RNA-silencing induces target gene relocation toward a specialized nuage domain

Yuchen Yang¹, David Grunwald¹, James R. Priess^{2,3} and Craig C. Mello^{1,4,5*}

Author Affiliations:

1. RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA 01605

2. Fred Hutchinson Cancer Research Center, Seattle, Washington, United States

3. Department of Biology, University of Washington, Seattle, Washington, United States

4. Program in Molecular Medicine, University of Massachusetts Medical School, 368 Plantation Street, Worcester, MA, 01605 United States

5. Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA, USA

* Correspondence, Craig.Mello@umassmed.edu.

Abstract

Argonaute small RNA pathways maintain genome integrity and fertility by enforcing the transgenerational silencing of transposons as well as many developmentally regulated germline genes. To propagate silencing, Argonaute pathways coordinate heterochromatin silencing with cycles of small RNA amplification. In animal germlines, mRNA surveillance is thought to occur within cytoplasmic perinuclear domains called nuage. In *C. elegans*, nuage granules called P granules surround each pachytene germline nucleus. P granules are known to host many of the Argonaute small RNA systems that carry out transcriptome surveillance, but what if any specific roles P granules might play in Argonaute silencing have remained mysterious. Here we show that RNAi triggers the enlargement of a single P granule, which uniquely accumulates target RNA. P granule enlargement occurs independently of, and precedes transcriptional silencing, which culminates in the relocalization of both alleles of the target gene to the inner nuclear membrane (INM) directly adjacent the specialized P granule. Similarly, we show that piRNA-mediated silencing, causes both alleles of a targeted gene to reside near the INM adjacent to a P granule that uniquely contains target RNA sequences. Our findings suggest that nuage and its associated Argonaute pathways re-organize the three dimensional location of their genomic targets to facilitate RNA-guided inheritance.

Introduction

In the *C. elegans* germline, two major Argonaute pathways initiated by different mechanisms—the piRNA pathway (Watanabe and Lin, 2014) mediated by the Piwi Argonaute PRG-1 and the dsRNA-initiated RNAi pathway (Fire et al., 1998) mediated by the Argonaute RDE-1 (Chen et al., 2005)—rely on a common set of conserved downstream small RNA and chromatin effectors that maintain heritable silencing (Billi et al., 2014; Holoch and Moazed, 2015; Weiser and Kim, 2019). Argonaute proteins and the machinery that produces their small RNA cofactors localize to P granules (Updike and Strome, 2010), where they are thought to act in mRNA surveillance and in the amplification of small-RNA silencing signals (Billi et al., 2014; Weiser and Kim, 2019). In the *C. elegans* meiotic germline, each P granule is localized over a cluster of nuclear pores (Pitt et al., 2000), where it has the potential to intercept nascent mRNA *en route* to the cytoplasm (Sheth et al., 2010). Although previous cytological studies demonstrated the rapid loss of target mRNA in the cytoplasm during RNAi (Fire et al., 1998; Sheth et al., 2010), changes in the P granules or associated RNAs during silencing were not reported and the localization of target DNA was not examined. Thus, the dynamics and spatial relationships between nuage and the mRNA and DNA targets of silencing have remained mysterious.

Recent advances in RNA and DNA FISH technology have greatly enhanced the ability to track molecules at single-molecule resolution (Beliveau et al., 2015; Fields et al., 2019; Ji and van Oudenaarden, 2012). For example, splinted fluorescent probes that assemble multiple fluorophores at a single target greatly amplify DNA FISH signals and permit detection of single genes (Beliveau et al., 2015; Fields et al., 2019). Here we utilize

these improved reagents to revisit the cytological events associated with Argonaute silencing.

Results and Discussion

To visualize the spatial and temporal response to RNAi, we exposed worms to dsRNA targeting an abundantly transcribed germline-expressed gene, *oma-1*, and monitored *oma-1* RNA- and DNA-FISH signals simultaneously over a time course of silencing. To distinguish cytoplasmic and nuclear signals in RNA + DNA FISH experiments, we marked the outer membrane of the nuclear envelope with a GFP::ZYG-12 fusion protein (Malone et al., 2003) (Figure 1A-1F), while P granules were identified with GFP::GLH-1 (Figure 1G-1L) tagged at the endogenous *glh-1* locus by CRISPR genome editing (Ghanta et al., 2020). We examined the pachytene region of the gonad, where germ cells are connected to a large, shared region of cytoplasm called the core. As expected from previous studies, *oma-1* mRNA in untreated animals was present at low levels in many P granules, and was abundant in the germ cell cytoplasm and gonad core (Figure 1J). A double dot of *oma-1* RNA signal was visible inside each germ nucleus throughout the pachytene region (Figure 1A, 1D and 1J). The nuclear RNA dots co-localized with *oma-1* DNA in simultaneous RNA/DNA FISH experiments (Figure 1A), suggesting that they represent transcription from the *oma-1* genes on the paired, homologous chromosomes.

As expected, RNAi induced a rapid, marked depletion of cytoplasmic *oma-1* mRNA (for example, compare Figure 1A to 1B). In the same gonads, however, an *oma-1* RNA signal appeared to increase within a single large, perinuclear focus adjacent to each

nucleus (Figure 1B and 1E). This perinuclear RNA signal was also present at 21 hours but was decreased in size (Figure 1C and 1F).

Prior to RNAi, the P granules varied in size (green signal in Figures 1G and 1J). However, after 6 hours of RNAi, one P granule on each nucleus appeared much larger than neighboring nuage (Figure 1H), and colocalized with the prominent, enlarged *oma-1* RNA focus (Figure 1K). Thus, RNAi appears to trigger differentiation between the P granules on the same nucleus, with target RNA accumulating in only one enlarged P granule. These results suggest that a single P granule becomes specialized for *oma-1* silencing.

The location of the *oma-1* RNA and DNA FISH signals within the nucleus exhibited dramatic changes during RNAi. Whereas prior to RNAi each pachytene nucleus exhibited two paired foci of DNA and the coincident RNA (Figure 1A), by 21 hours nuclei exhibited only a single focus of *oma-1* DNA and a coincident RNA signal (Figure 1C). These coincident signals were located near the INM (Figure 1F) and directly adjacent to the specialized P granule which, although now reduced in size, remained the only P granule that contained *oma-1* RNA (Figure 1I and 1L). The transition to a single focus of *oma-1* DNA and coincident RNA was evident in many nuclei by 6 hrs of *oma-1*(RNAi) (Figure 1B, inset ii), and when present this singular focus was always positioned near the INM adjacent the specialized P granule (Figure 1K, insets i and ii). Taken together these results suggest that RNAi causes the formation of a unique enlarged P granule where target RNA accumulates, and that formation of this specialized P granule precedes and possibly coordinates movement of the target DNA to the directly adjacent INM.

In order to determine whether genes in the RNAi pathway are required for the specialized P granule, we repeated the above assays in the mutant *rde-3*. RDE-3 is an effector of RNAi silencing that is required both in the animals exposed to dsRNA and for inherited silencing among their offspring (Chen et al., 2005). As expected, in *rde-3* mutants *oma-1* dsRNA did not trigger any of the cytological changes observed in wild-type worms (compare Figures 2A and 2B with Figures 2C and 2D, respectively). For example, the double dots of *oma-1* DNA did not relocalize toward the INM, low levels of *oma-1* RNA remained visible in many P granules, which appeared similar in size to those in untreated controls, and cytoplasmic *oma-1* mRNA remained abundant in the gonad core (Figure 2A and 2B and Supplemental Figure 2).

Nuclear effectors of RNAi are known to drive heterochromatin formation and several studies have linked heterochromatin formation to the localization of a silent gene near the INM (Reviewed in (Ahringer and Gasser, 2018)). We therefore wondered whether the nuclear effectors of the RNAi pathway are required for relocalization of the target genes, and whether targeting events at nuclear transcription sites might precede and drive P granule specialization. To explore these possibilities we examined the role of two genes, the nuclear WAGO Argonaute, *wago-9/hrde-1* and its downstream effector *nrde-2*. These genes are both required for establishment of heterochromatin at the target locus and for transgenerational inheritance of silencing induced by RNAi (Ashe et al., 2012; Buckley et al., 2012; Burton et al., 2011; Guang et al., 2010; Shirayama et al., 2012). Importantly, neither gene is essential for RNAi when a dsRNA trigger is present, but rather are only required for transmission of silencing to unexposed offspring. dsRNA triggered the loss of cytoplasmic *oma-1* mRNA in both mutants, similar to wild type, and the

accumulation of *oma-1* RNA in a single, enlarged P granule (Compare Figures 2E and 2G with Figure 2C). However, the nuclei retained two separate foci of DNA and coincident RNA that did not relocalize toward the INM even after 21 hours of RNAi (compare Figure 2F and 2H to Figure 2D). Moreover, by 21 hrs, the unique enlarged P granule had disappeared and *oma-1* RNA was detected in multiple smaller P granules (Fig 2F and 2H). Thus the nuclear events of RNAi are not required for the initial formation of the specialized P granule, but instead function to promote its maintenance and to coordinate the nuclear relocalization of the target genes into its proximity.

The *C. elegans* germline expresses tens of thousands of piRNAs that engage Piwi Argonautes to direct transgenerational silencing of transposons and many protein-coding genes (Billi et al., 2014). We wondered if piRNA silencing might also direct the coordination of the target loci and a specialized P granule. To address this possibility we examined the dosage compensation gene *xol-1*, which is a natural target of piRNA silencing (Tang et al., 2018). Mutations in the Piwi Argonaute, *prg-1*, cause *xol-1* expression in the germline and lead to dosage-compensation defects among progeny. We first examined *prg-1* mutants to determine where *xol-1* is expressed when derepressed. Although *xol-1* was expressed at lower levels than *oma-1*, we found that late pachytene nuclei in *prg-1* mutants had a pattern of *xol-1* DNA and RNA signals similar to the pattern seen for *oma-1* in wild-type animals prior to dsRNA exposure. For example, the nuclei each contained two foci of *xol-1* DNA and coincident RNA, and *xol-1* mRNA was visible in several perinuclear P granules (Figures 3A and 3B). In contrast, late pachytene germ cells in wild-type animals, where *xol-1* is repressed, exhibited a pattern of *xol-1* DNA and RNA FISH signals similar to that of silenced *oma-1* after 21 hours of

RNAi (Figure 3C and 3D). For example, the *xol-1* genes appeared as a single dot coincident with the *xol-1* nuclear RNA signal, and were localized adjacent to a P granule enriched for *xol-1* RNA. Thus, the cytology of the PRG-1-dependent OFF state of *xol-1* in wild-type animals resembles the dsRNA-induced OFF state of a gene targeted by RNAi.

We wondered whether different P granules become specialized to silence distinct targets. To address this possibility we decided to monitor both *oma-1* and *xol-1* simultaneously in wild-type animals over a time course of *oma-1*(RNAi). The *oma-1* and *xol-1* genes are on different chromosomes, and their respective nuclear signals of DNA and RNA generally remained well separated over the time course of RNAi (Figure 3E and 3F). For example at 6 hr of RNAi the enlarged, specialized P granule associated with *oma-1* silencing formed independently of the nuclear transcription site of *xol-1* and its associated P granule (Figure 3E), and at 21 hr of RNAi the *oma-1* genes were positioned near the INM independently of the *xol-1* DNA (Figure 3F). Together these findings suggest that each P granule promotes silencing of distinct genomic targets, and that the three dimensional positioning of the genome is stochastic at least with regard to the ultimate disposition of the silenced *oma-1* and *xol-1* genes at the INM.

In summary, we have shown that RNAi- and piRNA-mediated silencing trigger the specialization of a P granule. Nuclear silencing components direct the re-localization of the target genes into proximity of this specialized P granule but are not required for its formation. Each domain of nuage appears to serve distinct RNA targets which become spatially linked to their corresponding genomic DNA targets. After the target genes relocate, the nuage diminishes in size, perhaps reflecting the reduced transcriptional activity of the target locus. Thus our results suggest that the transcriptional silencing

machinery participates in a feedback loop that redirects the target loci and mRNA export to a single specialized P granule. The continued low-level transcription of the paired loci at the INM may serve to supply the adjacent specialized nuage with template RNA that feeds small RNA amplification and inherited silencing.

Thousands of *C. elegans* germline mRNAs are targeted by nearly one million distinct Argonaute guide complexes. How the system can simultaneously utilize this multitude of Argonautes to surveil gene expression has been entirely mysterious. Our findings suggest a partial answer to this mystery. Approximately 20 to 50 P granules surround each pachytene nucleus. Therefore, if each P granule is specialized to handle a subset of the RNA silencing load, each P granule need only harbor 50- to 20-thousand guide complexes, instead of nearly one million. Moreover by directing the co-localization of their target genes each P granule need service only 30 to 75 of the approximately 1500 protein coding genes that are targeted by the system transgenerationally (Gu et al., 2009).

Our findings suggest that each P granule is initially seeded with, or differentiates to contain, distinct RNA information and that P granules utilize this information to guide the transgenerational regulation of their target genes. During late oogenesis, some P granules dissociate from germ nuclei; the dissociated P granules mix in the shared core cytoplasm, and are taken up by enlarging oogonia (Wolke et al., 2007). P granules have liquid-like properties (Brangwynne et al., 2009) and in early embryos maternally-derived P granules appear to dissolve and reform in germline blastomeres (Seydoux, 2018). These embryonic P granules co-localize with parentally derived Argonaute guide complexes (Claycomb et al., 2009; Gu et al., 2009). Mutations that disrupt embryonic P granules impair transgenerational inheritance of Argonaute silencing signals (Dodson

and Kennedy, 2019; Ouyang et al., 2019). Thus it seems likely that when gene expression initiates in embryonic germ cell progenitors, inherited silencing signals in these embryonic P granules guide the relocalization of germline chromatin to initiate a new round of guide RNA biogenesis, nuage differentiation, and chromatin re-localization.

We do not know how P granules compartmentalize and transmit information across generations. For example, do Argonaute-guide complexes that assemble in each pachytene P granule, remain together, or do they become shuffled during the dynamics of early embryogenesis? Similarly, the questions of how nuage becomes specialized and how it orchestrates DNA relocalization will require further study. The image of nuage that emerges from these studies mirrors Darwin's notion of gemmules as the units of heredity (Choi and Mango, 2014; Liu, 2018)—seeds of parental information that accumulate in germ cells to guide inheritance.

Acknowledgements: We thank Susan Strome from UC Santa Cruz, and Karen Bennett from University of Missouri School of Medicine for kindly gifting reagents, Christina Baer from SCOPE at UMMS for support with the micromanager installation. We thank Darryl Conte, and members of the Mello and Grunwald labs for help with preparation of the manuscript. This work was supported by a Howard Hughes Medical Institute Investigatorship and NIH R37GM058800 (C.C.M), NSF grant 1614940 (D.G.), and NIH RO1GM098583 (J.R.P). . Some of the strains used in this work were provided by the Caenorhabditis Genetic Center, which is funded by the NIH National Center for Research Resources (NCRR).

Methods:

Worm culture and strains:

The wild-type strain N2 was used and nematodes were cultured as described (Brenner, 1974). The following transgenic strains were used: **JJ2101**: *zuls242*; [*nmy-2p::pgl-1::gfp* + *unc-119(+)*] (Sheth et al., 2010), **WH223**: *ojls9*; [*zyg-12::gfp* + *unc-119(+)*], **JJ2212**: [*nmy-2p::pgl-1::mrfp*]. and **WM455**, *gfp::flag::xol-1(ne4555)* (Tang et al., 2018). **WM704** *glh-1(ne4816[gfp::glh-1])* was generated by CRISPR genome editing as described in (Ghanta et al., 2020).

Sample preparation for combined FISH, GFP detection and Immunofluorescence

General techniques for gonad dissections were used as described (Raiders et al., 2018). Briefly, worms were rinsed free of bacteria, then collected in PBS containing 0.1 mM tetramisole (SIGMA L9756) as a paralytic. Once the worms stopped moving, they were placed in PBS for dissection. In some preparations, 0.1% Tween-20 was used to facilitate gonad transfer by micropipette. The dissected gonads were suspended briefly in buffer A (75 mM Hepes (pH 6.9), 40 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1 mM EGTA; all chemicals from Sigma), to which was added a equal volume of fixative buffer B (5% paraformaldehyde, 7.5 mM Hepes (pH 7.2), 12 mM NaCl, 1.0 mM KCl, 0.6 mM MgCl₂, 0.3 mM EGTA; all chemicals from Sigma). After fixing for 15 mins, the gonads were rinsed, then incubated in RNase-free PBS (ThermoFisher AM9624) containing 0.1% Triton-X100 (Sigma) for 10 mins. The gonads were transferred to room temperature MeOH for 10

mins, then placed in fixative buffer B for an additional 5 mins and rinsed in PBS. Gonads were then dehydrated and stored in EtOH.

RNA and DNA FISH

Gonads stored in EtOH were rehydrated in an EtOH/PBS series (10%, 20%, 60% EtOH), then rinsed and incubated in two changes of wash buffer C (10% formamide, 2XSSC [0.3M NaCL, 30 mM sodium citrate (7.0)], each rehydration and rinse step was 5 minutes. Then the sample was incubated with three changes of wash buffer C as follows: 60°C for 1-3 min, 70°C for 3 min, followed by 60°C for 10-20 min.

The wash buffer was replaced with a solution containing ~0.01µM oligo probes (RNA and primary DNA probes), 90% RNA FISH hybridization buffer (Stellaris SMF-HB-1), and 10% deionized formamide, and incubated at 37°C overnight in the dark. The samples were rinsed and incubated in wash buffer C for 10 mins at room temperature, then incubated in fresh wash buffer at 37°C for 1 hr. The wash buffer was removed and replaced with fresh wash buffer C at 37°C for 1 hr. Gonads were rinsed and kept in PBS for immunostaining experiments.

For combined RNA and DNA FISH experiments, the gonads were treated with an additional solution containing 0.01 µM Bridge oligo, 0.01µM detection oligo in 2XSSC, 30% formamide at room temperature for 3 hrs. Gonads were washed in prewarmed 2XSSC at 60°C for 20 min, followed by two washes in 2XSSCT (2XSSC with 0.3% Triton X-100) at 60°C for 5 min each, and a wash in 2XSSCT at 60°C for 20 min. Samples were then rinsed at room temperature in 2XSSC, then rinsed and kept in PBS.

Immunostaining

After the FISH protocol was complete gonads in PBS were incubated with primary antibodies diluted in PBS and 0.5 units/ μ l of RNasin (Promega N261A) at 4°C overnight. The antibody solution was removed and the slides rinsed in three changes of PBS, 5 min each. The gonads were then incubated in secondary antibodies diluted in PBS and 0.5 units/ μ l of RNasin at room temperature for 2 hours. The gonads were rinsed briefly in PBS, then immersed in mounting medium with DAPI (Vectashield H-2000) for imaging. The following antibodies were used: Rabbit anti-PGL-1, a gift from Susan Strome, (Kawasaki et al., 1998) Chicken anti-GLH-2, a gift from Karen Bennett (Gruidl et al., 1996).

Microscope settings and configuration:

All images were acquired on a Yokogawa CSU21 spinning disk confocal microscope mounted onto an Axiovert 200M and operated with Micromanager (Edelstein et al., 2010) software. The sample was mounted using a slide holder and motorized stage (ASI), and images were acquired with a Zeiss 63x (1.4 NA) Planapo oil immersion objective. Optical z stacks were collected at increments of 0.5 microns. The spinning disk unit added roughly a 2.5 x magnification factor resulting in a pixel size of ~100 nm in the image plane. Images were acquired with a Hamamatsu ImageEM (model C9100-13) series camera using an EM gain of 300 and set to free read mode. Light from four lasers in a custom laser module (made by Solamere) containing 405 nm, 488 nm, 561 nm and 640 nm laser lines was coupled into a monomode optical fiber and delivered to the Yokogawa scan head. The following bandpass filters (Chroma) were used as emission filters ET-460-50, ET-525-50, ET-605-52, ET-700-75. Laser power was adjusted for each sample to minimize sample bleaching. Typical acquisition times were between 400-500 msec/frame.

Image processing:

Composite images were stitched from 9 sub-images that have 50% overlapping with adjacent images (Preibisch et al., 2009), but subregions were cropped to size to show desired features in the final figure. Spatial offsets between color channels in lateral (x,y) and axial (z) directions were estimated using 500 nm sized four color-stained beads (Tetraspecks, Thermo Fisher, Catalog number T14792). The center plane is defined as the plane with the largest diameter signal within a bead and was determined manually in an unprocessed image stack. Raw image stacks, after axial offset correction, were filtered using a Gaussian kernel with a width $\sigma = 1$, a Laplacian of Gaussian (LoG3D filter (Sage et al., 2005)) followed by a Gaussian kernel with a width $\sigma = 1$. LoG3D is used for background suppression and not to localize the exact position of a spot. The sigma along the optical axis is taken as roughly half the number of slices the signal is visible in. This approach preserves the visual impression of signal dimension between the channels. The sigma for each channel used was: Ch1: $\sigma_{x,y} = 1$ pixel, $\sigma_z = 2$ pixel; Ch2: $\sigma_{x,y} = 1$ pixel, $\sigma_z = 2$ pixel; Ch3: $\sigma_{x,y} = 1$ pixel, $\sigma_z = 2$ pixel.

Figure legends:

Figure 1. Pachytene germ nuclei in adult hermaphrodite gonads examined at 0, 6, and 21 hours after exposure to *oma-1* dsRNA [*oma-1(RNAi)*]. Single gonads are shown in the paired panels A/D, B/E, C/F, G/J, H/K, and I/L. The larger image in each panel shows an optical z-projection of about 30 planes, corresponding to approximately half the thickness of the gonad. The three smaller insets show high magnifications of single optical planes through individual nuclei. (A-L) Confocal images of RNA FISH, DNA FISH, and fluorescent-protein signals (P granule = GFP:GLH-1 and Nuclear Env = ZYG-12::GFP). Signals were colored either green or magenta as indicated at top, such that coincident signals appear white. For example, perinuclear RNA in panel D is magenta, but the nuclear RNA appears white because it is coincident with the green DNA signal. Before, and at early timepoints in, *oma-1(RNAi)*, the pair of nuclear foci of DNA and coincident appear within a small radius around the synapsed, pachytene chromosomes, and appear randomly oriented with respect to the nuclear envelope (panels A and B). By 21 hrs, a single focus of DNA and coincident RNA appears juxtaposed to the envelope (panel C). Prior to RNAi most cytoplasmic *oma-1* mRNA in the gonad is contained in the shared core region underlying the nuclei and appears as a central, vertical band in panels A, D, and J.

Figure 2. Wild-type or mutant gonads exposed to *oma-1* dsRNA for 6hr or 21 hrs as indicated. Images are confocal images, as in Figure 1 with signals represented in green

or magenta, and merged, coincident signals in white. P granules were detected using a
antiserum specific for GLH-2.

Supplemental Figure 2. Wild-type or mutant gonads as indicated exposed to *oma-1*
dsRNA for 0hr. Images are confocal images, as in Figure 1 and 2.

Figure 3. (A-F) Merged confocal images as in Figures 1 and 2 of dissected gonads
isolated from strains that encode gfp fused to the endogenous *xol-1* gene. DNA and RNA
FISH signals were detected with both *xol-1* and *gfp* specific probes (see Methods). (A-D)
Merged confocal images of the pachytene zones in wt (A B) and *prg-1* mutants (C D).
Insets show single Z sections of representative nuclei. (E and F) Simultaneous detection
of P granules and *oma-1* and *xol-1* RNA (E) or DNA (F) in late pachytene wildtype gonads
after exposure to *oma-1* dsRNA for 6 hours (E) or 21 hours (F). The *oma-1* and *xol-1*
signals were in different regions of the nucleus, and in general were not both visible in a
single focal plane, therefore the separation of these signals in individual nuclei is
illustrated here by a projection of optical z planes through the entire nuclei (paired
arrowheads in E and F). The colocalization of *xol-1* RNA and P granule signal in (E) was
barely visible in this composite where contrast was adjusted to detect the much more
intense *oma-1* RNA P granule signal. P granules were detected using a mixture of
antisera specific for GLH-2 and PGL-1.

Figure 4. Model illustrating events in RNA silencing. In the top panels the initiation of
silencing induces clearance of cytoplasmic mRNA by cytoplasmic WAGO Argonautes

(yellow Argonautes in the model). By 6 hours, the RNA signal becomes limited to a single P granule which expands to support the amplification of small RNAs and to coordinate the programming of nuclear Argonautes (blue). Nuclear silencing in turn initiates heterochromatin formation and the capture of the target genes in proximity to the adjacent INM. After the target genes spatially connect to nuage, the nuage diminishes in size. When the nuclear RNAi machinery is disarmed by mutation (lower panel), a single enlarged P granule still forms, but the target genes fail to relocalize to the nuclear periphery, and mRNA once again accumulates in multiple P granules.

References:

- Ahringer, J., and Gasser, S.M. (2018). Repressive Chromatin in *Caenorhabditis elegans*: Establishment, Composition, and Function. *Genetics* 208, 491-511.
- Ashe, A., Sapetschnig, A., Weick, E.M., Mitchell, J., Bagijn, M.P., Cording, A.C., Doebley, A.L., Goldstein, L.D., Lehrbach, N.J., Le Pen, J., *et al.* (2012). piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* 150, 88-99.
- Beliveau, B.J., Boettiger, A.N., Avendano, M.S., Jungmann, R., McCole, R.B., Joyce, E.F., Kim-Kiselak, C., Bantignies, F., Fonseka, C.Y., Erceg, J., *et al.* (2015). Single-molecule super-resolution imaging of chromosomes and in situ haplotype visualization using Oligopaint FISH probes. *Nat Commun* 6, 7147.
- Billi, A.C., Fischer, S.E., and Kim, J.K. (2014). Endogenous RNAi pathways in *C. elegans*. *WormBook*, 1-49.
- Brangwynne, C.P., Eckmann, C.R., Courson, D.S., Rybarska, A., Hoege, C., Gharakhani, J., Julicher, F., and Hyman, A.A. (2009). Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science* 324, 1729-1732.
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* 77, 71-94.
- Buckley, B.A., Burkhart, K.B., Gu, S.G., Spracklin, G., Kershner, A., Fritz, H., Kimble, J., Fire, A., and Kennedy, S. (2012). A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. *Nature* 489, 447-451.
- Burton, N.O., Burkhart, K.B., and Kennedy, S. (2011). Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* 108, 19683-19688.

Chen, C.C., Simard, M.J., Tabara, H., Brownell, D.R., McCollough, J.A., and Mello, C.C. (2005). A member of the polymerase beta nucleotidyltransferase superfamily is required for RNA interference in *C. elegans*. *Curr Biol* 15, 378-383.

Choi, Y., and Mango, S.E. (2014). Hunting for Darwin's gemmules and Lamarck's fluid: transgenerational signaling and histone methylation. *Biochim Biophys Acta* 1839, 1440-1453.

Claycomb, J.M., Batista, P.J., Pang, K.M., Gu, W., Vasale, J.J., van Wolfswinkel, J.C., Chaves, D.A., Shirayama, M., Mitani, S., Ketting, R.F., *et al.* (2009). The Argonaute CSR-1 and its 22G-RNA cofactors are required for holocentric chromosome segregation. *Cell* 139, 123-134.

Dodson, A.E., and Kennedy, S. (2019). Germ Granules Coordinate RNA-Based Epigenetic Inheritance Pathways. *Dev Cell* 50, 704-715 e704.

Edelstein, A., Amodaj, N., Hoover, K., Vale, R., and Stuurman, N. (2010). Computer control of microscopes using microManager. *Curr Protoc Mol Biol Chapter 14*, Unit14 20.

Fields, B.D., Nguyen, S.C., Nir, G., and Kennedy, S. (2019). A multiplexed DNA FISH strategy for assessing genome architecture in *Caenorhabditis elegans*. *Elife* 8.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806-811.

Gruidl, M.E., Smith, P.A., Kuznicki, K.A., McCrone, J.S., Kirchner, J., Roussel, D.L., Strome, S., and Bennett, K.L. (1996). Multiple potential germ-line helicases are components of the germ-line-specific P granules of *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* 93, 13837-13842.

Gu, W., Shirayama, M., Conte, D., Jr., Vasale, J., Batista, P.J., Claycomb, J.M., Moresco, J.J., Youngman, E.M., Keys, J., Stoltz, M.J., *et al.* (2009). Distinct argonaute-mediated 22G-RNA pathways direct genome surveillance in the *C. elegans* germline. *Mol Cell* 36, 231-244.

Guang, S., Bochner, A.F., Burkhart, K.B., Burton, N., Pavelec, D.M., and Kennedy, S. (2010). Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. *Nature* 465, 1097-1101.

Holoch, D., and Moazed, D. (2015). RNA-mediated epigenetic regulation of gene expression. *Nat Rev Genet* 16, 71-84.

Ji, N., and van Oudenaarden, A. (2012). Single molecule fluorescent in situ hybridization (smFISH) of *C. elegans* worms and embryos. *WormBook*, 1-16.

Kawasaki, I., Shim, Y.H., Kirchner, J., Kaminker, J., Wood, W.B., and Strome, S. (1998). PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in *C. elegans*. *Cell* 94, 635-645.

Liu, Y. (2018). In Search of Darwin's Imaginary Gemmules. *Adv Genet* 101, 87-114.

Malone, C.J., Misner, L., Le Bot, N., Tsai, M.C., Campbell, J.M., Ahringer, J., and White, J.G. (2003). The *C. elegans* hook protein, ZYG-12, mediates the essential attachment between the centrosome and nucleus. *Cell* 115, 825-836.

Ouyang, J.P.T., Folkmann, A., Bernard, L., Lee, C.Y., Seroussi, U., Charlesworth, A.G., Claycomb, J.M., and Seydoux, G. (2019). P Granules Protect RNA Interference Genes from Silencing by piRNAs. *Dev Cell* 50, 716-728 e716.

Pitt, J.N., Schisa, J.A., and Priess, J.R. (2000). P granules in the germ cells of *Caenorhabditis elegans* adults are associated with clusters of nuclear pores and contain RNA. *Dev Biol* 219, 315-333.

Preibisch, S., Saalfeld, S., and Tomancak, P. (2009). Globally optimal stitching of tiled 3D microscopic image acquisitions. *Bioinformatics* 25, 1463-1465.

Raiders, S.A., Eastwood, M.D., Bacher, M., and Priess, J.R. (2018). Binucleate germ cells in *Caenorhabditis elegans* are removed by physiological apoptosis. *PLoS Genet* 14, e1007417.

Sage, D., Neumann, F.R., Hediger, F., Gasser, S.M., and Unser, M. (2005). Automatic tracking of individual fluorescence particles: application to the study of chromosome dynamics. *IEEE Trans Image Process* 14, 1372-1383.

Seydoux, G. (2018). The P Granules of *C. elegans*: A Genetic Model for the Study of RNA-Protein Condensates. *J Mol Biol* 430, 4702-4710.

Sheth, U., Pitt, J., Dennis, S., and Priess, J.R. (2010). Perinuclear P granules are the principal sites of mRNA export in adult *C. elegans* germ cells. *Development* 137, 1305-1314.

Shirayama, M., Seth, M., Lee, H.C., Gu, W., Ishidate, T., Conte, D., Jr., and Mello, C.C. (2012). piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* 150, 65-77.

Tang, W., Seth, M., Tu, S., Shen, E.Z., Li, Q., Shirayama, M., Weng, Z., and Mello, C.C. (2018). A Sex Chromosome piRNA Promotes Robust Dosage Compensation and Sex Determination in *C. elegans*. *Dev Cell* 44, 762-770 e763.

Updike, D., and Strome, S. (2010). P granule assembly and function in *Caenorhabditis elegans* germ cells. *J Androl* 31, 53-60.

Watanabe, T., and Lin, H. (2014). Posttranscriptional regulation of gene expression by Piwi proteins and piRNAs. *Mol Cell* 56, 18-27.

Weiser, N.E., and Kim, J.K. (2019). Multigenerational Regulation of the *Caenorhabditis elegans* Chromatin Landscape by Germline Small RNAs. *Annu Rev Genet* 53, 289-311.

Wolke, U., Jezuit, E.A., and Priess, J.R. (2007). Actin-dependent cytoplasmic streaming in *C. elegans* oogenesis. *Development* 134, 2227-2236.

Figure 1

<https://doi.org/10.1101/2021.03.09.434681>

Figure 2

Figure 3

Figure 4 Model

DNA/RNA

P-granule/RNA

rde-3

A

B

wt

C

D

wago-9

E

F

nrde-2

G

H