1 protects particular maternal mRNAs from degradation, in striking contrast to its more familiar role in promoting decapping. In the protozoan Plasmodium berghei, the CGH-1 orthologue (DOZI) is also required for female fertility and stabilization of certain mRNAs that are translationally silenced in female gametocytes, the female gamete precursor (Mair et al., 2006). Individual translationally suppressed mRNAs co-IP from gametocytes along with DOZI, and without DOZI, many mRNAs that are normally translationally silenced in the gametocyte are degraded. This striking similarity to our findings suggests that a protective function for CGH-1 in reproduction might be conserved. One possibility is that the degradation that occurs in the absence of CGH-1 is a “default” fate that eliminates errant mRNAs that have escaped from being appropriately localized or translationally silenced (Fig. 6 D).

It is an intriguing question how CGH-1 interacts with and protects particular mRNAs because DEAD box helicases generally bind mRNA nonspecifically (Rocak and Linder, 2004). This specificity might be provided by other CGH-1 complex components (Table I), by transient interactions with other factors, or by proteins that interact specifically with particular subgroups of CGH-1–associated mRNAs (Fig. 6 D). For example, in mammalian cells, the decapping machinery can be targeted to mRNAs by sequence-specific regulators (Franks and Lykke-Andersen, 2007). One possibility is that the CGH-1 complex is formed on mRNAs at germ (P) granules, at which some CGH-1 is found throughout oogenesis (Figs. 1 and S2 A). Some maternal mRNAs seem to associate with germ (P) granules by in situ hybridization (Lipshitz and Smibert, 2000; Lipshitz et al., 2006). Our results in C. elegans have shown that CGH-1 functions differently in the oogenic germ line and soma, and have distinguished three types of CGH-containing particles (germ [P] granules, storage bodies, and somatic P bodies; Fig. 1). In this simple metazoan, therefore, CGH-1 functions are tissue-specific and restricted with respect to its mRNA targets, at least during oogenesis. We conclude that CGH-1, PATR-1, and possibly other core P body mechanisms may have surprisingly complex functions during development. It will be important to elucidate how these mechanisms are targeted to individual mRNAs, and how CGH-1 and PATR-1 functions are regulated in different cellular and developmental contexts.

Materials and methods

Strains

WT [N2 Bristol] and mutant strains were maintained as in Brenner (1974). Deletion mutants were generated by the National BioResource Project for the Nematode, balanced, and maintained as strains patr-1(tm2402)/II; min-1[dpy-10[e128] mis-14] and dcp-2[m2420]/II; n-1[q5,15] (Table S1). Each was outcrossed at least seven times to N2. Additional strains used were: glp-4[bn2], unc-119(ed3), and cgh-1(ok492)III, fog-2(q71).

RNAi

Young adult hermaphrodites were injected with double-stranded RNA (~1 μg/μl total concentration) and allowed to lay eggs for 8–10 h before being transferred to new plates. F1 adult hermaphrodites were dissected and used for either gonad or embryo staining.

RIP-Chip

The CGH-1 RNP was immunoprecipitated from adult extracts as described previously (Boag et al., 2005) with the following modifications. Salmon sperm DNA–coated protein A beads (Millipore) were pretreated with 2 mg tRNA before addition to the protein lysate. After elution with CGH-1 peptide, RNA was purified from the eluted RNP mixture by extraction with Tri Reagent (Sigma-Aldrich) and subjected to one round of linear amplification using either the SuperScript System (Invitrogen) or as described previously (Baugh et al., 2001). Four independent co-IPs and linear amplifications were conducted, with microarray analysis performed as described previously (Reinke et al., 2004). We averaged the relative abundance of each gene across four independent CGH-1 versus IgG microarray experiments, and mRNAs with more than a threefold fold increase in CGH-1 IPs were defined as CGH-1–associated. SAGE data were obtained from the Genome BC C. elegans Gene Expression Consortium (http://elegans.bcgsc.bc.ca/).

Antibody production and microscopy

Rat polyclonal antibodies were raised against the 19 N-terminal amino acids of PATR-1 (Cocalico Biologicals, Inc.), and affinity purified (Sulforol; Pierce). Staining of patr-1[RNAi] or tm2402 gonads showed only a low level of diffuse background signal (Fig. S1 B). Gonad staining was performed as described previously (Navarro et al., 2001). For PATR-1[N] staining, embryos were fixed in methanol (~20°C) for 10 min and placed in cold acetone for a further 10 min, then washed, blocked, and stained similarly to gonads. Antibodies to POS-1 and GLH-2 were provided by Y. Kohara (National Institute of Genetics, Shizuoka, Japan) and K. Bennett (University of Missouri, Columbia, MO), and staining was carried out as described previously (Tabara et al., 1999). Secondary antibodies were species-specific Alexa A488 and A555 (Invitrogen), and samples were mounted in Vectashield mounting medium (Vector Laboratories). Confocal image acquisitions were performed at room temperature using a microscope.
Production and analysis of transgenic experiments were conducted. Abundance was determined using the Ct method (Applied Biosystems). Samples were run in duplicate, and relative levels were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3). RT-qPCR analysis was performed using RNA extracted from dissected gonads of WT and act-1 (mutant). This protocol provided consistent results across >50 individual gonads examined for each mRNA and genotype. Fluorescence RNA in situ hybridization was conducted at room temperature using an Axioplan 2 MOT (MDS Analytical Technologies). Live imaging of CGH-1::GFP–expressing embryos was conducted at room temperature using an Axioplan 2 MOT microscope equipped with an oil immersion Plan-Apochromat 1.4 NA 63x objective and controlled by AxioVision software, and images were acquired with a camera (AxioCam; all from Carl Zeiss, Inc.). Processing of all images was performed using Photoshop CS2 and Illustrator CS2 (both from Adobe).

Identification of additional CGH-1 complex proteins ATX2 and PAB-1 were identified here as corresponding to species that were previously detected in CGH-1 IPs (Boag et al., 2005). Briefly, CGH-1 and associated proteins were eluted from the protein A beads, separated on a 10% polyacrylamide gel, and stained. Individual bands were excised from the gel, digested with trypsin, and subjected to tandem mass spectrometry analysis. Proteins were identified by searching the National Center for Biotechnology Information Inr database using the Mascot program.

Immunoprecipitation and Western analysis IP of endogenous CGH-1 was conducted as described previously (Baog et al., 2005). For Western analysis of PATR-1 expression in N2 or patr-1(m2402), adult hermaphrodite worms or dissected N2 gonads were frozen and thawed in the presence of 1x reducing sample buffer and denatured at 95°C for 6 min. Samples were run on 10% SDS-PAGE gels and transferred to nitrocellulose membranes using standard methods. Proteins were detected with species-specific HRP-conjugated secondary antibodies. The relative abundance of PATR-1 in cgh-1(ok492) compared with WT was calculated by densitometry using ImageJ (National Institutes of Health). RT-qPCR analysis RNA was extracted from 1–day-old adults using Trizol Reagent and cDNA prepared from total RNA using SuperScript II (Invitrogen). Primer sequences were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi). RT-qPCR was performed using SYBR green PCR master mix (Applied Biosystems) and the ABI Prism 7700 Sequence Detection system (Applied Biosystems). Samples were run in duplicate, and relative abundance was determined using the ΔΔCt method (Livak and Schmittgen, 2001) and act-1 (actin) as the internal standard. For analysis of mRNA levels in cgh-1(ok492) and WT animals, three independent biological experiments were conducted for each mRNA. Additional experiments were performed using RNA extracted from dissected gonads of WT and cgh-1(ok492) adult hermaphrodites, and similar results were obtained. For comparison of mRNA levels in fog-2(q71) and WT animals, two independent experiments were conducted.

Production and analysis of transgenic CGH-1::GFP strains The CGH-1::GFP translational fusion construct pCGH-1::GFP-unc119 was constructed by cloning genomic fragments of 3,658 bp (predicted cgh-1 promoter and coding region) and 1,128 bp (the region 3' to the cgh-1 ORF) into the Nof and SacI sites, respectively, of the unc-119 rescue plasmid pBS-unc119-GFP. Transgenic animals were produced by biolistic transformation (Pratt et al., 2001). Animals were grown on nematode growth medium plates until the adult stage, then bleached to obtain embryos that were subsequently cultured for 3 days in S-basal medium containing HT115(DE3) bacteria. Gold beads that were coated with pCGH-1::GFP-unc119 were bombarded into these young adults using the PDS-1000/He with Hepta adaptor system (BioRad Laboratories). Animals were allowed to recover for 1 hr, transferred to NEM plates seeded with HT115(DE3) bacteria, grown at 25°C for 3 wk, and then screened for WT animals (unc-119 rescued). By genetic crossing, we obtained two strains in which the CGH-1::GFP transgene rescued cgh-1(ok492) animals to fertility. One of these strains (ldls100[P[cgh-1::GFP-3’UTR[cgh-1; unc-119[+]]) was analyzed in Fig. 2 E. Single focal plane images of transgenic embryos were acquired using a Axioskop-2 microscope (Carl Zeiss, Inc.).

Online supplemental material Fig. S1 diagrams the dcap-2(m2470) and patr-1(m2402) deletions, and shows how these mutations affect the organization of the gonad. A comparison of PATR-1 levels in WT, patr-1(m2402), and dissected gonads is also presented. Staining for endogenous CGH-1 and PATR-1 in patr-1(m2402) and dcap-2(m2470) is shown, demonstrating the specificity of the PATR-1 (N) antisera and the persistence of CGH-1 and PATR-1 foci in somatic tissues of dcap-2(m2470) adults. Fig. S2 shows representative images of cgh-1(ok492) and CAR-1 localization in N2, dcap-2(m2470), and patr-1(m2402) gonads, along with CAR-1 and GLH-2 staining in the null cgh-1(ok492) mutant. Fig. S3 provides supporting evidence that CGH-1 associates with and stabilizes a specific subset of maternal mRNAs. Table S1 compares offspring production in the WT (N2) and the predicted null mutants dcap-2(m2470) and patr-1(m2402). Table S2 lists the CGH-1–associated mRNAs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801183/DC1.

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Note added in proof: The Evans laboratory has shown that during C. elegans oogenesis, CGH-1 colocalizes with particles with the maternal mRNA ghp-1 and is required to maintain its stability under conditions in which oogenesis is arrested (Nobile, S.L., B.L. Allen, L.K. Goh, K. Nordick, and T.C. Evans. 2008. J. Cell Biol. 182: 559–572).

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