DEPS-1 is required for piRNA-dependent silencing and PIWI condensate organisation in *Caenorhabditis elegans*

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Membraneless organelles are sites for RNA biology including small non-coding RNA (ncRNA) mediated gene silencing. How small ncRNAs utilise phase separated environments for their function is unclear. We investigated how the PIWI-interacting RNA (piRNA) pathway engages with the membraneless organelle P granule in *Caenorhabditis elegans*. Proteomic analysis of the PIWI protein PRG-1 reveals an interaction with the constitutive P granule protein DEPS-1. DEPS-1 is not required for piRNA biogenesis but piRNA-dependent silencing: dep-1 mutants fail to produce the secondary endo-siRNAs required for the silencing of piRNA targets. We identify a motif on DEPS-1 which mediates a direct interaction with PRG-1. DEPS-1 and PRG-1 form intertwining clusters to build elongated condensates in vivo which are dependent on the Piwi-interacting motif of DEPS-1. Additionally, we identify EDG-1 as an interactor of DEPS-1 and PRG-1. Our study reveals how specific protein-protein interactions drive the spatial organisation and piRNA-dependent silencing within membraneless organelles.
he correct spatial organisation of molecules into organelles is essential for biological function. Recent studies reveal that membraneless organelles can be formed by proteins and nucleic acids condensing out of the bulk intracellular milieu, giving rise to liquid- or gel-like environments. These phase separated organelles, formed by proteins and nucleic acids, are sites for different aspects of eukaryotic RNA biology: the nucleolus is required for the assembly of ribosomes, stress granules allow for translational shunting of mRNAs during stress-response and processing bodies (P-bodies) organise small RNA-mediated regulation of mRNA. However, the molecular mechanisms of how RNAs and proteins assemble into phase separated organelles remain largely unexplored.

Small non-coding RNAs execute diverse biological functions. mRNAs are targeted for silencing by small RNAs based on Watson-Crick base-pair complementarity in complex with members of the Argonaute (Ago) protein family. Various Ago proteins associate with membraneless organelles such as the P-bodies and germ granules. Indeed, recently it has been shown that human Ago2 together with its binding partner TNRC6B can form biomolecular condensates entirely on their own. A recently discovered condensate, the Z granule, contains the Ago protein WAGO-4 to establish transgenerational inheritance (TEI) of exogenous RNAi in C. elegans. Hence, some small RNAs are routed through membraneless organelles. To further our understanding of how small RNAs operate within membraneless organelles, we turn to the piRNA pathway.

piRNAs associate with the PIWI clade proteins in the Argonaute family to repress transposable elements (TEs). Mutations in the piRNA pathway lead to varying degrees of infertility, indicating it plays an essential role in the survival of a species. For example, null mutations in each of the three piwi-coding genes lead to sterility in male mice. Depletion of the single functional PIWI protein in C. elegans leads to reduced fecundity; in humans, a mutation blocking the ubiquitination of the PIWI protein HIW1 has been implicated in azoospermia.

Mature piRNAs mediate transcriptional and post-transcriptional gene silencing. In C. elegans, piRNAs are 21 nt long with a 5′ preference for U, and contain 2-O-methylation at the 3′ end. piRNAs associate with the PIWI protein PRG-1 to scan for target mRNAs. These target mRNAs then serve as templates for the production of endo-siRNAs that are 22 nt long with a 5′ preference for G. The RNA-dependent RNA polymerases (RdRPs) EGO-1 and RRF-1 are known to form endo-siRNA condensates entirely on their own. DEPS-1 and PRG-1 are essential endo-siRNA factors. While at lower resolution PRG-1 as well as DEPS-1 appear to be condensates that overlap with each other, these condensates can be further resolved to clusters of proteins at higher resolution in the pachytene region. DEPS-1 forms a protein complex with PRG-1 in P granules and Sterile-1 was among the ten most enriched factors. DEPS-1 is not predicted to possess such domains despite its requirement for P granule integrity in C. elegans.

Results

DEPS-1 and PRG-1 form intertwining clusters. To further study the interaction between PRG-1 and DEPS-1, we asked whether the proteins co-localise in vivo. First, we confirmed that colocalisation in P granules by co-immunostaining transgenic animals expressing GFP-DEPS-1 and GFP-PRG-1 (Fig. 1a). In the adult germline, both proteins colocalise to P granules from the mitotic zone to the pachytene region. In the distal loop region, where oogenesis begins and P granules start to disperse from the nuclear membrane, a higher proportion of GFP-DEPS-1 starts to dissociate from the perinuclear region than PRG-1, suggesting the proteins are differentially regulated during a small temporal window. Given that PRG-1 binds to piRNAs to trigger secondary endo-siRNA biogenesis, we asked if DEPS-1 complexes colocalise with the mutator foci, which are biomolecular condensates that house essential endo-siRNA factors. While at lower resolution PRG-1 as well as DEPS-1 appear to be condensates that overlap with each other, these condensates can be further resolved to clusters of proteins at higher resolution in the pachytene region. Often these DEPS-1 and PRG-1 clusters weave around each other to form elongated condensates. In contrast, MUT-16 condensates do not resolve to smaller clusters and only juxtaposed close to the DEPS-1/PRG-1 complex (Fig. 1b). Consistent with previous findings, we investigated how DEPS-1 and PRG-1 clusters are positioned relative to the P granule protein PGL-1 in the pachytene region. We found that PRG-1 and PGL-1 form intertwining clusters while DEPS-1 and PGL-1 clusters overlap with each other (Supplementary Fig. S1g). ZNFX-1 forms condensates closely apposed to PGL-1 and MUT-16. ZNFX-1 is also found in close proximity to DEPS-1 clusters, consistent with DEPS-1 colocalisation with PGL-1 (Supplementary Fig. S1h).

DEPS-1 binds to PRG-1 via its PIWI Binding Site (PBS). To investigate if the DEPS-1/PRG-1 interaction is direct and RNA-independent, we purified recombinant RNA-free full-length DEPS-1 and PRG-1 as MBP-fusion proteins and tested for binding using microscale thermophoresis (MST). DEPS-1 and PRG-1 interact with a sub-micromolar dissociation constant (Fig. 1c, d and Supplementary Fig. S2a).
**Fig. 1** DEPS-1 binds to PRG-1 and mediates piRNA-dependent transgene silencing. 

**a** DEPS-1 and PRG-1 colocalise as peri-nuclear granules. C. elegans germlines expressing GFP-DEPS-1 were dissected and immunostained for GFP and PRG-1. PRG-1 and DEPS-1 colocalise in the proliferative zone, transition zone, pachytene, oocytes and embryos. A higher proportion of PRG-1 remains perinuclear in the loop region compared to DEPS-1. Scale bar = 3 μm.

**b** Colocalisation of PRG-1, DEPS-1 and MUT-16. Dissected germlines co-stained for PRG-1, RFP-DEPS-1 and GFP-MUT-16 were imaged and deconvoluted with Hyvolution settings. Two clusters with all three proteins present are shown. Scale bar = 0.25 μm.

**c** Domain/fragment architecture of PRG-1 and DEPS-1. Sequence alignment (Clustal W) of Ago binding motif II on *Drosophila melanogaster* GW182 and DEPS-1 PBS with flanking sequences. 

**d** MST measurement of fluorescently labelled DEPS-1 PBS motif peptide was incubated with unlabelled MBP-tagged PRG-1 PIWI domain. $K_{d}$ is 1.9 µM ± 98 nM. Representative of $n$ = 2 independent experiments. Data are presented as mean values ± SD of 3 technical replicates. 

**e** Mutations in *deps-1* lead to piRNA sensor transgene desilencing. Whitefield (right panel) and fluorescent (left panel) images of whole mounted animals show the piRNA sensor is efficiently silenced in wild-type animals and is desilenced in *prg-1*(*n4357*), *deps-1*(bn121 and bn124) and *deps-1*ΔPBS::rfp(mj608) mutants. Scale bar = 3 μm.
We analysed the effect of the PRG-1/DEPS-1 (Supplementary Fig. S2d). PRG-1 PIWI binds to full length DEPS-1 with the PRG-1 PIWI domain to be responsible for binding to full-length DEPS-1 required for normal piRNA pathway activity.

To identify which region of DEPS-1 is required for binding to PRG-1, we truncated DEPS-1 into three fragments of similar sizes and with fragment boundaries in regions lacking predicted secondary structures (Fig. 1c). We detected binding between PRG-1 PIWI and the N-terminal fragment of DEPS-1 (DEPS-1 ΔPBS) only with a K$_d$ of 151 ± 28 nM (Supplementary Fig. S2e, f). This is again in agreement with the binding between the full-length proteins indicating that DEPS-1 interacts with its N-terminal region with PRG-1 PIWI domain.

Given that DEPS-1 interacts with PRG-1 via PRG-1 PIWI and that the PIWI domains of PIWI and Ago families share similar folds overall, we next asked if DEPS-1 shares any characteristics with known protein interactors of the Ago PIWI domain. The GW182 proteins have been shown to bind to the Ago PIWI domain by its multiple GW motifs which fit into tryptophan-binding pockets. While DEPS-1 lacks any GW motifs, we noticed a degree of similarity between two short stretches of DEPS-1 and the D. melanogaster GW182 in our alignment (GW182), one at the N-terminal and the other at the C-terminal of DEPS-1. While the C-terminal region with similarity to dmGW182 is the poly-serine tail, the N-terminal region of DEPS-1 shares similarity with dmGW192’s Ago-binding motif II36,40 (Fig. 1c). Moreover, this N-terminal region is contained within DEPS-1 Δfrags1 which binds to PRG-1 PIWI. We therefore generated a peptide for part of this sequence (DEPS-1 peptide; Fig. 1c) to test its binding with PRG-1 PIWI. DEPS-1 peptide binds to PRG-1 PIWI with a K$_d$ of 1.9 ± 0.1 μM indicating this Ago-binding motif II-like region of DEPS-1 is indeed responsible for PRG-1 interaction (Fig. 1e). We have termed this motif the PIWI-binding site (PBS).

Upon removal of the PBS, PRG-1 PIWI fails to bind to DEPS-1 Δfrags1 (Supplementary Fig. 2g).

DEPS-1 is required for piRNA-dependent silencing. Using the piRNA sensor, we next asked if the DEPS-1 functions in the piRNA pathway in vivo. The piRNA sensor is a genetic tool consisting of a GFP- or mcherry- tagged histone 2B (H2B) with a piRNA target site at its 3’ end, rendering its expression dependent on the piRNA pathway. We analysed the effect of the PRG-1/DEPS-1 binding by removing the PBS from endogenous DEPS and replacing it with a 5x glycine residue-linker via CRISPR-Cas9 gene editing (henceforth referred to as DEPS-1 ΔPBS) as well as two DEPS-1 null alleles (bn121 and bn124). Crossing DEPS-1 mutants with piRNA sensor animals, we found that the piRNA sensor is de-silenced in both DEPS-1 null mutants, as well as the DEPS-1 ΔPBS mutant, as in the prg-1(n4357) mutant, indicating that DEPS-1 and specifically its PBS is required for piRNA-dependent silencing (Fig. 1f, Supplementary Fig. S3a). Correspondingly, small RNAs targeting the piRNA sensor are reduced in DEPS-1 Δnull mutants (Supplementary Fig. S3b). Hence, DEPS-1 binding to PRG-1 is required for normal piRNA pathway activity.

Having seen that DEPS-1 ΔPBS has a similar effect on piRNA function as DEPS-1 null mutation, we tested if DEPS-1 ΔPBS is also resistant to germline RNAi as observed in the DEPS-1 Δnull mutants.

PRG-1 condensate organisation needs PRG-1 and DEPS-1 binding. Having identified the PRG-1 binding site on DEPS-1 and shown that it is required for piRNA-dependent silencing, we asked how the removal of this site affects DEPS-1 localisation. Live imaging of GFP-DEPS-1 ΔPBS expressing animals in the pachytene region revealed that in the absence of PBS, DEPS-1 becomes diffused in the cytoplasm and forms fewer granules (Fig. 2a, Supplementary Fig. S3d; expression of GFP-DEPS-1 ΔPBS is at ~70% of the wild-type protein). We imaged the condensates at a high resolution to inspect how the DEPS-1 and PRG-1 cluster organisation is affected. As shown before wild-type DEPS-1 and PRG-1 clusters intertwine each other to form elongated condensates. While DEPS-1 ΔPBS localises to PRG-1 condensates when DEPS-1 is able to associate with the peri-nuclear region, PRG-1 condensates contain either very little or no DEPS-1 ΔPBS (Supplementary Fig. S3e). Furthermore, PRG-1 clusters do not intertwine with DEPS-1 ΔPBS. We measured the length of the PRG-1 condensates along the peri-nuclear edge and found that PRG-1 condensates (with and without DEPS-1 ΔPBS in deps-1 ΔPBS and deps-1 null null) become more compacted compared with the PRG-1/DEPS-1 WT elongated condensates (Fig. 2b, Supplementary Fig. S3f, g). Hence, the peri-nuclear organisation of PRG-1 clusters is maintained by its direct interaction with DEPS-1.

PRG-1 and DEPS-1 intrinsically form clusters in vitro. We wondered if the ability to form these small clusters of DEPS-1 and PRG-1 is intrinsic to these proteins. We fluorescently labelled recombinant MBP-tagged DEPS-1 and PRG-1 full length proteins for high-resolution confocal imaging. We found that PRG-1, but not DEPS-1, was able to form small clusters (Fig. 2c). To mimic the crowded environment of the P granule, we incubated the proteins with 5% PEG2000. In the presence of the molecular crowding agent, DEPS-1 are able to form clusters. Furthermore, these in vitro clusters are of similar size to the in vivo clusters (~250 nm in diameter). This suggests the formation of these sub-organelle clusters are intrinsic to the protein sequences of DEPS-1 and PRG-1. We incubated the two proteins together in the presence of PEG2000 to see if they form intertwining clusters similar to those observed in vivo. Indeed, the DEPS-1 and PRG-1 clusters associate with each other (Fig. 2d, Supplementary Fig. S3h). However, they do not form the elongated structures observed in vivo, indicating the elongation is dependent on interactions with other intracellular components. Finally, the association between DEPS-1 and PRG-1 clusters can be disrupted by the presence of DEPS-1 ΔPeptide but not by the addition of an unrelated, human PGFR2 peptide, which is unable to compete with DEPS-1 for binding to PRG-1 (Fig. 2d). Hence, DEPS-1 and PRG-1 clusters association requires the PBS motif on DEPS-1.

EDG-1 binds DEPS-1 and PRG-1 and modifies DEPS-1 condensates. Despite the uncoupling of DEPS-1 from PRG-1 by the deletion of PBS, DEPS-1 ΔPBS and PRG-1 remain in the same P granules at a low level. We therefore wondered if other proteins form a complex with them. We performed a yeast-two-hybrid (Y2H) screen using full length DEPS-1 as a bait. We obtained one high-confidence candidate - the putative protein encoded by B0035.6. Y2H data indicated that B0035.6 interacts with DEPS-1 via its C-terminal region (Supplementary Fig. S4a). B0035.6 has no predicted conserved domain structures but a low similarity to human MEG-3. B0035.6 was also one of the significant interactors of PRG-1 in our proteomic analysis (Supplementary Data 1).
Fig. 2 PRG-1 and DEPS-1 intertwining organisation is dependent on PBS on DEPS-1. a Live worm imaging of the germline of wild-type animals (N2) (left), animals expressing wild-type GFP-DEPS-1 (ax2063; GFP-DEPS-1WT, middle) or GFP-DEPS-1 with mutated PBS (mj608; GFP-DEPS-1ΔPBS, right). GFP-DEPS-1ΔPBS forms fewer granules and instead is diffused in the cytoplasm compared with GFP-DEPS-1WT. Scale bar = 3 µm. b DEPS-1 and PRG-1 condensates are malformed in gfp::deps-1ΔPBS(mj608) mutant. Dissected germlines co-stained for PRG-1 and GFP-DEPS-1 were imaged and deconvoluted with Hyvolution settings. Two selected clusters of each genotype were enlarged to show differences between the wild-type and PBS mutant form of GFP-DEPS-1. Top panels: gfp::deps-1WT(ax2063); Bottom panels: gfp::deps-1ΔPBS(mj608). The length of PRG-1 condensate along the perinuclear membrane was measured manually. Bar graph represents twenty PRG-1 condensates measured in two germlines (total n = 40 for each genotype). Scale bar = 0.25 µm. Data represented here as mean values ± SD. Kolmogorov–Smirnov test was performed, **p value < 0.005. Source data are provided as a Source Data file.

c Recombinant DEPS-1 and PRG-1 form small clusters similar to in vivo proteins. 1.7 µM MBP-tagged DEPS-1 (top panel) and 0.6 µM MBP-tagged PRG-1 were labelled with Atto-488 or −594, respectively. While DEPS-1 was only able to form small protein clusters in the presence of 5% PEG2000, PRG-1 formed clusters even in absence of PEG2000. Scale bar = 0.5 µm. d Intertwining clusters of recombinant DEPS-1 and PRG-1 is dependent on the PBS motif on DEPS-1. 3.4 µM Atto-488 labelled MBP-tagged DEPS-1 was incubated with 0.6 µM Atto-594 labelled MBP-tagged PRG-1 in the presence of 5% PEG2000. The two protein clusters associate with each other. When PRG-1 was preincubated with 2 mM of a peptide containing the DEPS-1 PBS motif, the two proteins failed to co-localise. An unrelated peptide (2 mM FGFR2 peptide) does not disrupt DEPS-1 and PRG-1 clusters association. Scale bar = 5 µm. e edg-1 was knocked down via RNAi in animals expressing deps-1::gfp(ax2063). Animals were dissected for germline staining of GFP in the pachytene region. DEPS-1 forms brighter granules upon edg-1 knockdown. Scale bar = 3 µm. f DEPS-1 condensates are more intense upon RNAi knockdown of edg-1. n = 10 per genotype. Data represented here as mean values ± SD. Two-sided t-tests were performed (**p value < 0.005). Source data are provided as a Source Data file.
and Supplementary Fig. S4b), suggesting DEPS-1, B0035.6 and PRG-1 form a trimeric complex. We then performed RNAi knock-down of B0035.6 and tested if it is required for the normal formation of DEPS-1 or PRG-1 condensates. Reducing B0035.6 expression level lead to enlarged DEPS-1 condensates (Fig. 2e, f), but not PRG-1 condensates nor the P granule protein PGL-1 (Supplementary Fig. S4c). We therefore named B0035.6 as Enlarged Deps Granules-1 (edg-1). Given the dependence of the piRNA pathway on the mutator foci, we tested if MUT-16 condensation was affected and found that they are not (Supplementary Fig. S4c). Hence, while edg-1 is found to be an interactor for both DEPS-1 and PRG-1, it specifically modulates DEPS-1 condensation. However, it is unclear whether the change in DEPS-1 condensate mediated by edg-1 knockdown is due to a change in DEPS-1 protein level.

DEPS-1, PRG-1 and mutator condensates are interdependent. piRNA function requires protein factors in the P granule as well as the mutator foci; we investigated how DEPS-1, PRG-1 and MUT-16 condensates are affected by mutations in deps-1, prg-1 and mutator genes. In the pachytene region, deps-1Δ and deps-1ΔPBS mutations lead to PRG-1 condensates becoming brighter as reflected by higher condensate intensity, suggesting either more proteins are present in the condensates or PRG-1 becomes more densely packed (Fig. 3a, d and Table 1). Since deps-1 mutations have been shown to alter the levels of the mRNA and proteins of P granule factors35, we investigated if deps-1 mutations also affect prg-1. No significant differences in either mRNA or protein products of prg-1 in deps-1 mutants were detected (Supplementary Fig. S5a, b, c). Hence, the effects of deps-1 on PRG-1 condensate are solely in the subcellular distribution of the protein.

**Fig. 3 Morphologies of PRG-1, DEPS-1 and MUT-16 condensates are interdependent.** a GFP-MUT-16 and PRG-1 localisations were examined in prg-1 (n4357), deps-1Δm(bn121) and deps-1ΔPBS(mj605) mutants. The common genotypes of strains used are mut-16(pk710); gfp::mut-16(mgSi2) which is denoted as ‘wild-type’. Additional mutations upon this common genotype are indicated on the left. C. elegans germlines were dissected and immunostained for GFP and PRG-1. Scale bar = 3 µm. b GFP-DEPS-1 and PRG-1 localisations were examined in prg-1(n4357), mut-16(pk710), mut-2(ne298) and mut-15(tm1358) mutants. ‘wild-type’ indicates the common genotype of gfp::deps-1(ax2063) among the strains used and additional mutations are indicated on the left. C. elegans germlines were dissected and immunostained for GFP and PRG-1. Scale bar = 3 µm. c Fold change in DEPS-1 condensate density in wild-type (n = 41), prg-1 (n = 13), mut-16 (n = 11), mut-2 (n = 7), mut-15 (n = 9) animals. d Fold change in PRG-1 condensate density in wild-type (n = 37), mut-15 (n = 9), mut-16 (n = 11), deps-1Δm (n = 12), deps-1ΔPBS (n = 3), mut-2 (n = 7) animals. e Fold change in MUT-16 condensate density in wild-type (n = 25), prg-1 (n = 5), deps-1Δm (n = 9), deps-1ΔPBS (n = 5) animals. c-e Data are presented as mean values ± SD. Source data are provided as a Source Data file.
### Table 1 Condensate defects in mutator

| Relevant mutation | Control strain genotype (Fig. 3a and Table 1) | Test strain genotype (Fig. 3a, bottom panel and Table 1) | Intensity | Area | Circularity |
|-------------------|-----------------------------------------------|--------------------------------------------------------|-----------|-------|-------------|
| mut-16            | deps-1null(bn124)                             | mutants (31 of 2012 genes)                              | +         | +     | +           |
| mut-2             | mutants (447 genes)                            | mutants (447 out of 2012 genes)                         | +         | +     | +           |
| mut-16            | deps-1null(bn124)                             | mutants (447 out of 2012 genes)                         | +         | +     | +           |
| mut-2             | mutants (761 out of 2012 genes)                | mutants (761 out of 2012 genes)                         | +         | +     | +           |
| PBS               | mutants (704 genes; Hypergeometric Test: p < 10^{-29}) | mutants (704 genes; Hypergeometric Test: p < 10^{-29}) | +         | +     | +           |

Intriguingly, *deps-1* mutants contain fewer and brighter MUT-16 condensates despite DEPS-1 being a P granule protein (Fig. 3a, Table 1 and Supplementary Fig. S5d). However, whether the change in MUT-16 condensates is due to protein expression level or localisation defects remains to be determined.

**Table 1** Condensate defects in *mutator* strain genotypes. Two-sided *t*-tests were performed unless marked with * for which Kolmogorov Smirnov tests were performed.

**deps-1** is required for 22Gs against some piRNA targets. Having observed that *deps-1* mutations desilence the piRNA sensor, we analysed the small RNA populations in *deps-1* mutants. We first examined the effects of the *deps-1null* mutant on piRNA abundance. We sequenced the small RNA population from 5′- independent libraries and show that the *deps-1* mutant has a comparable level of 21U population as in wild-type animals (Fig. 4a). We then examined the abundance of secondary siRNAs (22Gs). We found 2012 genes with over 50 reads per million on average across all samples. We observed a significant overlap of 259 genes with greater than twofold reduction in 22Gs levels in *deps-1* mutants (447 genes) and *prg-1* mutants (704 genes; Hypergeometric Test: p < 10^{-29}; Supplementary Fig. 6a). In addition, we examined the effect of *deps-1* on a published list of piRNA targets, of which 173 exceeded the 50 reads per million threshold, and observed an enrichment for piRNA targets having a > twofold reduction in 22Gs levels (106 out of 447 genes) in *deps-1* mutants (Hypergeometric Test: p < 10^{-30}; Fig. 4b and Supplementary Fig. 6b). This indicates that *deps-1* is required for the accumulation of 22Gs on a subset of *prg-1* targets.

**deps-1** functions in multiple small RNA pathways. We next asked if other small RNA populations are affected in *deps-1* mutants. We observed an overlap of 428 genes between the genes that show greater than twofold reduction in secondary siRNA levels in *deps-1* mutants (447 out of 2012 genes) and *mut-16* mutants (761 out of 2012 genes; Hypergeometric Test: p < 10^{-194}; Supplementary Fig. 6a). Similarly, we found 31 repetitive elements with greater than twofold reduction in *deps-1* mutants (31 out of 2012 genes) which also show greater than twofold reduction in *mut-16* mutants (64 out of 2012 genes; Hypergeometric Test: p < 10^{-30}; Supplementary Fig. 6a).
Fig. 4 deps-1 regulates 22Gs against piRNA targets and other endo-siRNAs. a Small RNAs were sequenced in animals containing piRNA sensor (mjIs144; denoted as wild-type) alone, or in the presence of deps-1(bn124) or prg-1(n3457) mutations. deps-1 mutant expresses similar level of 21Us as in wild-type animals, whereas 21Us in prg-1(n3457) mutant is significantly diminished compared with wild type and deps-1 mutant (one-sided t-test, p value < 10^{-10}). n = 2 biologically independent samples. Centre line indicates the median, outer boxes represent the 25th and 75th percentiles, whiskers indicate the distance 1.5 times distance between the 25th and 75th percentiles or are limited to the most extreme observation. Outliers are marked if they are greater or less than the whiskers. Cluster analysis of 5 small RNA pathways: piRNA targets (Fig. 4a), repetitive elements (Fig. 4b), and wago targets (Fig. 4d), in the indicated mutants compared to wild type. Wild-type denotes animals expressing piRNA sensor (mjIs144); deps-1 mutant (one-sided t-test, p value < 10^{-10}); mut-16 target, mRNAs of 13 genes are significantly downregulated, whereas 32 genes are significantly upregulated (mutant/wild-type) 0.58; One-sample t-test, p value < 10^{-10}; Supplementary Fig. 6g). In addition, we found in general when small RNAs are decreased, their target mRNAs are more likely to be downregulated (R^2 = 0.58; One-sample t-test p < 0.01; Fig. 5a) in deps-1 null mutant and vice versa (One-sample t-test p < 0.1), suggesting the previously described effects on mRNA are mediated by the perturbations in small RNA populations. It remains to be determined which Argonuates are responsible for the changes in

Small RNAs target P granule-associated genes. Having observed changes in secondary endo-siRNAs in deps-1 mutants, we asked whether changes in small RNA in our data correlate with previously published changes in mRNA expression.

Test: p < 10^{-8}; Supplementary Fig. 6c). deps-1 also affects wago targets (30 out of 425 wago targets show > twofold reduction in 22Gs level in deps-1 mutant; Hypergeometric Test: p < 10^{-8}; Supplementary Fig. 6c). However, unlike mut-16 in which 22 out of 23 ergo-1 targeting 22Gs have > twofold reduction (Hypergeometric Test: p < 10^{-8}), deps-1 has limited effects on ergo-1 targets (7 out of 23 genes; Hypergeometric Test: p < 0.2; Supplementary Fig. 6e). Lastly, deps-1 does not affect the 22Gs of csr-1 targets because we observed a significant reduction in the number of overlapping genes between the csr-1 targets (4 out of 162 genes) and genes with > twofold reduction in secondary siRNA levels in deps-1 mutants (447 out of 2012 genes; Hypergeometric Test: p < 10^{-13}; Supplementary Fig. 6f). Therefore deps-1 functions in multiple (but not all) germline small RNA pathways which suggests the possibility that deps-1 might interact with other Ago proteins.
mRNAs and 22Gs given *deps-1* is involved in multiple small RNA pathways.

As most germline-expressed genes are targeted by endo-siRNA, it follows that P granule proteins may also be targeted by endo-siRNAs (8986 genes out of 11,088 genes expressed in germline are targeted by endo-siRNA). We obtained a list of P granule factors from AmiGO under the GO term 'P granule' and manually curated the list to remove protein isoforms of the same gene. Comparing the P granule list with known endo-siRNA targets of various small RNA pathways, we observed that 55 out of 63 P granule factors are endo-siRNA targets (Fig. 5a and Supplementary Data 2) and that 10 out of 63 P granule factors are whose 22Gs are differentially regulated in *deps-1* mutant (Fig. 5b).

As expected, P granule factors are not more likely to be targeted by endo-siRNA than other germline expressed genes in general (Hypergeometric Test: $p < 0.5$) or affected in *deps-1* mutant (Hypergeometric Test: $p < 0.1$). Spike et al. show that RDE-4, a protein essential for RNAi, is downregulated in *deps-1* null mutant which is likely the cause of *deps-1* mutants being RNAi resistant. Hence, some of the effect of *deps-1* mutations on small RNA functions could be indirect and that *deps-1* has a regulatory role on proteins with a direct role in these small RNA pathways.

**Discussion**

Small RNA pathways associate with membraneless organelles. Here we reveal a role for the P granule factor DEPS-1 in the piRNA pathway, functioning as a link between piRNAs and...
secondary endo-siRNAs. Specifically, DEPS-1 directly binds PRG-1 through a conserved PIWI binding site (PBS) regulating 22Gs homeostasis. Furthermore, the direct interaction with DEPS-1 is important for the organisation of PRG-1 sub-organellar clusters within the P granule (Fig. 5c).

It has long been known that various small RNA machineries, such as Argonaute proteins, localise to P granules. However, it is only recently that a role for the P granule in ensuring appropriate small RNA-mediated silencing has been shown. Using mutant animals that lack P granules in the C. elegans primordial germ cells, these studies elegantly show that P granules are required for transgenerational inheritance of RNAi44,50,51. The direct interaction between PRG-1 and DEPS-1 provides an example for how small RNAs and P granules can be biochemically coupled.

The piRNA pathway is dependent on protein factors both in P granules and mutator foci. Despite PRG-1 forming aberrant condensates in the deps-1 null mutant, the piRNA population is normal in these animals suggesting that 21Us are still able to associate with PRG-124,26. In contrast, deps-1 mutations reduce endo-siRNAs of piRNAs origin and lead to brighter downstream of normal level of 22Gs, but not 21Us, against piRNA targets places staining intensity55; differences in translational activity between rearrangements as well as exchange of materials with their surfaceless organelles is thought to facilitate dynamic internal homoeostasis. Furthermore, the direct interaction with DEPS-1 is critical in fine-tuning multiple piRNA and endo-siRNA pathways merits further study.

We have identified EDG-1 as an interacting partner of DEPS-1 and PRG-1. Interestingly, knockdown of edg-1 specifically affects DEPS-1 condensates and not PRG-1 or the constitutive P granule protein PGL-1. Whether and how edg-1 regulate small RNA pathways remain to be determined. Indeed, whether EDG-1 is localised to P granules requires further investigation.

Percinuclear germ granules are conserved features throughout the animal kingdom, and are sites of RNA metabolism and RNA-mediated gene regulation.9,33. The liquid-like property of membranless organelles is thought to facilitate dynamic internal rearrangements as well as exchange of materials with their surroundings. Recently, a non-dynamic, gel-like scaffold has been found to envelope the liquid-core of P granules54; under electron microscopy, the crest and the base of P granules show distinct staining intensity; differences in translational activity between the periphery and the core of the P-bodies have been observed in Drosophila oocytes56. These suggest subdomains exist within membranless organelles to support or as a result of their functional complexities. We observe here that PRG-1 and DEPS-1 condensates are formed from smaller clusters of proteins that intertwine. Whether this organisation of PRG-1 and DEPS-1 substructures within the P granule is the result or reflective of the piRNA pathway activity is unclear. In this respect, Wan et al. show that ZNFX-1 forms a condensate that areas appose to both the P granules and Mutator foci14, while Ishidate et al. demonstrated that ZNFX-1 interacts with Ago proteins and promotes the Mutator foci machinery to position to the 3' end of mRNAs12. Together, their studies provide evidence that there is a correlation between zones of intense molecular activities and protein localisation.

While much focus has been placed on how proteins drive phase-transition in RNP foci formation, a flurry of recent studies investigated the importance of RNAs in the formation of RNP foci. Langdon et al. show that the secondary structure of mRNAs plays essential roles in specifying distinct Whi3-containing RNPs57. Furthermore, RNA:protein ratios determine phase-transition events in proteins prone to solid aggregation58. Given that a myriad of small RNA pathways are routed through the P granules and mutator foci in C. elegans25, it will be important to decipher how the various RNA species contribute to the formation of these organelles.

**Methods**

**Immunoprecipitation for mass spectrometry.** Synchronised wild-type N2 and prg-1(n4357) animals were grown to 1 day-old adults at 20 °C on HB101. After use, animals thoroughly to remove debris. Animals were resuspended in lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% NP-40) and snap frozen in liquid nitrogen. The samples were then lysed by bead-beating, followed by centrifugation at 4 °C to remove insoluble debris. Anti-PRG-1 antibody (Custom) or rabbit IgG was pre-coupled to protein A/G matrix (Thermo Scientific). Samples were incubated with the superimposed of worm lysates in B2.1 (DE3) at 37 °C. Briefly, 10 mL PRG-1 with N2 lysates, three biological replicates of anti-PRG-1 with prg-1(n4357) lysates and three biological replicates of anti-IgG with N2 lysates). The immunoprecipitates were then washed with 3 × 1 ml of lysis buffer and eluted in elution buffer (8 M urea, 10 mM HEPES pH 8.0) with shaking at room temperature for 30 min.

**LS-MS/MS.** Briefly, proteins eluted from immunoprecipitations were reduced and alkylated. Quantified proteins were then digested consecutively in solution using Lys-C and trypsin (both at 1:50 enzyme:substrate ratio)59. Peptides were desalted, dried and re-dissolved in 5% acetic acid. RPLC was performed using a Dionex RSLC nano HPLC (Thermo Scientific). Peptides were injected onto a 75 μm × 2 cm PepMap-C18 pre-column and resolved on a 75 μm × 50 cm RP-C18 EASY-Spray temperature-controlled integrated column-emitter (Thermo Scientific) using a 4-h multistep gradient from 5% B to 35% B with a constant flow of 200 nl min⁻¹ as described previously. The mobile phases were: 2% acetonitrile (ACN) incorporating 0.1% formic acid (FA) (solvent A) and 80% ACN incorporating 0.1% FA (solvent B). The spray was initiated by applying 2.5 kV to the EASY-Spray emitter and the data were acquired on a Q-Exactive Orbitrap plus (Thermo Scientific) under the control of Xcalibur software in a data-dependent mode selecting the 15 most intense ions for HCD-MS/MS. The survey scan was acquired over an m/z range of 350–1600 with a 70,000 resolution, AGC target of 1E6 ions and a maximum IT of 20 ms. The subsequent MS2 scans were acquired over an m/z range of 200–200 m/z at 17.500 resolution, an AGC target of 1E5 and 60 ms maximum IT. Peptide ions were isolated with 1.4 Th precursor ion isolation window and fragmented using HCD with normalised collision energy (NCE) of 27. Data have been deposited in PRIDE (project accession code: PXD016838).

**Mass spectrometry data analysis.** Raw MS data processed by MaxQuant51. iBAQ values were divided by the total sum of intensity of each sample52. These normalised values were then log₂ transformed to obtain normality and the resulting values were used for student's t-test. To identify proteins enriched in immunoprecipitated PRG-1 from wild-type animals, the medians of the transformed values were used for fold-change calculations.

**Molecular cloning and recombinant protein expression.** All PRG-1 and DEPS-1 constructs were cloned using restriction enzymes into the phmAL-CSX vector. Recombinant proteins were expressed in BL21 (DE3) at 37 °C. The subsequent MS2 scans were acquired over an m/z range of 350–1600 with a 70,000 resolution, AGC target of 1E6 ions and a maximum IT of 20 ms. The subsequent MS2 scans were acquired over an m/z range of 200–200 m/z at 17.500 resolution, an AGC target of 1E5 and 60 ms maximum IT. Peptide ions were isolated with 1.4 Th precursor ion isolation window and fragmented using HCD with normalised collision energy (NCE) of 27. Data have been deposited in PRIDE (project accession code: PXD016838).

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Protein labelling. 100 μM of purified proteins were incubated with 1 mM Atto fluor dye for 2 h in the dark. Free dye was subsequently removed from labelled proteins using a 25-25 desalting column.

Peptide sequences. DPES-1 peptide: IPLKKFGEVILVNISEDCHDHK FGR2 peptide: PDFFSQPAPVHKLTKRIP

Microscale thermophoresis (MST). Proteins/peptides were labelled with atto-488 NHS ester (Sigma, 41698). Free dye was separated from labelled peptide using G25 desalting columns. Unlabelled proteins were serial diluted 1:2 and incubated with a constant amount of labelled protein. MST assays were carried in an instrument supplemented with 0.01% NP-40. Fluorescence was monitored throughout the assay (5 s laser off, 30 s laser on, 5 s laser off). The apparent dissociation constant (K_D) was calculated by the law of mass action using data from either thermophoresis or thermophoresis with temperature jump.

Small RNA library preparation. Synchronised animals were grown to 1-day-old adults 20 °C. After being washed with M9 to remove bacteria, animals were resuspended in TBSure (Bioline, BIO-38033). Animals were lysed with 5× freeze-thaw cycles in liquid nitrogen. Total RNA was isolated by chloroform extraction. For 5′-independent libraries, 5 μg of total RNA was treated with 5′ polyphosphatease (Epicerin, RP0902H). Small RNAs were indexed using the TruSeq small RNA sample kit (Illumina). and size selected by gel separation in 6% TBE gels (Life Tech) and subsequently purified.

Small RNA analysis. Small RNA sequencing results were obtained from https://basespace.illumina.com/ as fastq files after demultiplexing. Sequencing data is available in the European Nucleotide Archive under study accession number PRJEB31348 (Table Data 4). 3′ Adapter, reads below 18 nt length and reads with a length of 50 or 42 nt were removed using cutadapt. Remaining reads were performed on normally distributed data. Kolmogorov-Smirnov tests, a non-parametric test used on data not normally distributed (which is mostly MUT-16 quantification). For fold change calculations, average intensity of all controls of one experiment was obtained and used to calculate the fold change of the individual mutant germline within the same experiment.

Western blotting. Proteins from 75–150 μg of worm lysates were resolved by SDS-PAGE and transferred onto PVDF membrane. Antibodies used: Antibodies used: anti-PRC-1 (Custom, 1:1000); anti-tubulin (Sigma, DM1A; 1:1000); anti-DEPS-1 (custom, kind gifts of Strome lab, 1:50).

RNAi. Adult animals were bleached to obtain embryos which then hatched and synchronised in M9 for 24–48 h at 20 °C. L1 animals were fed with bacteria expressing control dsRNA or dsRNA against edg-1. 1-day old adults were subsequently dissected for germline imaging.

General animal maintenance. Animals were fed with HB101 and maintained at 20 °C (unless stated otherwise) on NGM plates. Strains used in this study are listed in Supplementary Data 3.

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Author contributions

Conceptualisation, E.A.M., A.A. and K.M.S.; experiments, K.M.S., F.B., D.B., A.A., C.C.L., D.M., A.S. and N.D.; image analysis tool development R.B.; bioinformatics, F.B.; data analysis, K.M.S. and F.B.; supervision, E.A.M., A.L., J.E.L. and K.M.S.; funding acquisition, E.A.M. and J.E.L.; writing—original draft, K.M.S., F.B. and E.A.M.; writing—review and editing, K.M.S., F.B., A.A. and E.A.M.

Competing interests

The authors declare no competing interests.

Additional information

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