PGL proteins self associate and bind RNPs to mediate germ granule assembly in *C. elegans*

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Germ granules are germ lineage–specific ribonucleoprotein (RNP) complexes, but how they are assembled and specifically segregated to germ lineage cells remains unclear. Here, we show that the PGL proteins PGL-1 and PGL-3 serve as the scaffold for germ granule formation in *Caenorhabditis elegans*. Using cultured mammalian cells, we found that PGL proteins have the ability to self-associate and recruit RNPs. Depletion of PGL proteins from early *C. elegans* embryos caused dispersal of other germ granule components in the cytoplasm, suggesting that PGL proteins are essential for the architecture of germ granules. Using a structure–function analysis in vivo, we found that two functional domains of PGL proteins contribute to germ granule assembly: an RGG box for recruiting RNA and RNA-binding proteins and a self-association domain for formation of globular granules. We propose that self-association of scaffold proteins that can bind to RNPs is a general mechanism by which large RNP granules are formed.

Introduction

In many organisms, germ cells have electron-dense cytoplasmic organelles, generally referred to as "germ granules," which are believed to play roles in germ cell specification and differentiation (Eddy, 1975; Saffman and Lasko, 1999). They are large RNA-enriched nonmembranous organelles, and historically called by diverse names such as P granules in *Caenorhabditis elegans,* polar granules in *Drosophila*, and germinal granules in *Xenopus* (Eddy, 1975). In animals in which the germline is preformed (including the above three organisms), germ granules are maternally inherited by the fertilized egg and then specifically segregated to the germ lineage during early embryogenesis. Germ granules contain specific mRNAs and proteins, some of which are conserved among species (e.g., *Drosophila* Vasa [Hay et al., 1988a,b; Lasko and Ashburner, 1988] and its homologues [Raz, 2000]), but many appear to be species specific. Despite the evolutionary divergence of their molecular composition, the majority of the protein components of germ granules are implicated in various aspects of RNA metabolism, which has led to the prediction that the common biochemical function of germ granules is to regulate the translation efficiency and/or stability of mRNAs in the germline (Seydoux and Braun, 2006; Strome and Lehmann, 2007).

In *Caenorhabditis elegans*, germ granules are called P granules, and some of the specific mRNAs and ∼40 protein components (mostly RNA-binding proteins) of P granules have been identified (Strome, 2005). Among them, GLH-1–4 (*Drosophila* Vasa homologues; with DEAD-box helicase motifs; Gruidl et al., 1996; Kuznicki et al., 2000), PGL-1 and PGL-3 (*C. elegans*–specific germ granule components; each with an RGG box [multiple Arg-Gly-Gly repeats]; Kawasaki et al., 1998, 2004), and DEPS-1 (Spike et al., 2008) are the only known components that exclusively localize to P granules at all developmental stages; other components localize to P granules transiently, and many of them are present in somatic lineages as well (Strome, 2005). Although many P-granule components have been identified, how the granules are assembled (and disassembled in somatic cells) is not well understood.

Here we established an assay to analyze the granule-forming ability of P-granule components using cultured mammalian

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Abbreviation used in this paper: PABP, poly(A) binding protein.

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Table I. **Percentage of CHO cells with granules containing the indicated P-granule components**

P-granule component	Localization to P granules	Motifs	[a]Formation of amorphous aggregates	[b]Formation of globular granules	[c]Colocalization with PGL-3 granules
			%	%	%
PGL-3	Constitutive	RGG box	4	96	N.A.
PGL-1	Constitutive	RGG box	10	82	100
GLH-1	Constitutive	DEAD box, 4 CCHC fingers	2	0	4
GLH-3	Constitutive	DEAD box, 2 CCHC fingers	4	0	20
MEX-1	Transient	2 CCCH fingers	20	0	100
MEX-3	Transient	2 KH domains	4	0	92
POS-1	Transient	2 CCCH fingers	50	0	82
OMA-1	Transient	2 CCCH fingers	8	0	90
OMA-2	Transient	2 CCCH fingers	10	0	92
SPN-4	Transient	RNP motif	8	0	98
GLD-1	Transient	KH domain	10	0	92
CGH-1	Transient	DEAD box	16	0	80
GLD-3	Transient	KH-related domains	4	0	16
IFE-1	Transient	Translation initiation factor 4E	0	0	6

[a]Percentage of cells in which the expressed GST-tagged P-granule components formed amorphous aggregates.

[b]Percentage of cells in which the expressed GST-tagged P-granule components formed globular granules.

[c]Percentage of cells in which the expressed GST-tagged P-granule components colocalized with the granules formed by 6xHis-tagged PGL-3.

50 cells were randomly selected in each expression experiment, and the number of the cells forming granules was counted. In the coexpression experiments, we counted cells with granules that contained both PGL-3 and the corresponding component. N.A., not applicable.

cells and somatic *C. elegans* cells. We show that PGL proteins autonomously form cytoplasmic granules in heterologous cells, and that RNA and some protein components are recruited to granules in a manner dependent on the RGG box. In contrast, GLH proteins do not exhibit autonomous granule formation ability. Our findings demonstrated that PGL proteins play a crucial role as scaffolds in the assembly of P granules in *C. elegans.*

Results and discussion

To identify the components that play a major role in P-granule assembly, each component of P granules was examined for its ability to form granules in cultured mammalian cells in which no other *C. elegans* proteins were present. 14 P-granule components were used for this study: four were constitutive P-granule components—PGL-1 (Kawasaki et al., 1998), PGL-3 (Kawasaki et al., 2004), GLH-1 (Gruidl et al., 1996), and GLH-3 (Kuznicki et al., 2000)—and ten were transient components: MEX-1 (Guedes and Priess, 1997), MEX-3 (Draper et al., 1996), POS-1 (Tabara et al., 1999), OMA-1 (Detwiler et al., 2001; Shimada et al., 2002), OMA-2 (Detwiler et al., 2001; Shimada et al., 2002), SPN-4 (Ogura et al., 2003), GLD-1 (Jones et al., 1996), GLD-3 (Eckmann et al., 2002), CGH-1 (Navarro et al., 2001), and IFE-1 (Amiri et al., 2001; Table I).

Although the majority (12/14) of the P-granule components were scattered in the cytoplasm or formed amorphous aggregates of diverse shapes with unclear boundaries, GST-tagged PGL-1 and PGL-3 formed globular granules—either spherical or ellipsoidal shape with clear boundaries, reminiscent of P granules—in the cytoplasm of CHO cells (Fig. 1, A–C; Table I; and Fig. S1). Granules were detected in 92% (46/50) and 100% (50/50) of the GST::PGL-1– and GST::PGL-3–positive cells, respectively. Notably, two other constitutive P-granule components, GLH-1 and GLH-3, were dispersed in the cytoplasm. PGL-1 and PGL-3 are paralogues with 77% amino acid similarity and they interact directly with each other (Kawasaki et al., 2004). When coexpressed in CHO cells, they colocalized in the same granules (Fig. 1 D), suggesting that both of these PGL proteins participate in globular granule assembly. Granule formation by PGL-3 was confirmed in four other mammalian cell lines (NIH3T3, HeLa, MDCK, and HEK293; unpublished data). We refer to granules formed by PGL-1 and/or PGL-3 as "PGL granules."

To examine whether PGL proteins can form granules in *C. elegans* cells in the absence of germ cell–specific factors, PGL-1 and PGL-3 were individually expressed in *C. elegans* somatic cells. In embryonic somatic cells (PGLs expressed via the *pes-10* promoter; Seydoux and Fire, 1994) as well as in adult pharyngeal cells (PGLs expressed via the *myo-2* promoter; Okkema et al., 1993), GFP::PGL-1 and GFP::PGL-3 formed cytoplasmic granules (Fig. 1, E and F; and unpublished data). In contrast, GFP and GFP::GLH-1 were dispersed in the cytoplasm, and no granules were detected (Fig. 1, G and H; and unpublished data). Thus, consistent with the results obtained with CHO cells, PGL proteins, but not GLH-1, have the ability to form granules autonomously in the absence of other germline-specific factors in *C. elegans*.

These results raised the possibility that PGL proteins serve as a scaffold for P-granule formation in *C. elegans*. To test this possibility, we next examined whether PGL granules recruit other known P-granule components in CHO cells. PGL granules were stained positively by the DNA/RNA-specific dye, SYTOX, and this staining disappeared when the fixed cells were pretreated with ribonuclease A (RNase A; Fig. 2, A and B), suggesting that PGL granules contain RNA. In addition, endogenous poly(A)-binding protein (PABP), which normally localizes throughout the cytoplasm (Fig. 2 C), was also enriched in PGL

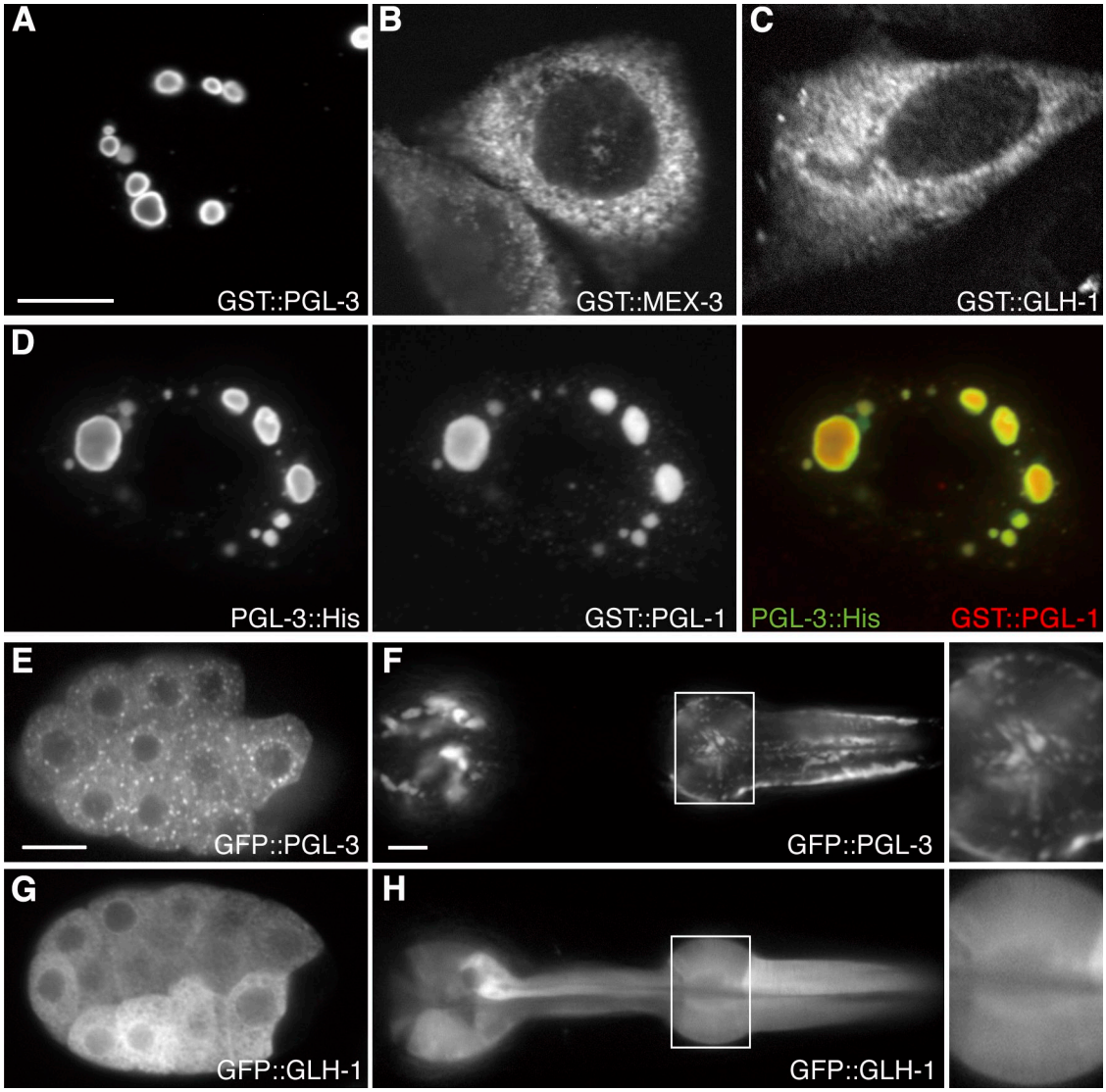

Figure 1. **PGL proteins autonomously form globular granules.** (A–D) Immunofluorescence images of CHO cells expressing *C. elegans* P-granule components. (A) GST::PGL-3, (B) GST::MEX-3, (C) GST::GLH-1, (D) coexpression of PGL-3::6×His and GST::PGL-1. (E–H) Transgenic *C. elegans* expressing GFP-tagged proteins. (E and F) GFP::PGL-3 ectopically expressed via the *pes-10* (E) or *myo-2* (F) promoter. (G and H) GFP::GLH-1 ectopically expressed via the *pes-10* (G) or *myo-2* (H) promoter. (E and G) An ∼50-cell embryo. (F and H) Adult pharynx. Bars, 10 µm.

granules (Fig. 2 D). Furthermore, when coexpressed with PGL-3, 8 of 12 P granule components showed substantial co-localization with the granules formed by PGL-3 (Table I and Fig. S2). For example, when MEX-3 was expressed by itself or co-expressed with LacZ, it was generally dispersed in the cytoplasm (Fig. 1 B). When MEX-3 was coexpressed with PGL-3, however, 92% (46/50) of the cells had PGL granules that contained MEX-3 (Table I and Fig. 2 E). GLD-3, and the constitutive P-granule components GLH-1 and GLH-3, were recruited to PGL granules in <25% of cells (Table I, Fig. 2 F, and Fig. S2). More efficient recruitment of GLH-1 and GLH-3 to PGL granules is observed in *C. elegans* intestinal cells, perhaps revealing an effect of cell origin or type on this association (Updike et al., 2011). IFE-1 was recruited to PGL granules only when they contained PGL-1 (Fig. S2), consistent with previous reports that PGL-1, but not PGL-3, directly binds to IFE-1 (Amiri et al., 2001; Kawasaki et al., 2004). Thus, in mammalian cells, PGL proteins autonomously form RNP granules that contain endogenous RNA, PABP, and certain coexpressed *C. elegans* P-granule components.

To test whether the PGL proteins serve as the scaffold for P-granule assembly in *C. elegans*, we depleted the PGL proteins from early *C. elegans* embryos via a combination of genetic mutations and RNAi, and then examined the localization of other P-granule components. In wild-type early embryos, POS-1 and MEX-3 (both transient P-granule components) are dispersed in the cytoplasm of somatic cells, whereas in the germ lineage they form granules that predominantly colocalize with PGL proteins, with some diffuse signal still detectable in the cytoplasm (Fig. 3, B and D; Draper et al., 1996; Ogura et al., 2003). In *pgl-1(RNAi);pgl-3(RNAi)* embryos in which both PGL-1 and PGL-3 were undetectable, granules formed by POS-1 were vastly decreased compared with wild-type embryos (Fig. 3 C; number of granules of >1.5-µm diameter in

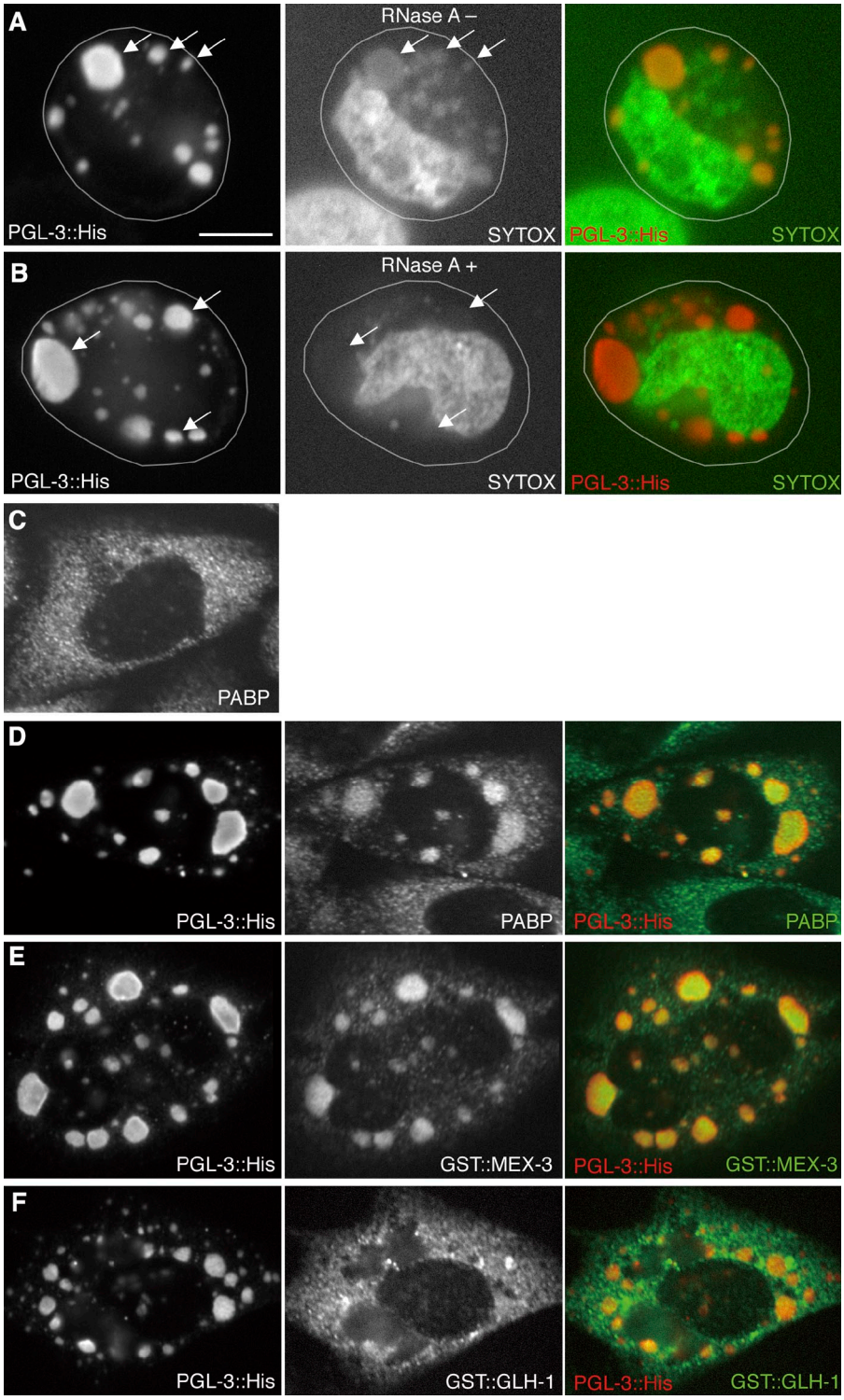

Figure 2. **PGL proteins recruit other P-granule components in CHO cells.** (A and B) SYTOX staining of DNA and RNA as shown without (A) or with (B) ribonuclease A treatment. (C–F) Immunofluorescence images of CHO cells. (C) Endogenous poly(A)-binding protein (PABP) in a cell not expressing PGL-3. (D) A cell expressing PGL-3::6×His; PGL-3::6×His, and endogenous PABP is shown. (E) A cell coexpressing PGL-3::6×His and GST::MEX-3. (F) a cell coexpressing PGL-3::6×His and GST::GLH-1. Bar, 10 µm.

P2 cell [mean ± SD]: wild type, 40.0 ± 10.0 [n = 8]; *pgl-1(RNAi);pgl-3(RNAi)*, 12.4 ± 3.5 [n = 7]). Granules formed by GEP::MEX-3 were also significantly decreased (Fig. 3 E; wild type, 48.3 ± 9.0 [n = 4]; *pgl-1(RNAi);pgl-3(RNAi)*, 13.8 ± 3.6 [n = 6]). We speculate that the POS-1– or MEX-3–containing granules in the absence of PGL proteins are different types of RNP granules, such as P bodies (Gallo et al., 2008). These results support the model that PGL granules recruit other components to assemble P granules.

Localization of GLH proteins was also affected by depletion of PGL proteins. In wild-type embryos, GLH-1 almost completely colocalizes with PGL granules (Fig. 3 F; Gruidl et al., 1996). When both PGL-1 and PGL-3 were depleted, however, GLH-1 was dispersed in the cytoplasm of both germline and somatic embryonic cells (Fig. 3 G), suggesting that PGL proteins are essential for GLH-1 to be incorporated into granular structures. GLH-1 and GLH-4 have partially redundant functions that are necessary for PGL proteins to form granular

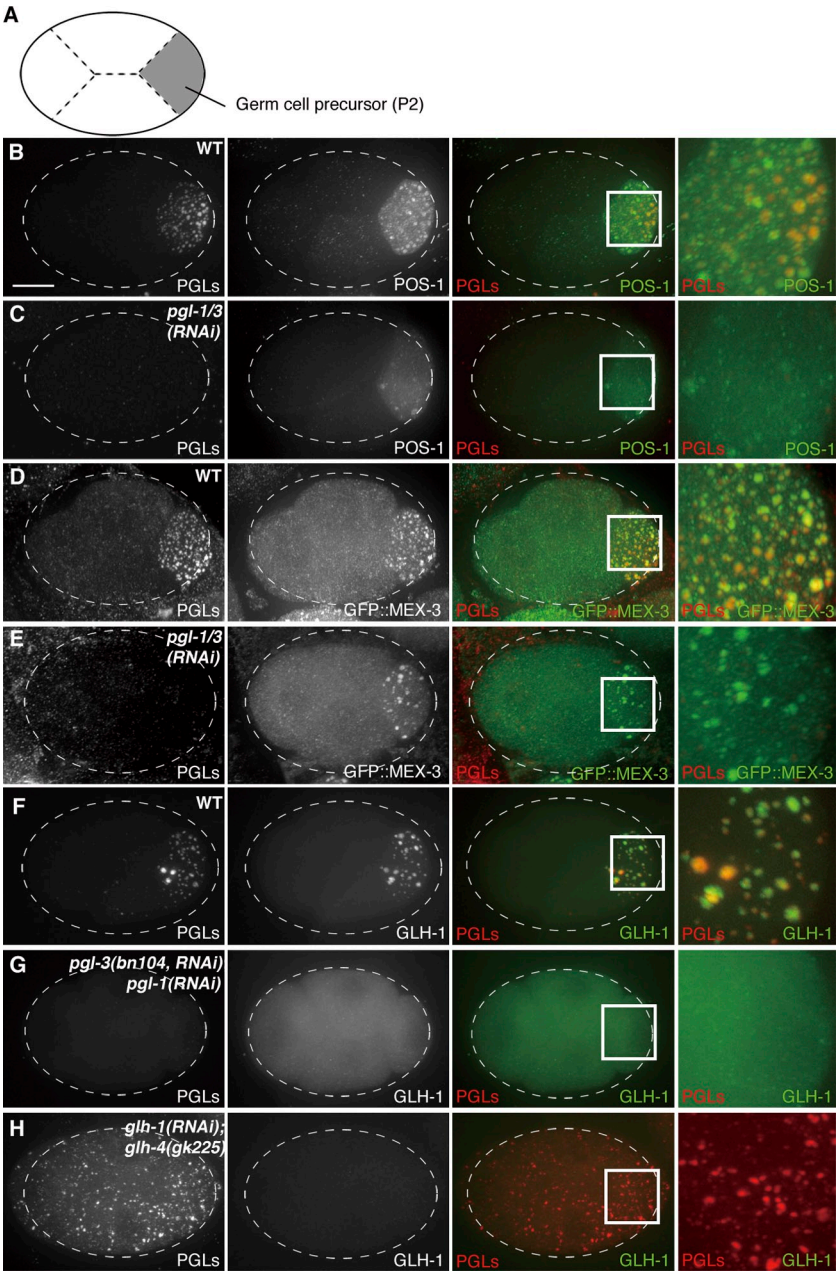

Figure 3. **PGL proteins function as the scaffold for P-granule assembly.** (A) Schematic representation of a 4-cell stage *C. elegans* embryo. (B–H) Immunofluorescence images of 4-cell stage *C. elegans* embryos. Maximum projections of confocal Z-series images that cover whole embryos are shown. In each image, the protein that was detected is indicated. Bar, 10 µm. (B, D, and F) Control wild-type embryos. (C, E, and G) *pgl-1(RNAi);pgl-3(RNAi)* or *pgl-1(RNAi);pgl-3(bn104, RNAi)* embryos. (H) *glh-1(RNAi);glh-4(gk225)* embryo. PGLs: both PGL-1 and PGL-3 detected by the mixtures of monoclonal antibodies KT3 (anti-PGL-3) and KT4 (anti-PGL-1). GFP::PGL-3ΔRGG and GFP::MEX-3 were detected by anti-GFP. POS-1 and GLH-1 were detected by anti-POS-1 and anti-GLH-1, respectively.

structures in the *C. elegans* adult germline (Kawasaki et al., 1998; Kuznicki et al., 2000). We confirmed this in early embryos; in *glh-1(RNAi) glh-4(gk225)* embryos in which both GLH-1 and GLH-4 were undetectable, PGL-3 was dispersed in both somatic and germline cytoplasm, and cytoplasmic granules smaller than wild-type P granules were detected throughout embryos (Fig. 3 H). These results demonstrate that PGL and GLH proteins are mutually required to form granular structures and indicate that, although PGL proteins have the ability to self-aggregate, GLH proteins are essential in the early germline to maintain the multicomponent granular structures formed by PGL proteins.

To understand the molecular mechanism by which PGL proteins mediate RNP granule formation, we performed a structure–function analysis of PGL-3 in CHO cells (Fig. 4 A). The only recognizable motif in PGL-1 and PGL-3 is a C-terminal RGG box that acts as an RNA-binding domain (Godin and Varani, 2007). PGL-3 lacking the RGG box (PGL-3ΔRGG; lacking amino acid residues 633–693) formed globular granules approximately the same size as those formed by the full-length PGL-3 (Fig. 4, B and C). Endogenous RNA, however, was undetectable in the granules formed by PGL-3ΔRGG (unpublished data), and endogenous PABP and other coexpressed P-granule components (MEX-1, MEX-3, POS-1, OMA-1, OMA-2, SPN-4, GLD-1, and CGH-1) were not present in the granules (Fig. 4 C and Fig. S3). These results indicate that the RGG box of PGL-3 is dispensable for the formation of globular granules but necessary to capture and incorporate RNA and RNA-binding proteins into the granules.

Although PGL-3(1–318) did not form globular granules on its own (Fig. 4 D), when coexpressed with the full-length PGL-3, PGL-3(1–318) colocalized with granules formed by the

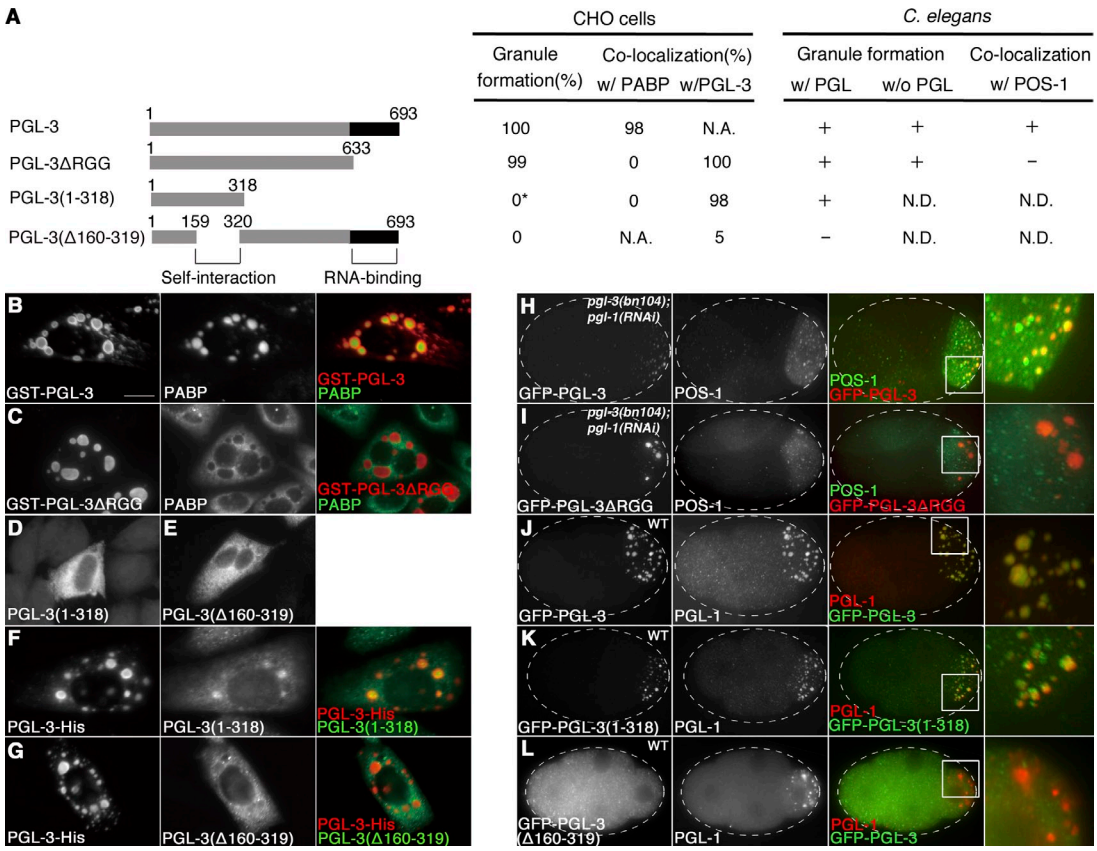

Figure 4. **PGL-3 has two distinct domains for self-interaction and recruitment of RNA and RNA-binding proteins.** In each image, the protein that was detected is indicated. (A) Summary of the structure–function analysis of PGL proteins in CHO cells and *C. elegans* embryos. N.A., not applicable. N.D., not determined. *, 50% of cells formed small amorphous aggregates. (B–G) Immunofluorescence images of CHO cells. (B) A cell expressing GST::PGL-3; endogenous PABP was colocalized with GST::PGL-3 granules. (C) A cell expressing GST::PGL-3ΔRGG; endogenous PABP was excluded from GST::PGL-3ΔRGG granules. (D) A cell expressing GST::PGL-3(1–318). (E) A cell expressing GST::PGL-3(Δ160-319). (F) A cell coexpressing GST::PGL-3(1–318) and PGL-3::6×His; GST::PGL-3(1–318) were recruited to PGL-3::6×His granules. (G) A cell coexpressing GST::PGL-3(Δ160-319) and PGL-3::6×His; GST::PGL-3(Δ160-319) were not recruited to PGL-3::6×His granules. (H and I) *pgl-1(RNAi);pgl-3(bn104)* 4-cell stage embryo expressing GFP::PGL-3 (H) or GFP::PGL-3ΔRGG (I), stained by anti-GFP and anti-POS-1. (J–L) Wild-type *C. elegans* 4-cell stage embryos expressing GFP-tagged PGL-3 variants, stained by anti-GFP and anti-PGL-1 (KT4 mAb). (J) GFP::PGL-3, (K) GFP::PGL-3(1–318), (L) GFP::PGL-3(Δ160-319). Bars, 10 µm.

full-length PGL-3 (Fig. 4 F), suggesting that this fragment contains a region for self-interaction. On the other hand, PGL-3(Δ160-319) did not form globular granules (Fig. 4 E) and was not recruited to the granules formed by full-length PGL-3 (Fig. 4 G). It has been shown that PGL-1 directly interacts with PGL-1 and PGL-3 in vitro (Kawasaki et al., 2004). Thus, it is likely that residues 160–319 of PGL-3 comprise a region essential for self-interaction of PGL proteins.

We next tested whether the domains identified using mammalian cells have the predicted functions in *C. elegans* embryos. First, truncated PGL-3 proteins tagged with GFP were maternally expressed in wild-type worms (i.e., containing endogenous PGL-1 and PGL-3), and their localization was examined during early embryogenesis. As expected, PGL-3Δ RGG and PGL-3(1–318) exhibited granular structures in germline cells (Fig. 4, J and K; and unpublished data), suggesting that these truncated proteins and endogenous wild-type PGL proteins jointly formed granules. PGL-3(Δ160-319) was not incorporated into P granules and was dispersed in the cytoplasm of both germline and somatic cells in the early embryos (Fig. 4 L), consistent with the results from the CHO cell experiments.

Next, to investigate the importance of the RGG box for recruitment of other P-granule components in *C. elegans* embryos, GFP::PGL-3 or GFP::PGL-3ΔRGG was expressed in the absence of endogenous PGL-1 and PGL-3 (*pgl-1(RNAi);pgl-3(bn104)*) in the early embryos, and we examined colocalization of GFP::PGL-3 or GFP::PGL-3ΔRGG with a transient P-granule component, POS-1. GFP::PGL-3 formed granules in the germ line cells and these granules contained POS-1 (Fig. 4 H). GFP::PGL-3ΔRGG also formed granules in germ line cells, but these granules did not contain POS-1 (Fig. 4 I). As in *pgl-1;pgl-3* embryos (Fig. 3, C and E), POS-1–containing granules were significantly decreased, and they did not contain GFP::PGL-3ΔRGG (Fig. 4 I). These results further confirmed the finding in CHO cells that the RGG box of PGL-3 is dispensable for granule formation but essential for recruitment of other P-granule components (presumably RNPs) in *C. elegans*.

Our results reveal that PGL-3 proteins have two distinct regions for RNP granule assembly: one for globular granule assembly via self-interaction, and the RGG box for recruiting RNA and RNA-binding proteins (Fig. 4 A). Because RGG boxes of various proteins have been shown to bind RNA with low sequence preference (Godin and Varani, 2007), PGL proteins

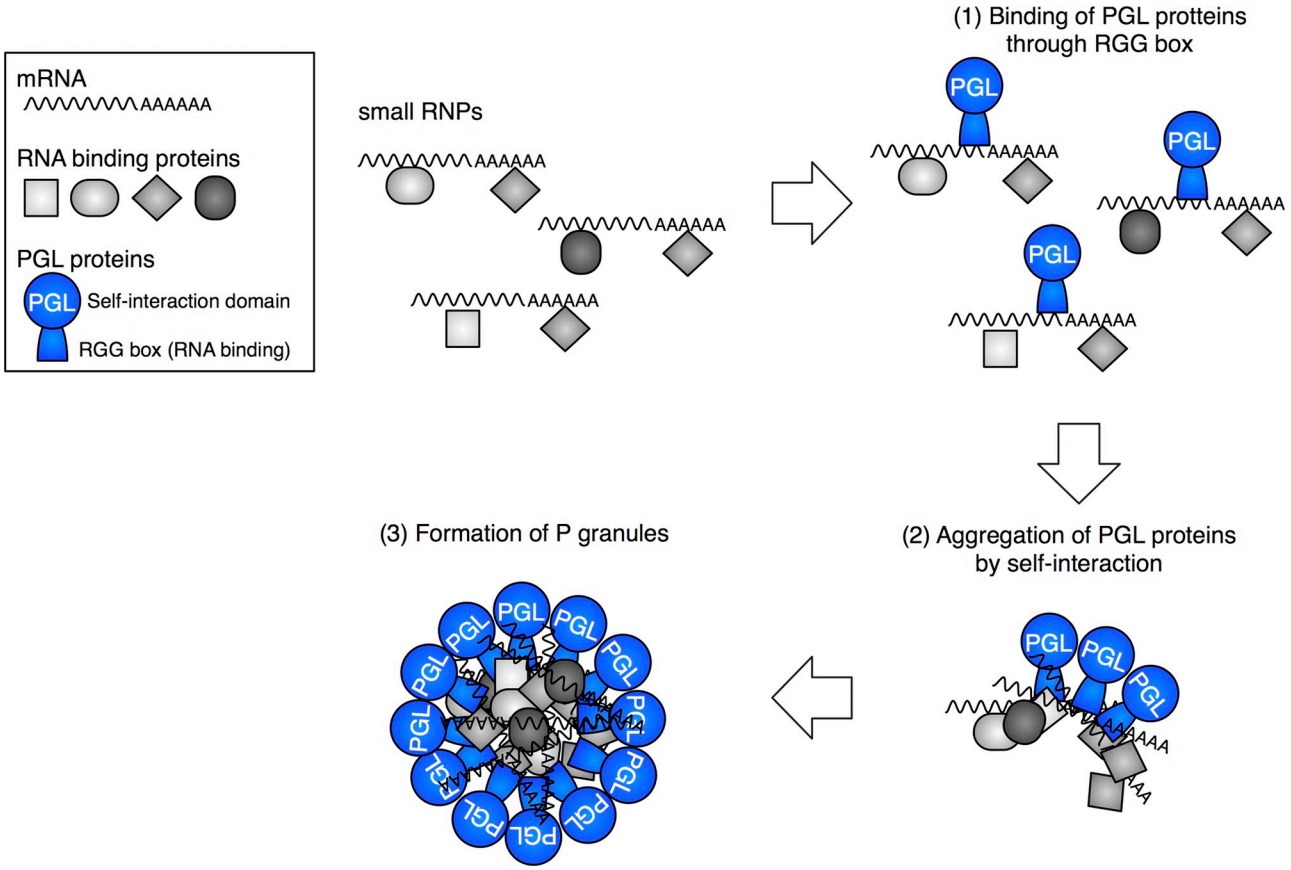

Figure 5. **A two-step model of P-granule formation.** (1) PGL proteins bind to diverse small mRNPs through RGG boxes. (2) PGL proteins self-aggregate through direct interaction between self-interaction domains. (3) P granules are assembled as large RNPs.

are likely to bind via the RGG box to diverse mRNAs and/or mRNA protein complexes (mRNPs) that contain transient protein components of P granules. As most of the transient P-granule components contain various types of RNA-binding motifs and all of the tested components in this study were excluded from granules formed by PGL-3ΔRGG in CHO cells (Fig. S3), we speculate that this RNA-dependent recruitment is the major mechanism of incorporation of transient components into P granules. Because some RNA-binding proteins (e.g., GLH-1) were not recruited to PGL-3 granules, there seems to be some specificity for the recruitment of RNPs, but how it is achieved is currently unclear (some P-granule components, such as IFE-1, appear to be recruited to PGL granules by direct protein–protein interactions [Amiri et al., 2001]). These properties of PGL proteins raises a two-step model for P-granule formation: in the first step, PGL proteins bind to various mRNPs through interaction with RGG boxes; in the second step, globular granules are formed by the self-interaction domain of PGL proteins (Fig. 5); the order of the two steps can be opposite.

A recent report showed that P granules in early *C. elegans* embryos are in dynamic equilibrium with their soluble components, and a conceptual model was proposed in which assembly and disassembly of P granules are regulated by the concentration of soluble P-granule components and their saturation point, which would be lower in the germ lineage than in the soma (Brangwynne et al., 2009). Our data indicate that PGL proteins are the key components directing P-granule assembly/disassembly, and that GLH proteins may be involved in shifting the equilibrium toward granule assembly, possibly by lowering the saturation point of free PGL proteins in the germ lineage.

An essential role for scaffolding proteins in RNP granule assembly has been reported. For example, in the case of formation of processing bodies (P-bodies, a class of cytoplasmic RNP granules) in yeast, a multi-step assembly mechanism was proposed: initially, an individual mRNA associates with various proteins to form an mRNP, and then individual mRNPs form P-bodies via self-aggregation of a scaffolding protein(s)—Edc3p and/or Lsm4p (Decker et al., 2007). In *Drosophila*, Tudor protein is thought to function as a scaffold for germ granule (polar granule) assembly (Arkov et al., 2006). Because some germ plasm–specific proteins and RNAs can form small RNPs ("preparticles") even in the absence of Tudor, it was proposed that the role of Tudor in germ plasm formation may be to bind these small RNPs and assemble them into a larger granule (Arkov et al., 2006). Thus, we propose that self-association of scaffold proteins that can bind to RNPs might be one of the general mechanisms by which large RNP granules are formed.

Materials and methods

Cloning and constructs of tagged P-granule components

The full-length coding region of each P-granule component was PCR amplified from the corresponding cDNA clones (from Y. Kohara, National Institute

of Genetics, Mishima, Japan) or adult *C. elegans* cDNA library (from Y. Iino, University of Tokyo, Tokyo, Japan; Hayashizaki et al., 1998). Two of the subsequent plasmids were subjected to site-directed mutagenesis (*mex-1* and *gld-3*) to generate the wild-type sequence using the QuikChange XL Site-Directed Mutagenesis kit (Agilent Technologies) because the original yk cDNA clones contained mutations. Truncated versions of *pgl-3* were PCR amplified from the plasmid containing the full-length *pgl-3*. Generally, amplified cDNA fragments were cloned into the pDONR201 entry vector (Invitrogen) by the BP recombination reaction according to the manufacturer's instructions. The PCR templates to amplify the full-length coding regions and the expression vectors used in this study are listed in Table S1. Primer sequences used for PCR reactions are available upon request.

For constitutive expression of P-granule components in mammalian cells, the amplified cDNA fragments were cloned into expression vectors containing the cytomegalovirus promoter (pDEST26 for the N-terminal 6xHis tag, pDEST27 for the N-terminal GST tag, or pcDNA-DEST40 for the C-terminal 6xHis tag) by the Gateway system (Invitrogen). pcDNA/GW-40/lacZ for expressing control LacZ::6×His was purchased from Invitrogen.

For expression of PGL-3, MEX-3, and the PGL-3 variants in the *C. elegans* germline and early embryos, the coding regions were cloned into the plasmid pID3.01B (from G. Seydoux, Johns Hopkins University School of Medicine, Baltimore, MD) for the GFP tag. These vectors contained the *pie-1* promoter and *pie-1* 3′-untranslated region for germline expression. For expression of GFP, GFP-tagged PGL-1, PGL-3, and GLH-1 in *C. elegans* somatic cells, the coding regions were cloned into plasmid pPD118.33 and pPD134.96 (both from A. Fire, Stanford University School of Medicine, Stanford, CA) to allow expression of N-terminal GFP-tagged proteins under the control of the *myo-2* and *pes-10* promoters, respectively, and the *let-858* 3′-untranslated region. The CFP-coding region in pPD134.96 was replaced with the GFP-coding region derived from pPD118.26 (from A. Fire).

Expression and immunostaining of the tagged proteins in cultured mammalian cells

CHO cells were transfected using TransIT-LT1 transfection reagents (Takara Bio Inc.). HEK293, HeLa, MDCK, and NIH3T3 cells were transfected using a Microporator-Mini (MP-100; Digital Bio). Cells were cultured on poly-D-lysine–coated slides for 24 h, fixed in 10% formaldehyde for 20 min, and permeabilized with 0.5% (wt/vol) Triton X-100 for 25 min. Slides were blocked with 3% (vol/vol) goat serum in phosphate-buffered saline containing 0.03% (wt/vol) Triton X-100 for 1 h before incubation with primary and secondary Alexa Fluor–conjugated antibodies (Invitrogen). GST-tagged and His-tagged proteins were detected with anti-GST (antibody B-14; Santa Cruz Biotechnology, Inc.) and anti-His (MBL), respectively. A monoclonal antibody (mAb) against PABP (clone 10E10; Sigma-Aldrich) was used to stain endogenous PABP. The following secondary antibodies were used: goat anti–mouse IgG(H+L) Alexa Fluor 488 and 594, goat anti–rabbit IgG(H+L) Alexa Fluor 488 and 594, and donkey anti–rat IgG(H+L) Alexa Fluor 594 (Invitrogen). After final washes, each sample was mounted with VECTASHIELD (Vector Laboratories). For staining nucleic acids, 100 nM SYTOX (Invitrogen) was added to the slides after incubation with secondary antibodies. RNase A (Fermentas) treatment was conducted by incubating slides in 25 µg/ml RNase A solution for 1 h before incubation with primary antibodies.

Worm strains and transgenic lines

C. elegans strains were derived from the wild-type Bristol strain N2 and cultured by standard techniques (Brenner, 1974). *unc-119*(*ed3*), *pgl-3*(*bn104*), and *glh-4*(*gk225*) strains were provided by the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN).

Expression constructs under the control of the *pie-1* promoter were transformed into *unc-119*(−) worms by microparticle bombardment (Praitis et al., 2001) and chromosomal integrants were obtained. For somatic expression of PGL-1, PGL-3, and GLH-1, expression plasmids (derived from pPD118.33 or pPD134.96) were cotransformed into wild-type worms with selection marker plasmid pRF4 containing *rol-6(su1006)* by microinjection (Mello et al., 1991) and worms having extrachromosomal arrays were obtained. The GFP signal was observed in adult worms or embryos carrying the transgene. The amount and size of the proteins induced under the *pes-10* promoter were confirmed by Western blotting.

Immunostaining of worm embryos

Embryos were immunostained as described previously (Takeda et al., 2008). In brief, embryos were collected by cutting gravid hermaphrodite adults, permeabilized by freeze-cracking, and fixed with methanol–acetone (−20°C methanol for 5 min, −20°C acetone for 5 min). The samples were rehydrated before antibody staining by passing the slide through an acetone series (90, 70, 50, and 30%) at room temperature followed by transfer into PBS + 0.5% (wt/vol) Tween 20 (PBST). For immunostaining, the slides were incubated for 1–2 h at room temperature in PBST containing 0.5% BSA + 0.5% skim milk. Endogenous PGL-3 was visualized with mAb KT3 (1:4 dilution; Takeda et al., 2008) or polyclonal rat or rabbit anti-PGL-3 (1:20,000, MBL; generated using His-tagged PGL-3 as antigen). Endogenous PGL-1 was visualized with mAb KT4 (1:4 dilution; Takeda et al., 2008), mAb K76 (1:500 dilution; Wood et al., 1984), or rabbit polyclonal anti-PGL-1 (1:10,000; MBL; generated using GST-tagged PGL-1 as antigen). Specificity of the PGL-3 antibodies and PGL-1 antibody generated in this study was confirmed by Western blotting and immunostaining of samples from wild type and *pgl-3* or *pgl-1* mutants. Anti-GFP (1:200 dilution; MBL) was used to visualize GFP::MEX-3. Anti-GLH-1 (Kawasaki et al., 2004) was used at 1:2,000 dilution. For secondary antibodies, goat anti–mouse IgG(H+L) Alexa Fluor 488 and 594, goat anti–rabbit IgG(H+L) Alexa Fluor 488 and 594, donkey anti–rat IgG(H+L) Alexa Fluor 594, and goat anti–mouse IgM Alexa Fluor 594 (Invitrogen) were used. Secondary antibodies (1:100–1:200 dilution) were preadsorbed with *C. elegans* acetone powder and incubated for 1 h at room temperature. After final washes, each sample was mounted with VECTASHIELD (Vector Laboratories).

Microscopy

Immunofluorescence images of cultured cells and embryos were acquired with a DSU disk-scanning confocal microscope system (BX61; Olympus) with a 100× objective lens (U-PlanApo Oil Iris3-SP, 1.35 NA). For each cultured cell and embryo, Z-series images (0.5–1.0-µm steps) were acquired and projected using a maximum intensity algorithm to produce a single integrated image using MetaMorph software (Molecular Devices). Images were processed with Photoshop (Adobe) software.

RNAi

RNAi was performed by the soaking method described previously (Maeda et al., 2001). In brief, L4 worms were soaked in dsRNA solution (~2 µg/ml for each RNA species) for 24 h and then cultured on plates for 24 h at 25°C. Adult worms were then cut open, and embryos were immunostained. dsRNA was transcribed in vitro from cDNA clones yk847b03 for *pgl-1*, yk1437a06 for *pgl-3*, and yk514b11 for *glh-1*.

Online supplemental material

Fig. S1 shows immunofluorescence images of CHO cells coexpressing GST-tagged P-granule components and LacZ::6xHis. Fig. S2 shows immunofluorescence images of CHO cells coexpressing GST-tagged P-granule components and PGL-3::6xHis or PGL-1::6xHis and PGL-3::6xHis. Fig. S3 shows immunofluorescence images of CHO cells coexpressing GST-tagged P-granule components and 6xHis::PGL-3ΔRGG. Table S1 lists the PCR templates and expression vectors used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201010106/DC1.

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