Perinuclear P granules are the principal sites of mRNA export in adult *C. elegans* germ cells

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**SUMMARY**
Germline-specific granules of unknown function are found in a wide variety of organisms, including *C. elegans*, where they are called P granules. P granules are cytoplasmic bodies in oocytes and early embryos. Throughout most of the *C. elegans* life cycle, however, P granules are associated with clusters of nuclear pore complexes (NPCs) on germ cell nuclei. We show that perinuclear P granules differ from cytoplasmic P granules in many respects, including structure, stability and response to metabolic changes. Our results suggest that nuclear-associated P granules provide a perinuclear compartment where newly exported mRNAs are collected prior to their release to the general cytoplasm. First, we show that mRNA export factors are highly enriched at the NPCs associated with P granules. Second, we discovered that the expression of high-copy transgenes could be induced in a subset of germ cells, and used this system to demonstrate that nascent mRNA traffics directly to P granules. P granules appear to sequester large amounts of mRNA in quiescent germ cells, presumably preventing translation of that mRNA. However, we did not find evidence that P granules normally sequester aberrant mRNAs, or mRNAs targeted for destruction by the RNAi pathway.

**KEY WORDS:** P granules, Export, mRNA, *C. elegans*

**INTRODUCTION**

Germ cells or germ cell precursors in animals ranging from nematodes to mammals have distinctive cytoplasm, called germ plasm, with unique organelles called P granules in *C. elegans* (reviewed by Seydoux and Braun, 2006). Genetic transplantation studies in *Drosophila* showed germ plasm is both necessary and sufficient for germline development (reviewed by Extavour and Akam, 2003). Although similar transplantation studies have not been possible in *C. elegans*, P granules contain many proteins that are essential for germline development (reviewed by Strome, 2005). Moreover, loss of P granules in *mex-3;gld-1* mutants is associated with a germline teratoma phenotype, where germ cells differentiate inappropriately as muscles and neurons (Ciosk et al., 2006).

P granules are best known as cytoplasmic organelles in oocytes and in early embryos, where they localize asymmetrically to germ cell precursors (Strome, 2005). Once the germ lineage is established, however, P granules associate with nuclei and remain perinuclear throughout development (Strome, 2005). Cytoplasmic P granules are highly dynamic, with rapid growth, fusion, and shrinkage behaviors reminiscent of liquid droplets; the dynamics of perinuclear P granules have not been determined (Brangwynne et al., 2009). Diverse proteins have been shown to be enriched in perinuclear and/or cytoplasmic P granules, such as components of the RNAi pathway (DRR-3, EGO-1, CSR-1, PRG-1 and WAGO-1); components of the spliceosome (Sm proteins); components of processing bodies (P-bodies) and stress granules; PGL-1, a protein that binds an isoform of the mRNA cap-binding protein eIF4E; the poly(A) binding protein PAB-1; the DEAD box RNA helicase GLH-1; and PIE-1, a protein involved in transcriptional repression during early embryogenesis (Amiri et al., 2001; reviewed by Anderson and Kedersha, 2009; Mello et al., 1996; reviewed by Updike and Strome, 2009). The diversity of P granule components and the phenotypes of mutants defective in individual components do not provide a simple, single model for P granule function. Moreover, most P granule components are localized to additional nuclear or cytoplasmic compartments, and it is not known whether P granule localization is crucial for the function of any component.

The finding that most P granule proteins have RNA binding motifs suggests a role in RNA metabolism (Updike and Strome, 2009). Previous studies showed that perinuclear P granules in quiescent, non-ovulating gonads contain high levels of mRNA, although it is not known how this stored mRNA traffics to P granules (Schisa et al., 2001). Yeast P-bodies can provide temporary storage for non-translated mRNA, in addition to serving as sites for mRNA degradation, and P granules contain the P-body-associated proteins CGH-1 and DCAP-2 (reviewed by Rajayaguru and Parker, 2009). However, P granules lack additional proteins that are found in P-bodies, and germ cells contain other granules that more closely resemble conventional P-bodies (Anderson and Kedersha, 2009).

Although perinuclear P granules might store RNA in quiescent gonads, their relationship to nascent mRNA in normal gonads is not clear. Perinuclear P granules are localized at clusters of nuclear pores, where they might interact with newly exported RNAs (Pitt et al., 2000). However, normal gonads incubated with \(^{3}H\)-uridine show no enrichment of label in perinuclear P granules. Second, specific mRNAs examined by in situ hybridization show little or no enrichment in perinuclear P granules (Schisa et al., 2001). Thus, nascent mRNA might either transit through perinuclear P granules very rapidly upon export or be exported through nuclear pore complexes (NPCs) that are not associated with perinuclear P granules. The present study provides direct evidence that nascent mRNAs are exported predominantly through the NPCs associated with perinuclear P granules and that the nascent mRNA transits through perinuclear P granules before entering the general cytoplasm.
cytoplasm. Moreover, we show that perinuclear P granules, which are normally found in actively transcribing adult germ cells, differ in several respects from cytoplasmic P granules, which are found in transcriptionally quiescent oocytes and early embryos.

MATERIALS AND METHODS

**C. elegans** strains

Nematodes were cultured and manipulated genetically as previously described (Brenner, 1974). Unless otherwise indicated, experiments were performed on the N2 strain (var. Bristol), maintained at 20°C and analyzed as 1-day-old adults. The following strains and alleles were used: T3735 (hsp-16-2::GFP::unc-54 3’t UTR); BS3426 {rme-2(b1005) unc-24(e138)/unc-5(e53) lin-45(ax19) (IV)}; J5369 {mex-3(zeu155)/hT1; him-5(e1490)V}; RB912 {ddx-19(ok783)II}; CB2303 {ced-1(e1735)/I}; MT5811 {ced-1(e1735)/I; ced-3(m717)/IV}; JJ2101 [zuk242(tmy-2::PGL-1::GFP::unc-119)]; and JJ2072 [zuk237 (58g11.2::F58G11.2::GFP::unc-119)]. For extrachromosomal transgenic strains, wild-type worms were injected with either zuk218 [pUS24 (hsp-16.2::GFP::zuk-2 3’t UTR)] or zuk224 [pCL25 (hsp-16.2::GFP::pos-1 3’t UTR)] to obtain strains JJ1944, JJ1940 and JJ1959, respectively. For extrachromosomal transgenic arrays, wild-type worms were injected with plasmid plus a dominant rol-6 cotransformation marker (Mello and Fire, 1995). Strain JJ2072 with an integrated transgene zuk237 was created by microparticle bombardment of unc-119 worms (Praitsis et al., 2001).

**Time-lapse imaging and photobleaching experiment**

Confocal, live imaging was performed as previously described (Tenlen et al., 2008; Wolke et al., 2007). The following settings were used for acquisition: exposure, 400 milliseconds; laser intensity, 82%; gain, 1; step size, 1 μm; acquisition interval, 15 seconds. Particles were tracked using Imaris Image Analysis (5.5.3) software (Lammermann et al., 2008).

Photobleaching experiments were performed and analyzed as previously described (Tenlen et al., 2008). Twenty images (0.5 second intervals) were acquired before photobleaching; recovery was measured with 67 images (40 minutes) as homogenous, fibrillar granular bodies by electron microscopy, which time they detach and become cytoplasmic (Fig. 1A) (Strome, 2005). Cytoplasmic P granules in oocytes and early embryos appear as homogenous, fibrillar granular bodies by electron microscopy, whereas perinuclear P granules can contain a distinct electron-dense base approximately 70-100 nm from the nuclear rim (Fig. 1E-G) (Pitt et al., 2000; Wolf et al., 1983). We examined the ultrastructure of perinuclear P granules in fourth larval stage (L4) germ cells and in adult germ cells in mitosis and at all stages of meiosis. In addition to the electron-dense base, we found that P granules have an electron-dense ovoid structure that we term the crest at their periphery (Fig. 1D; see also Fig. S1A in the supplementary material). Germ nuclei in the mitotic zone often contain small P granules with a crest but no obvious base; we presume that these are newly formed P granules (Fig. 1C,D). The crest appears to decrease in size and the base becomes less evident or disappears in late diplotene and diakinetic oogonia when P granules detach from the envelope (Fig. 1H-I).

We wanted to determine whether perinuclear P granules exhibited the same dynamic behaviors of rapid growth, fusion and shrinkage described for cytoplasmic P granules (Brangwynne et al., 2009). Spinning disk confocal microscopy was used to generate movies of live pachytenic-stage germ cells expressing the P granule component PGL-1 fused to Green Fluorescent Protein (GFP). Fifty perinuclear P granules that were selected at random for analysis could be tracked for 15-40 minutes before nuclear rotations or occasional body movements of the anesthetized animals shifted the P granule out of the visual field. During the period of observation, however, none of the selected P granules appeared to fuse or show marked changes in

**RESULTS**

**Perinuclear P granules are asymmetric structures with dynamic components**

Each of the two arms of the *C. elegans* hermaphrodite gonad is a cylindrical array of germ cells progressing from mitosis through meiosis (Fig. 1A). Each germ cell has an opening connecting it with the shared, cytoplasmic core of the gonad; materials flow along the core toward enlarging oocytes at the end of the gonad. P granules are perinuclear in mitotic and meiotic germ cells until oogenesis, at which time they detach and become cytoplasmic (Fig. 1A) (Strome, 2005). Cytoplasmic P granules in oocytes and early embryos appear as homogenous, fibrillar granular bodies by electron microscopy, whereas perinuclear P granules can contain a distinct electron-dense base approximately 70-100 nm from the nuclear rim (Fig. 1E-G) (Pitt et al., 2000; Wolf et al., 1983). We examined the ultrastructure of perinuclear P granules in fourth larval stage (L4) germ cells and in adult germ cells in mitosis and at all stages of meiosis. In addition to the electron-dense base, we found that P granules have an electron-dense ovoid structure that we term the crest at their periphery (Fig. 1D; see also Fig. S1A in the supplementary material). Germ nuclei in the mitotic zone often contain small P granules with a crest but no obvious base; we presume that these are newly formed P granules (Fig. 1C,D). The crest appears to decrease in size and the base becomes less evident or disappears in late diplotene and diakinetic oogonia when P granules detach from the envelope (Fig. 1H-I).
size (Fig. 2A, A'). We next asked whether PGL-1 itself was a stable component of perinuclear P granules by FRAP (fluorescence recovery after photobleaching) experiments. Individual photobleached P granules showed very rapid recovery of PGL-1::GFP fluorescence ($t_{1/2}$ recovery = 19±7 seconds, $n=9$; Fig. 2B-2B'). Thus, perinuclear P granules are asymmetric, relatively stable bodies that contain at least some highly dynamic components such as PGL-1.

PGL-1 is present throughout the germ cell cytoplasm but is enriched in P granules under normal growth conditions. However, PGL-1 disappears from perinuclear P granules in late stages of spermatogenesis and in pachytene germ cells of quiescent, non-ovulating gonads (Amiri et al., 2001; Schisa et al., 2001). To ask whether decreased transcriptional activity might trigger the loss of PGL-1 from perinuclear P granules, we injected young adult gonads with the transcriptional inhibitor $\alpha$-amanitin. PGL-1::GFP disappeared progressively from P granules in pachytene germ cells beginning at 30 minutes (Fig. 2D, E; see also Fig. S2A in the supplementary material; $n=10$ gonads). Animals depleted in NXF-1, an essential mRNA export factor in C. elegans (Tan et al., 2000), showed a similar, pachytene-specific, loss of PGL-1 (Fig. 2H).

We found that the loss of perinuclear PGL-1 in spermatogonia was associated with a marked decrease of immunostaining with H14 (see Fig. S3A, B in the supplementary material), an antibody that recognizes the phosphorylated carboxy-terminus of active RNA polymerase (Seydoux and Dunn, 1997). Similarly, we found that germ cells at early stages of apoptosis lost PGL-1 from perinuclear P granules and that this loss was associated with decreased staining with H14 (see Fig. S3C-F in the supplementary material). Thus, although PGL-1 is a prominent component of cytoplasmic P granules in oocytes and early embryos that are transcriptionally quiescent, transcriptional activity is required for PGL-1 to associate with perinuclear P granules in multiple examples of germ cells.
mRNA export factors are concentrated at P granule-associated NPCs

To examine the relationship between perinuclear P granules and nascent mRNA, we wanted markers to visualize both the cytoplasmic face of NPCs and the location of mRNA export factors. Previous studies visualized presumptive NPCs with the antibody mAb414, which recognizes several yeast and mammalian nucleoporins with phenylalanine-glycine (FG) repeats (Davis and Blobel, 1986; Radu et al., 1995; Shah et al., 1998). However, mAb414 stains multiple proteins of unknown identity in C. elegans immunoblots (Geles and Adam, 2001), and some C. elegans proteins that are not nucleoporins contain FG repeats, notably the P granule protein GLH-1 (see below). Therefore, we generated monoclonal antibodies that recognize the C. elegans nucleoporin NPP-9, a predicted export factor DDX-19 and a known mRNA export factor NXF-1 (see Fig. S4A-G in the supplementary material).

NPP-9 is an FG repeat-containing nucleoporin homologous to vertebrate Nup358/RanBP2, a major component of the eight fibers that extend from the cytoplasmic face of NPCs (Wu et al., 1995; Yokoyama et al., 1995). αNPP-9 stained the rim of all somatic and germ cell nuclei examined (Fig. 3A,B; see also Fig. S4A in the supplementary material). As P granules detach from the nuclear envelope during oogenesis, they appear to retain material stained by αNPP-9 (Fig. 3C,D) (see also Pitt et al., 2000). Cytoplasmic foci of PGL-1 were observed infrequently in the gonad core prior to P granule detachment (box e in Fig. 3B); however, these foci were not associated with NPP-9 (Fig. 3E). These foci might form de novo in the core or result from P granule fragmentation during germ cell apoptosis. NPP-9 was concentrated in large patches on germ nuclei that we presume are clustered NPCs, and was present in much smaller punctae that might be individual, or small groups of, NPCs (Fig. 4A). Co-staining for PGL-1 showed that a P granule was present on essentially all of the large patches of NPP-9; in a data set of 100 large patches (NPP-9 surface area=1.5±0.7 μm²), 99% were associated with a P granule. Small NPP-9 punctae (<0.8 μm²) were not associated with typically sized P granules (Fig. 4B). We hereafter refer to the large clusters of NPCs associated with P granules as Pg-NPCs (P granule-associated NPCs) to distinguish these from other NPCs. Pg-NPCs appeared to be distributed randomly on the surface of most germ nuclei. In L4 germ nuclei and in the zygote/early pachytene adult nuclei, however, only NPCs were present where a prominent lobe from the nucleolus contacted the envelope (0/20 and 0/53 lobes were adjacent to Pg-NPCs in L4s and adults, respectively; see Fig. S1, open arrows, in the supplementary material).

DDX-19 is a predicted DEAD-box helicase related to the mRNA export factors DDX19 in mammals and Dbp5p in yeast (Arur et al., 2009; Hodge et al., 1999; Schmitt et al., 1999; Tseng et al., 1998). αDDX-19 showed low levels of staining at the nuclear rim and in the cytoplasm of somatic cells but much stronger staining on germ nuclei; staining diminished or disappeared on late oogonial nuclei that cease transcription (Fig. 3A). At high magnification, DDX-19 appeared concentrated in patches on both adult and larval germ nuclei (Fig. 3F, Fig. 4A). Co-staining with αNPP-9 and αDDX-19 showed that the vast majority of DDX-19 was localized at Pg-NPCs: in the data set of 100 large patches of NPP-9 described above, 94% of the P granule-positive patches colocalized with a patch of DDX-19 (Fig. 3F, Fig. 4A,B). The profile of each DDX-19 patch typically was smaller than the associated patch of NPP-9 but similar to that of PGL-1 (Fig. 4A,B). In cross-sections of nuclei, DDX-19 appeared concentrated between the zones of PGL-1 and NPP-9, forming a tripartite sandwich (Fig. 3F); remarkably, P granules initially maintained a similar, tripartite appearance after detaching from oogonia nuclei (Fig. 3G and inset).

NXF-1 is the ortholog of the mRNA export factors NXF1/TAP in humans and Mex67p in yeast, and depletion of NXF-1 causes poly(A)-containing mRNA to be retained in C. elegans germ nuclei (Tan et al., 2000). αNXF-1 showed strong staining at the nuclear rim of somatic cells and germ cells (Fig. 5A). Co-staining experiments with αPGL-1 and αNXF-1 showed that nearly all the NXF-1 at the rim of germ nuclei is present at Pg-NPCs: surface views of nuclei showed a P granule in close association with the prominent patches of NXF-1 (Fig. 5B, arrow) and cross-sections showed that NXF-1 was concentrated at the bases of P granules (Fig. 5C, arrow). In addition to staining at the nuclear rim, αNXF-1 stained foci at the center of most germ nuclei, a region occupied by the nucleolus (Fig. 5C, arrowhead). NXF-1 localization at the nuclear rim was very
similar to NPP-9 localization (Fig. 5D). Indeed, the αNXF-1 and αNPP-9 antibodies showed strong competition for staining on formaldehyde-fixed nuclei, necessitating the use of a denaturing fixative.

Nascent mRNA is exported primarily from Pg-NPCs and enters P granules

The above results show that two factors associated with mRNA export are localized predominantly at Pg-NPCs, strongly suggesting that Pg-NPCs are the principal sites of mRNA export under normal growth conditions. To examine nascent mRNA directly, we developed a technique for inducing and visualizing transgene expression in germ cells. Promoters for heat shock genes such as hsp-16.2 are used to induce expression in somatic cells (Fire et al., 1990), but endogenous hsp-16.2 is poorly expressed in germ cells (our unpublished data). Although high-copy transgenic arrays can greatly amplify somatic expression, such arrays are subject to repeat-induced silencing in germ cells (Kelly et al., 1997). Consistent with this view, we found that a high-copy transgene containing the hsp-16.2 promoter, the coding region for GFP and an unc-54 3’UTR [hsp-16.2::gfp::unc-54 (3’UTR)] (Link et al., 1999) showed no detectable mRNA expression in most germ cells following heat shock. Surprisingly, however, mRNA from this transgene was expressed at very high levels in late pachytene/diplotene germ cells; we refer to this region of the gonad as the expression zone (Fig. 6A,C).

Several experiments were performed to characterize the induction of transgene-derived mRNA in the expression zone; in brief, these results suggest that the expression zone contains primarily sense, full-length mRNA (see Fig. S5 in the supplementary material). To examine nascent mRNA traffic, transgenic animals were heat-shocked at 34°C for 30 minutes, allowed to recover for 0 to 60 minutes, then processed for FISH (fluorescence in situ hybridization) and immunocytochemistry. Immediately following heat shock (t=0 minutes), transgenic mRNA appeared at high levels in two intranuclear foci that probably represent positions of the integrated transgene on the paired chromosomes (see legend to Fig. S5 in the supplementary material). Lower levels of mRNA were present throughout the nucleoplasm but not detected in the cytoplasm (Fig. 6B,C; see also summary in Table S1 in the supplementary material). By 10-15 minutes, the nascent mRNA showed additional localization to numerous perinuclear foci that co-localized with the P granule marker GLH-1 (Fig. 6E,F). Optical cross-sections showed that the nascent mRNA extended throughout P granules (Fig. 6G) and was largely outside the zone of NPP-9 (Fig. 7B). Between 15 and 30 minutes, the level of nascent mRNA decreased in P granules and increased in the cytoplasm and gonad core (Fig. 6D; data not shown). By 45 minutes, some mRNA appeared in late oogonia and oocytes, presumably through cytoplasmic flow (see below; data not shown). For comparison, measured rates of cytoplasmic flow in the core (~7 μm/minute) (Wolke et al., 2007) would be expected to transport materials the
distance from the expression zone to the oocytes in about 25-30 minutes. Finally, mRNA levels in all regions of the gonad diminished markedly by 60-90 minutes (see Fig. S5 in the supplementary material). We observed essentially identical temporal and spatial patterns of mRNA localization for transgenes encoding GFP with 3′UTRs from mRNAs that are either (1) transcribed and translated in the gonad, (2) transcribed but not translated in the gonad or (3) not normally expressed in the gonad (see Table S2 in the supplementary material). Thus, nascent mRNAs in general appear to localize transiently to P granules before dispersing in the cytoplasm.

**Perinuclear localization of nascent mRNA depends on P granule proteins**

Transient, perinuclear localization might result from specific mechanisms moving newly exported mRNA into and through P granules. Alternatively, localization might result simply from pulsed mRNA moving through clustered NPCs; if so, cells lacking P granules might show analogous localization. We examined the localization of nascent mRNA in intestinal cells; these polyploid cells produce much more transgene-derived mRNA than germ cells and have a high density of NPCs comparable to NPC clusters on germ nuclei (Fig. 6A; data not shown) (see also Pitt et al., 2000). Nascent mRNA was detected in the nucleoplasm and intestinal cytoplasm by 15 minutes post-heat shock but showed no obvious enrichment near the zone of NPP-9 (Fig. 7A). By contrast, germ cells in the same animals showed mRNA concentrated near the NPP-9 zone, within P granules (Fig. 7B). We next examined mRNA localization in germ cells depleted of GLH-1 by glh-1(RNAi); this treatment results in small, abnormal P granules that lack multiple P granule components (Gruidl et al., 1996; Kawasaki et al., 1998; Schisa et al., 2001). Similar to intestinal cells, the glh-1(RNAi) germ cells showed little mRNA accumulation near the zone of NPP-9, in contrast to mock-treated controls (Fig. 7C-D’; see Table S1 in the supplementary material).

**P granules do not sequester mRNAs that are targeted for degradation by the RNAi or NMD pathways**

Because the double-stranded RNA-specific endonuclease Dicer is a component of nuclear-associated chromatoid bodies in mouse germ cells (Kotaja et al., 2006), we wanted to determine if mRNA...
Fig. 7. Perinuclear mRNA localization requires P granules. (A) Intestinal cell 15 minutes after heat shock, showing little or no mRNA (green) adjacent to the NPP-9 zone (red). (B) Germ cell in the same animal showing perinuclear foci of RNA (green, left panel) adjacent to the NPP-9 zone (red) and coincident with P granules (green, right panel). (C,E) Single pachytene germ cell from a heat-shocked, glf-1(RNAi) animal. Staining as indicated for transgenic mRNA, NPP-9 and DNA. Note the very low level of perinuclear mRNA (arrow; shown at high magnification in C). (D,E) Germ cell from mock-treated control animal stained as indicated and showing a high level of perinuclear mRNA (arrow; shown at high magnification in D'). Scale bars: 2.5 μm in A-C; 2.5 μm in B,E-G.

destined for degradation by the RNAi pathway is sequestered or degraded in P granules. We used the pos-1 gene to analyze the RNAi pathway, as pos-1 is abundant in germ cells and highly sensitive to RNAi (Tabara et al., 1998). We first established that dsRNA targeting the pos-1 3’UTR was effective in depleting endogenous pos-1 mRNA within 24 hours (data not shown). Next, worms expressing a hsp-16.2::GFP transgene with the pos-1 3’UTR were treated with the same dsRNA for 24 hours, then heat shocked to induce mRNA expression. As observed under non-RNAi conditions, the mRNA passed through P granules into the cytoplasm by 30 minutes (Fig. 8A,B; see Table S1 in the supplementary material).

P granules contain some proteins found in P-bodies (see Introduction), and yeast mRNAs with premature stop codons accumulate in P-bodies (Sheth and Parker, 2006). To determine whether nonsense-containing mRNAs accumulate in P granules, we analyzed mex-3 and rme-2 mRNAs that are normally translated in oocytes (with cytoplasmic P granules) and late pachytene germ cells (perinuclear P granules), respectively. mex-3(zu155) and rme-2(h1005) mutants have mRNAs with premature stop codons and can be suppressed by inhibiting the NMD (nonsense-mediated decay) pathway (Fig. 8C-E) (Lee and Schedl, 2004). As expected for NMD, the mex-3 and rme-2 mutant mRNAs were present initially at wild-type levels but disappeared from oocytes and the pachytene zone, respectively, where translation normally ensues (compare Fig. 8F with 8G and Fig. 8H with 8I). We found that the mutant mRNAs showed no obvious enrichment in perinuclear or cytoplasmic P granules either before, or within, the degradation zones (Fig. 8F’-H’). Indeed, overlapping or adjacent foci of mRNA and PGL-1 staining were observed much more frequently for the abundant wild-type mRNAs than for the mutant mRNAs, presumably reflecting their relative abundance (compare Fig. 8F’ with 8G’).

Multiple P granule proteins contain FG repeats

mRNA export through NPCs involves export factors such as NXF1 that bind clustered FG repeats on nucleoporins such as Nup358/RanBP2 (reviewed in Tran and Wente, 2006), and we noted previously that the GLH family of P granule proteins also contain clustered FG repeats (Fig. 9A) (Schisa et al., 2001). We noticed that one of the four predicted isoforms of C. elegans DDX-19 has a cluster of 20 FG-repeats, including 16 repeats of a GxFG motif found in a subset of nucleoporins (Fig. 9A). By contrast, human DDX19 has only a single FG sequence, and yeast Dpb5p has only two, widely separated FG sequences (GenBank accession numbers BC003626 and U28135). Nearly all of the FG repeats in the C. elegans DDX-19 isoform are encoded by a single, alternatively spliced fourth exon (Fig. 9A). We found that ddx-19 mRNA containing the fourth exon is abundant in germ cells but not detected above background in somatic cells (Fig. 9B,C).

Proteomic analyses in yeast and mammals have provided lists of all putative nucleoporins and major NPC-associated proteins (Cronshaw et al., 2002; Rout et al., 2000). We searched the C. elegans proteome for examples of predicted proteins with clustered GxFG repeats that are not known nucleoporins. In addition to the
GLH proteins and the DDX-19 isoform, a cluster of GxFG repeats were present in PQN-75, a novel protein, and in F58G11.2, a predicted RNA helicase with no clear mammalian ortholog. A genomic in situ hybridization database indicated that both of the latter genes are highly enriched in gonads (NEXTDB Ver.4.0 at http://nematode.lab.nig.ac.jp/db2), and we confirmed this result for F58G11.2 (Fig. 9D,E). Despite several attempts, we failed to recover viable strains expressing GFP fused to PQN-75. However, worms expressing GFP fused to F58G11.2 showed variable localization to P granules in adult gonads and consistent localization to cytoplasmic P granules in early embryos (Fig. 9F) and to the nuclear-associated P granules in later embryos (Fig. 9G). We conclude that P granules contain at least three components with clustered GxFG repeats – the GLH family, DDX-19 and F58G11.2.

DISCUSSION

Pg-NPCs are principal sites of mRNA export in germ cells

We have shown that perinuclear P granules and cytoplasmic P granules differ in both morphology and dynamics. Although cytoplasmic P granules exist in transcriptionally quiescent oocytes, our study demonstrates that perinuclear P granules and their associated NPCs (Pg-NPCs) are the major sites of mRNA export in germ cells. We showed here that known and predicted mRNA export factors are localized at Pg-NPCs in both larval and adult germ cells under normal growth conditions (Figs 3, 5). Furthermore, we showed that heat shock can induce expression of high-copy, transgenic arrays in a subset of germ cells, and that most of this nascent mRNA appears to be exported through Pg-NPCs (Fig. 6). We do not yet know why the transgenes are expressed only in these germ cells. However, the spatial, temporal, and genetic parameters of expression parallel the normal pattern of X chromosome-specific expression, suggesting a shared mechanism of activation or derepression (Kelly et al., 2002).

If most nascent mRNA is exported through Pg-NPCs, other NPCs might have distinct functions. For example, NPCs that are adjacent to the asymmetrically positioned nucleolus in yeast nuclei lack proteins such as Mlp1p and Mlp2p that prevent the export of non-splcified mRNAs (Galy et al., 2004; Strambio-de-Castillia et al., 1999); this functional asymmetry suggests that the nucleolus-associated NPCs might be specialized for transport in ribosome biogenesis (Tran and Wente, 2006). Interestingly, we found that NPCs, but not Pg-NPCs, were present where a lobe of the nucleolus contacts the nuclear envelope in some germ cells (see Fig. S1 in the supplementary material). Moreover, [3H]-uridine-labeled C. elegans gonads do not show an enrichment of label on perinuclear P granules (Schisa et al., 2001), and analogous labeling studies on C. bergerac gonads showed that most of this label is incorporated in rRNA rather than mRNA (Starck and Brun, 1977; Starck et al., 1983). If rRNA is not exported through Pg-NPCs, this might explain why perinuclear P granules in old, quiescent gonads accumulate large quantities of mRNA but lack detectable rRNA (Schisa et al., 2001).

Newly exported mRNA traffics through P granules

As viewed by electron microscopy, the cytoplasmic fibrils of NPCs are approximately 50 nm in length (Stoffler et al., 1999), whereas the electron-dense base of the P granule begins approximately 70 nm from the nuclear rim. As observed by immunocytochemistry, the mRNA export factor NXF-1 is concentrated below the P granules and is essentially coincident with NPP-9, a presumptive component of the NPC fibrils. Thus, mRNA released from an NPC should be at or near the electron-dense base of a P granule. P granules extend 300-400 nm from the nuclear rim into the cytoplasm, well beyond the predicted terminus of NPC fibrils, and our immunoostaining experiments show that the P granule proteins GLH-1 and PGL-1 localize predominantly outside the zone of NPP-9 (Figs 6, 7). Thus, our FISH analysis allows us to conclude that (1) the perinuclear foci of newly exported RNA are clearly outside of NPCs (Fig. 7) and (2) that these foci overlap extensively with P granules (Fig. 6). Thus, most mRNAs enter a distinct compartment, the P granule, after their export from germ nuclei. We argue that perinuclear localization does not result simply from the pulsed generation of heat-shock-induced mRNA because this localization does not occur in intestinal cells lacking P granules or in germ cells lacking a subset of P granule proteins (Fig. 7). Instead, we propose that perinuclear localization results because nascent mRNAs move through P granules, and that this movement is significantly slower than diffusion in the surrounding cytoplasm.

Fig. 9. FG-repeat proteins in P granules. (A) Diagram showing FG repeats (vertical bars) in the nucleoporin NPP-9 and in four additional C. elegans proteins encoded by gonad-enriched mRNAs. (B, C) In situ hybridization for sense (B) and antisense (C) ddx-19 (exon 4) mRNA. (D, E) In situ hybridization for sense (D) and antisense (E) F58G11.2 mRNA. (F) Two-cell embryo showing colocalization of F58G11.2::GFP with cytoplasmic P granules (arrow). (G) Fifty-cell embryo showing colocalization of F58G11.2::GFP with P granules (arrow). Scale bars: 25 μm.
Movement of mRNA through P granules might be similar to transport through the NPC. Free diffusion through the NPC is restricted because FG repeat nucleoporins form a hydrophilic meshwork in the central channel (reviewed by D’Angelo and Hetzer, 2008). The P granule protein GLH-1 contains a cluster of FG repeats, and we showed here that two additional P granule-enriched proteins (F58G11.2 and a germline-specific isoform of DXD-19) contain similar repeats. The presence of multiple FG repeat proteins in P granules raises the possibility that P granules confront newly exported mRNA with a second hydrophobic barrier to free diffusion. Indeed, we speculate that exposed hydrophobic domains of P granule proteins might underlie the unusual fusion behaviors of cytoplasmic P granules after detachment from the nuclear envelope (Brangwynne et al., 2009).

Transport through the NPC involves factors such as NXF1 that bind specifically to FG repeats, possibly dissolving the local hydrophilic meshwork (Ribbeck and Gorlich, 2001; Rout et al., 2000). In future studies, it will be interesting to determine whether mRNA movement through P granules involves analogous, dedicated transport factors or a sequence of random bind-and-release interactions with RNA-binding components of P granules. The finding that P granules have asymmetric morphology raises the possibility some of these components have ordered positions that contribute to mRNA movement or release.

P granules: assembly sites versus disposal centers

Materials diffusing randomly in germ cell cytoplasm would quickly exit into the gonad core, where they would be swept away toward distant oogonia (Wolke et al., 2007). Thus, perinuclear P granules might provide a crucial compartment to assemble complexes between nascent mRNA and regulatory small RNAs or proteins before the mRNA is diluted in the core cytoplasm. The germline teratoma phenotype of mex-3; gld-1 double mutants that lose P granules appears to result in part from the premature expression of somatic-specific transcription factors that normally function in early embryogenesis (see Introduction; Ciosk et al., 2006). Thus, the mutant germ cells might express the appropriate regulatory molecules but be inefficient in assembling complexes with target mRNAs in the absence of P granules. Totipotent germ cells could be particularly sensitive to the inappropriate expression of these transcription factors, as the misexpression of such factors can readily respecify the fates of multipotent early embryonic cells (Zhu et al., 1998).

We did not find evidence that P granules sequester or degrade mRNAs targeted by the RNAi pathway. Instead, the targeted mRNA moved through P granules into the general cytoplasm, similar to all other transgenic mRNAs examined in our study (Fig. 6). Conversely, nonsense-containing mutant mRNAs did not appear to relocate from the cytoplasm into P granules prior to or during degradation. However, we cannot exclude the possibility that degradation occurs so rapidly in P granules that relocation could not be detected. For example, although some yeast mRNAs targeted for NMD become visibly enriched in P-bodies, others do not (Sheth and Parker, 2006).

Although P granules do not appear to sequester mRNAs targeted for degradation, they store large amounts of mRNA in gonads that are not ovaulating and can release this mRNA if ovulation resumes (Schisa et al., 2001). We favor the hypothesis that P granules are sequestering nascent mRNA in the quiescent gonads but could not test this directly with our heat shock system as these conditions prevented transgene expression (our unpublished observations). However, we showed that the localization of at least two P granule components, PGL-1 and GLH-2, is dependent on active transcription in pachytene germ cells, indicating that the composition, and possibly function(s), of P granules can be regulated by physiological state.

In conclusion, our results draw several basic distinctions between perinuclear P granules and cytoplasmic P granules. We suggest that perinuclear P granules are compartments where newly exported mRNAs find appropriate regulatory molecules before being released to the general cytoplasm. In this view, P granules might not have a unitary function in mRNA metabolism, but rather contribute to multiple facets of mRNA regulation. Clearly, much remains to be learned in future studies about the structure, composition and physiology of these interesting organelles.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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