

Nuclear Argonaute protein NRDE-3 switches small RNA partners during embryogenesis to mediate temporal-specific gene regulatory activity

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eLife Assessment

The study by Chen and Phillips provides evidence for a dynamic switch in the small RNA repertoire of the Argonaute protein NRDE-3 during embryogenesis in *C. elegans*. The work is supported by **convincing** experimental data, shedding light on RNA regulation during development. While the functional relevance of this process warrants further investigation, this study provides **valuable** insights into small RNA pathways with broader implications for developmental biology and gene regulation in other systems.

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Abstract

RNA interference (RNAi) is a conserved gene regulation mechanism that utilizes the Argonaute protein and their associated small RNAs to exert regulatory function on complementary transcripts. While the majority of germline-expressed RNAi pathway components reside in perinuclear germ granules, it is unknown whether and how RNAi pathways are spatially organized in other cell types. Here we find that the small RNA biogenesis machinery is spatially and temporally organized during embryogenesis. Specifically, the RNAi factor, SIMR-1, forms visible concentrates during mid-embryogenesis that contain an RNA-dependent RNA polymerase, a poly-UG polymerase, and the unloaded nuclear Argonaute protein, NRDE-3. We also observe that many other RNAi factors form foci in embryonic cells distinct from “SIMR granules”, including the Argonaute protein CSR-1, underscoring a potential role for cytoplasmic concentrates of RNAi factors to promote gene regulation in embryos. Curiously, coincident with the appearance of the SIMR granules, the small RNAs bound to NRDE-3 switch from predominantly CSR-class 22G-RNAs to ERGO-dependent 22G-RNAs. Prior work has shown that NRDE-3 binds ERGO-dependent 22G-RNAs in the somatic cells of larvae and adults to silence ERGO-target genes; here we demonstrate that NRDE-3-bound, CSR-class 22G-RNAs repress transcription in oocytes. Thus, our study defines two separable roles for NRDE-3, targeting germline-expressed genes during oogenesis to promote global transcriptional repression, and switching during embryogenesis to repress recently duplicated genes and retrotransposons in somatic cells, highlighting the plasticity of

Introduction

Precise gene expression is essential for organisms at all developmental stages. Small RNAs and their partners, the Argonaute (AGO) proteins, play an important role in regulating gene expression by targeting and silencing complementary nucleic acid sequences. This small RNA-mediated gene silencing process is known as RNA interference (RNAi) (Fire et al. 1998 [↗](#)). The nematode *Caenorhabditis elegans*, distinguished by its expanded Argonaute family and intricate RNAi pathway, is a well-established model organism to study the RNAi pathway. *C. elegans* has 19 functional Argonaute proteins and various classes of small RNAs (Yigit et al. 2006 [↗](#); Seroussi et al. 2023 [↗](#)), which is greatly expanded compared to 8 Argonaute proteins in mammals, 5 in *Drosophila melanogaster*, and 1 in *Schizosaccharomyces pombe* (Höck and Meister 2008 [↗](#)). This expansion of the Argonaute family in nematodes may be linked to the diversity of habitats in which nematodes reside and environmental cues to which they must respond. First, RNAi has been well-studied in plants for its role as an antiviral defense mechanism (Ding et al. 2004 [↗](#)); and like plants, worms lack an adaptive immune system, making the RNAi system a primary means to respond to viral intruders (Félix et al. 2011 [↗](#); Ashe et al. 2013 [↗](#); Sarkies and Miska 2013 [↗](#)). Second, nematodes have a specialized nucleic acid transporter required for the uptake of double-strand (ds)RNA from the intestinal lumen (McEwan et al. 2012 [↗](#); Winston et al. 2007 [↗](#)), indicating that environmental sensing mediated by ingested dsRNA is an important aspect of nematode physiology (Sarkies and Miska 2013 [↗](#)). Lastly, it has been proposed that Ago diversity and rapid evolution could be linked to the environmental plasticity of nematodes, including the capacity for parasitism and challenges of invading and colonizing a host (Buck and Blaxter 2013 [↗](#)). Regardless of the evolutionary origin for the expansion of RNAi pathway proteins in nematodes, these pathways are not only important for a response to the environment, but are essential for the regulation of thousands of endogenous genes. Therefore, untangling the details of RNA silencing in *C. elegans* will shed light on the mechanisms of small RNA-mediated gene regulation in *C. elegans* and other organisms.

Argonaute proteins can be subdivided into three clades. Proteins are grouped into the AGO and PIWI clades based on their similarity to *Arabidopsis thaliana* AGO1 and *Drosophila melanogaster* PIWI, respectively. The third, WAGO, clade represents a nematode-specific expansion of the Argonaute protein family (Yigit et al. 2006 [↗](#)). While small RNAs bound by the AGO- and PIWI-clade Argonaute proteins tend to be processed from longer, precursor transcripts, the WAGO-clade Argonaute proteins bind 22-nucleotide, 5'-triphosphorylated small RNAs (22G-RNAs) with which are each de novo synthesized by RNA-dependent RNA polymerases (RdRPs) (Gu et al. 2009 [↗](#); Pak and Fire 2007 [↗](#); Aoki et al. 2007 [↗](#)). However, even within the WAGO clade, each of the 11 Argonaute proteins exhibits specificity for a unique group of 22G-RNAs and exhibits distinct tissue and developmental expression patterns (Seroussi et al. 2023 [↗](#)). For example, WAGO-1 binds 22G-RNAs that target transposons, pseudogenes, and aberrant transcripts, and silences genes post-transcriptionally in the germline cytoplasm (Gu et al. 2009 [↗](#)), while CSR-1 binds 22G-RNAs targeting germline-expressed genes, functioning to clear maternal mRNA in early embryos while licensing and tuning gene expression in the adult germline (Quarato et al. 2021 [↗](#); Gu et al. 2009 [↗](#); Claycomb et al. 2009 [↗](#)). Other WAGO Argonautes, such as SAGO-1 and SAGO-2, function exclusively in somatic cells and play roles in regulating endogenous genes, exogenous RNAi, and immunity (Seroussi et al. 2023 [↗](#)). Unique amongst the WAGO Argonautes for their nuclear localization are HRDE-1 and NRDE-3, which are thought to silence genes co-transcriptionally in germline and soma respectively, and are required for the inheritance of RNA silencing signals from parents to offspring (Buckley et al. 2012 [↗](#); Guang et al. 2008 [↗](#)). Despite extensive characterization of the *C. elegans* Argonaute proteins, we still know little about the factors that promote the spatiotemporal expression of each Argonaute protein and the mechanisms that

promote Argonaute-small RNA binding specificity. Furthermore, most Argonaute-small RNA sequencing experiments have been performed at a single time point, usually in adult *C. elegans*, meaning that we have little understanding as to how the RNA targets of each Argonaute protein change across development.

In the *C. elegans* germline, many of the RNAi components, including Argonaute proteins, RdRPs, and other small RNA processing machinery, localize within phase-separated germ granules. Often, proteins acting in different functional branches of the RNAi pathway seem to reside in separate compartments of the germ granules, suggesting that there are specialized areas within the germ granules where distinct molecular reactions occur. Presently, six sub-compartments of the germ granule have been identified in *C. elegans*: P granules, Z granules, *Mutator* foci, SIMR foci, and more recently, E granules and D granules (Brangwynne et al. 2009 [↗](#); Phillips et al. 2012 [↗](#); Wan et al. 2018 [↗](#); Manage et al. 2020 [↗](#); Chen et al. 2024 [↗](#); Huang et al. 2024 [↗](#)). Processing bodies (P-bodies), a well-characterized condensate that contains proteins associated with mRNA turnover and translationally repressed mRNAs, also closely associate with germ granules (Du et al. 2023 [↗](#)). These germ granule compartments are situated at the cytoplasmic side of the nucleus, proximal to nuclear pores. However, the mechanisms governing their spatial organization remain unknown. Moreover, with the majority of studies focusing on mechanisms of RNA silencing and germ granule organization in the germline, there is limited understanding of how each of these germ granule compartments assembles and functions in embryos. It has been observed that in *C. elegans* embryogenesis, the primordial germline cell P4 divides into Z2 and Z3 progenitor germ cells (PGCs) at around the 100-cell stage, coinciding with the demixing of Z granules from P granules, the appearance of *Mutator* foci and SIMR foci, and the initiation of germ cell transcription (Updike and Strome 2010 [↗](#); Uebel et al. 2021 [↗](#); Wan et al. 2018 [↗](#); Seydoux and Dunn 1997 [↗](#)). Together, the assembly of this more complex germ granule organization coinciding with a burst of transcription from the germ cells, may indicate that these multi-compartment structures are necessary to monitor the newly synthesized germline transcripts. Yet even these limited studies of RNAi pathway factors in embryos fail to address a role for ribonucleoprotein granules in RNA silencing in the soma.

Here, we discovered that SIMR-1, the founding component of the germline SIMR foci, is also found in cytoplasmic granules in the somatic cells of *C. elegans* embryos. These embryonic “SIMR granules” additionally contain factors involved in 22G-RNA amplification and associated with the nuclear Argonaute protein, NRDE-3. However, NRDE-3 itself only associates with the SIMR granules when not bound to small RNAs. Strikingly, the SIMR granules exhibit temporal dynamics where they first appear in early embryogenesis (around the 8-cell stage), peak around the 100-cell stage, and have mostly disappeared by the comma stage of embryogenesis. Curiously, these embryonic SIMR granules are by no means the only RNAi-related embryonic granules, as numerous other RNAi factors are found in separate granules in embryos, including components of the CSR pathway, the Argonaute CSR-1 and its RdRP EGO-1. Interestingly, clearance of many of these granules, including the embryonic SIMR, CSR, Z and P granules, is regulated by a common mechanism of autophagic degradation. Furthermore, by sequencing the small RNAs bound by NRDE-3 in early and late embryogenesis, we found that the formation of the SIMR granules coincides with a switch in NRDE-3 small RNA targets, from CSR-class 22G-RNAs to ERGO-class 22G-RNAs. Together, our data demonstrates that NRDE-3 has two separate functions, first acting in oogenesis to repress RNA Polymerase II and promote genome-wide transcription repression, and second acting downstream of ERGO-1 to transcriptionally silence retrotransposons, pseudogenes, and aberrant transcripts. Further, the SIMR granules themselves appear to be sites of NRDE-3-bound 22G-RNA biogenesis and loading and may contribute to the efficiency or specificity of Argonaute-small RNA interactions during embryogenesis.

Results

SIMR-1 and ENRI-2 localize to cytoplasmic granules during embryogenesis

In previous work, we sought to identify proteins that associate with SIMR-1 and ultimately found that SIMR-1 associates with HRDE-2 and the nuclear Argonaute protein, HRDE-1, to promote correct HRDE-1 small RNA binding in germ cells (Chen and Phillips 2024a [↗](#)). In that work, we also identified another nuclear Argonaute protein, NRDE-3, as an interactor of SIMR-1. NRDE-3 has been shown previously to bind ERGO-dependent 22G-RNAs and silence ERGO-target genes in somatic cells (**Figure 1—figure supplement 1 A-B** [↗](#)). To delve further into this potential interaction between SIMR-1 and NRDE-3, we first systematically compiled a list of the protein interactions identified from previous studies for both SIMR-1 and NRDE-3 (**Figure 1A** [↗](#)). Interestingly, the HRDE-2 paralog, ENRI-2, had been shown to interact with both SIMR-1 and NRDE-3 in embryos by immunoprecipitation (IP) followed by mass spectrometry (mass-spec), and another HRDE-2 paralog, ENRI-1, was similarly shown to interact with only NRDE-3 (Lewis et al. 2020 [↗](#)). These findings suggest that SIMR-1, NRDE-3, ENRI-2, and possibly ENRI-1 proteins may function together in the somatic nuclear RNAi pathway, analogous to the roles of SIMR-1, HRDE-1, and HRDE-2 in the germline nuclear RNAi pathway.

Here, we first aimed to address whether and where NRDE-3, SIMR-1, ENRI-1, and ENRI-2 colocalize. NRDE-3 has previously been shown to be expressed in the nucleus of most somatic cells (Guang et al. 2008 [↗](#)). Until recently, all characterization of NRDE-3 was done using a low-copy, integrated transgenic strain in which the nuclear localization was not visible until the ~30-80-cell stage of development, and it was presumed that this localization reflected the endogenous NRDE-3 localization (Guang et al. 2008 [↗](#); Lewis et al. 2020 [↗](#)). However, a more recent study constructed an endogenously-tagged NRDE-3 strain using CRISPR and found that NRDE-3 additionally localizes to the nucleus of oocytes and early embryos (Seroussi et al. 2023 [↗](#), 2022 [↗](#)). In a recent study, we addressed this discrepancy, and demonstrated that the older, transgenic NRDE-3 construct is targeted for RNAi-mediated gene silencing in germ cells and early embryos (Chen and Phillips 2024b [↗](#)). SIMR-1 is a component of the SIMR foci, a sub-compartment of germ granules, that appears as punctate foci at the periphery of *C. elegans* germ cells starting in embryos through the adult stage (Manage et al. 2020 [↗](#); Uebel et al. 2021 [↗](#)). ENRI-1 has been reported to localize to the cytoplasm of oocytes and embryos while ENRI-2 localized to both the nucleus and cytoplasm, varying depending on developmental stage (Lewis et al. 2020 [↗](#)). With these four proteins showing distinct localization patterns from one another, it was unclear how these proteins could physically interact.

To investigate where and how these interactions might potentially occur, we chose to initially examine localization of these proteins in the germline of adult *C. elegans* using the endogenously-tagged NRDE-3 strain which is visible starting in late pachytene. As expected based on previous work, NRDE-3 localizes to the nucleus of germ cells, while SIMR-1 is found in the cytoplasm in SIMR foci, a compartment of the germ granule (**Figure 1—figure supplement 2A** [↗](#)) (Seroussi et al. 2022 [↗](#), 2023 [↗](#); Manage et al. 2020 [↗](#)). Next, we decided to examine NRDE-3 and SIMR-1 localization in embryos, carefully dividing the embryos into distinct developmental stages, from 4-cell to comma stage. As expected, we found that NRDE-3 is consistently localized to the nucleus in all embryonic stages (**Figure 1B** [↗](#)). Interestingly, we observed that SIMR-1 forms granules in the cytoplasm of somatic cells during some embryonic stages (**Figure 1B** [↗](#)). By quantifying the total number of granules per embryo across embryonic development, we found that the “SIMR granules” first appear around the 8-cell stage and reach a peak at approximately the 100-cell stage, coinciding with the division of the germline precursor cell P_4 into the primordial germ cells Z_2 and Z_3 (Wang and Seydoux 2013 [↗](#)). Subsequently, the number of SIMR granules decreases, and in late

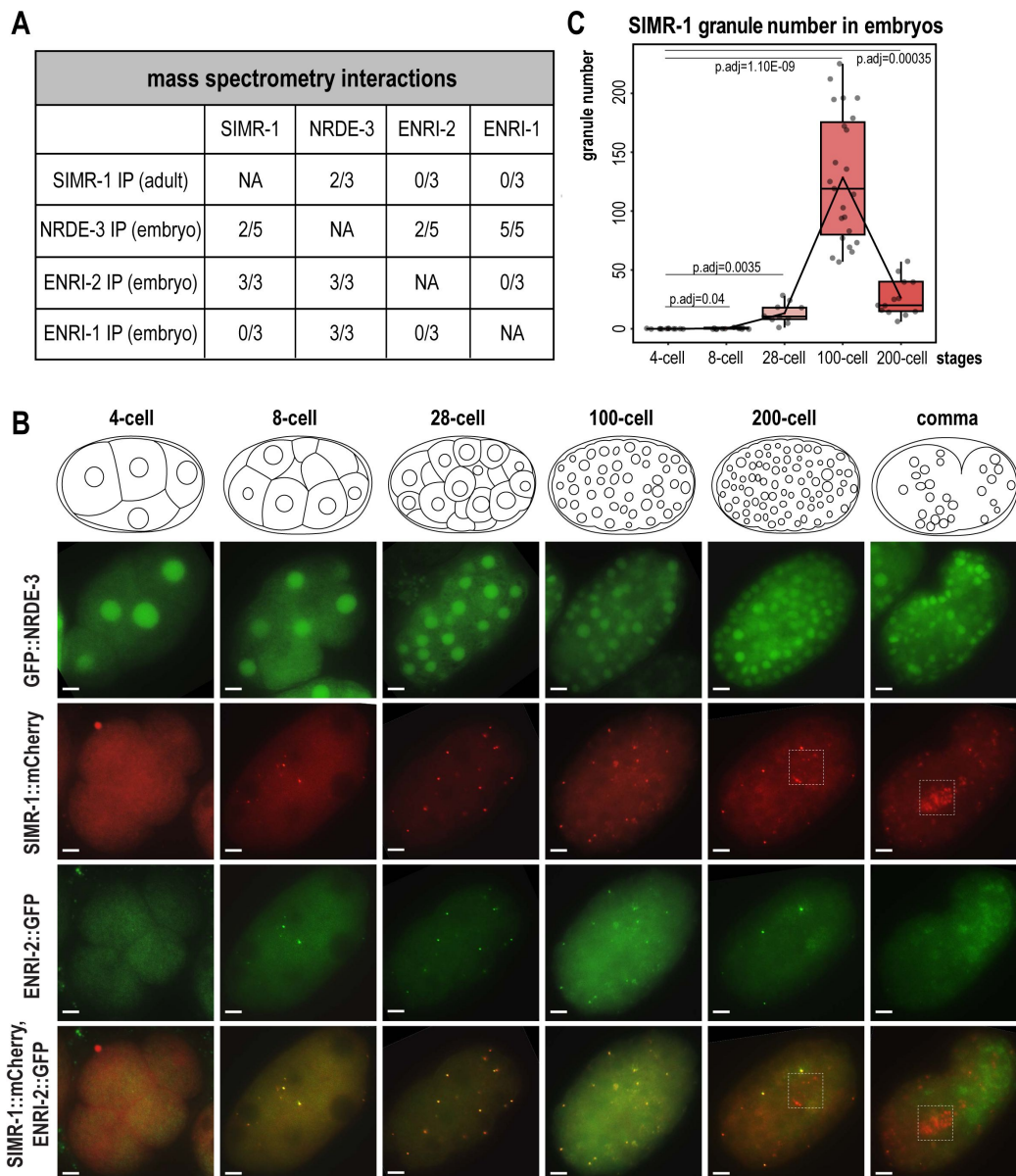


Figure 1.

SIMR-1 and ENRI-2 colocalize at somatic granules in embryos.

A. Summary of IP-mass spectrometry interactions detected between NRDE-3, ENRI-2, ENRI-1, and SIMR-1 from previously published studies (Chen and Phillips, 2024, Lewis *et al.*, 2021). The number of replicates from which the interaction was detected relative to the total number of replicates performed is indicated.

B. Live imaging of GFP::3xFLAG::NRDE-3 and SIMR-1::mCherry::2xHA; ENRI-2::2xTy1::GFP embryos at different stages (4-cell, 8-cell, 28-cell, 100-cell, 200-cell, and comma). Boxes identify the location of Z_2a and Z_3p primordial germ cells, showing that SIMR-1 is present in germ granules while ENRI-2 is not. At least five individual embryos were imaged for each genotype and stage. Scale bars, 5 μ m.

C. Box plot of SIMR-1::mCherry::2xHA granule number quantification at different embryonic stages (4-cell, 8-cell, 28-cell, 100-cell, and 200-cell). At least ten individual embryos at each stage were used for quantification. Each dot represents an individual embryo, and all data points are shown. Bolded midline indicates median value, box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range. Lines connect the mean granule number for each stage, illustrating the change in number of SIMR granules across the developmental stages of the embryo. Two-tailed t-tests were performed to determine statistical significance and p-values were adjusted for multiple comparisons. See Materials and Methods for a detailed description of quantification methods.

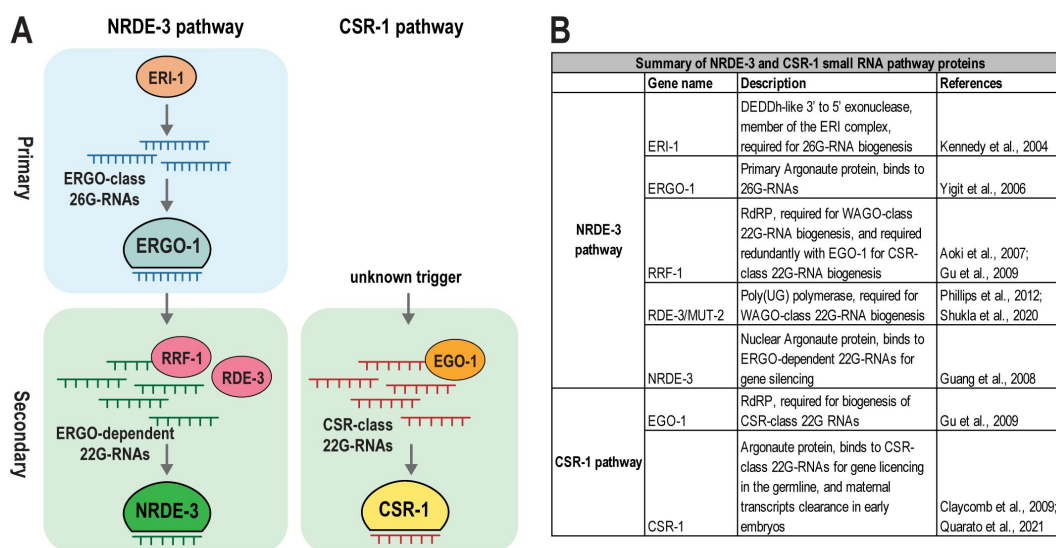


Figure 1—figure supplement 1.

Summary of NRDE-3 and CSR-1 small RNA pathway and components.

A. Illustration of NRDE-3 and CSR-1 pathways in *C. elegans*. In the NRDE-3 pathway, primary ERGO-class 26G-RNAs are synthesized by the ERI complex, including ERI-1. The primary Argonaute protein, ERGO-1, is loaded with the ERGO-class 26G-RNAs. The secondary ERGO-dependent 22G-RNAs, a subset of the WAGO-class 22G-RNAs, are synthesized from ERGO-1-targeted mRNAs. Their synthesis depends on the Mutator complex, which includes the RNA-dependent RNA polymerase, RRF-1, and the poly(UG) polymerase, RDE-3. They are subsequently loaded into NRDE-3 and other cytoplasmic WAGO-family Argonaute proteins to promote gene silencing. In the CSR-1 pathway, the CSR-class 22G-RNAs are synthesized by the RNA-dependent RNA polymerase, EGO-1. CSR-1 is loaded with CSR-class 22G-RNAs to promote gene silencing in the germline and clearance of maternal transcripts in early embryos.

B. A table summarizing the function of some key proteins in NRDE-3 and CSR-1 pathways.

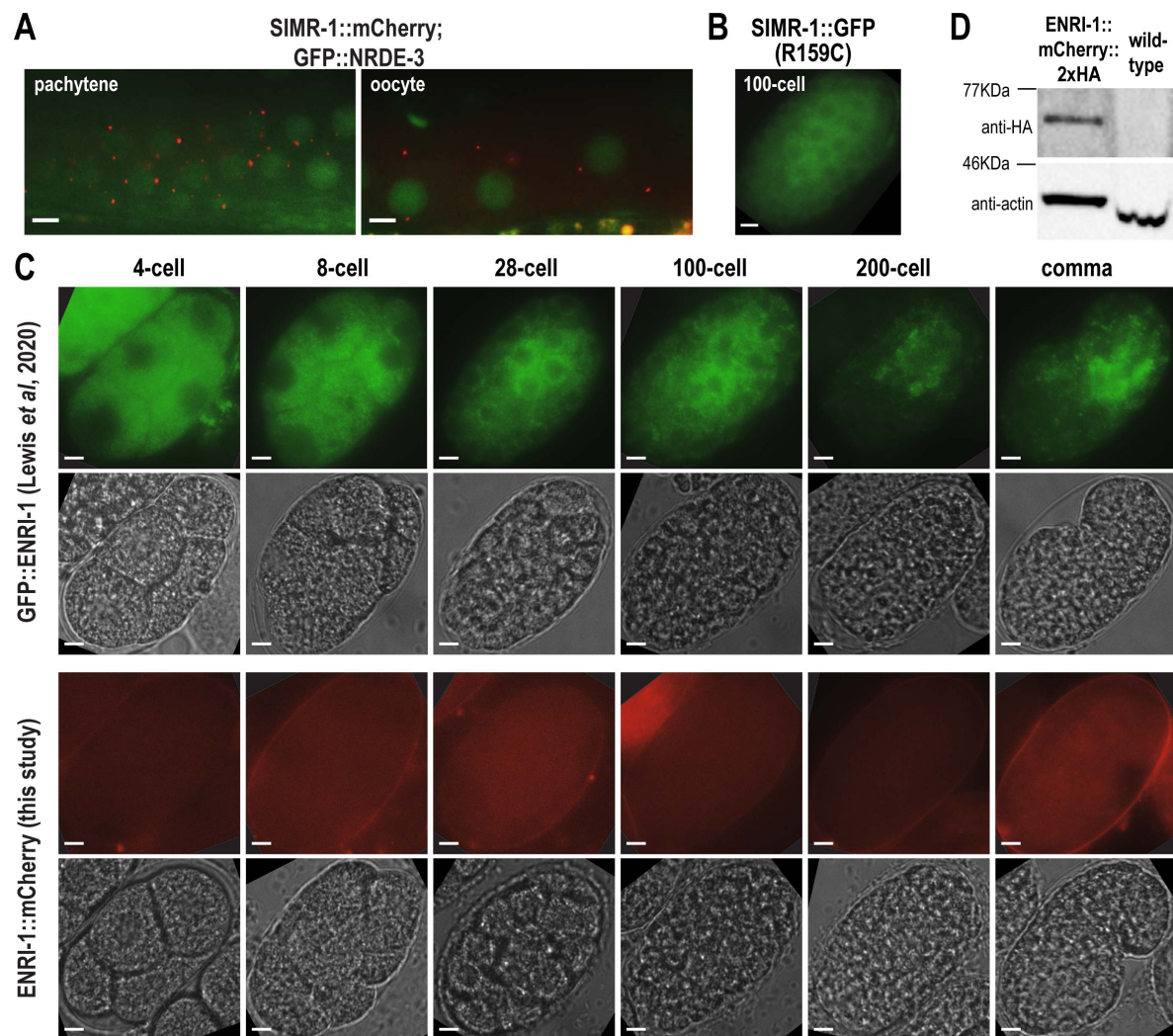


Figure 1—figure supplement 2.

Expression of NRDE-3, SIMR-1, and ENRI-1.

A. Live imaging of the germline from SIMR-1::mCherry::2xHA; GFP::3xFLAG::NRDE-3 day-one-old adult animals. Late pachytene (left) and oocyte (right) regions are shown, revealing that SIMR-1 and NRDE-3 do not colocalize in the germline. At least seven individual germlines were imaged. Scale bars, 5 μ m.

B. Live imaging of SIMR-1::GFP(R159C)::GFP::3xFLAG embryo at 100-cell stage, showing that SIMR-1(R159C) fails to not localize to cytoplasmic granules. At least five individual embryos were imaged. Scale bars, 5 μ m.

C. Live imaging of 2xTy1::GFP::ENRI-1 (top two rows) and ENRI-1::mCherry::2xHA (bottom two rows) embryos at different stages (4-cell, 8-cell, 28-cell, 100-cell, 200-cell, and comma). Fluorescence and brightfield channels are shown, demonstrating that ENRI-1 does not show prominent localization in embryos. At least seven individual embryos were imaged for each genotype and stage. Scale bars, 5 μ m.

D. Western blot of ENRI-1::mCherry::2xHA (left lane) and wild-type (right lane) gravid adult animals (72 hours at 20°C after L1 arrest), showing that the ENRI-1::mCherry::2xHA is expressed in this strain. Anti-HA-HRP and anti-actin antibodies were used for the western blot. Source data of the original blot is provided.

embryos, SIMR-1 localizes primarily to the germ granules surrounding the two germ cells, as previously observed (Uebel et al. 2021 [↗](#)) (**Figure 1B–C** [↗](#)). We had previously shown that the Tudor domain of SIMR-1 was important for its assembly into germline SIMR foci. Therefore, we next explored the requirement for the Tudor domain in assembling SIMR-1 cytoplasmic granules in embryos (Manage et al. 2020 [↗](#)). We found that the Tudor domain mutant, SIMR-1(R159C), fails to assemble in cytoplasmic granules in the embryos (**Figure 1—figure supplement 2B** [↗](#)), indicating that, similar to germline SIMR foci, the Tudor domain is also required for assembly of the cytoplasmic SIMR granules in embryos.

We next focused on ENRI-1 and ENRI-2 and observed that ENRI-2 shows similar cytoplasmic granule localization and colocalizes with SIMR-1 in embryos, but it does not localize to the germ granules in Z_2 and Z_3 , suggesting that the activity of ENRI-2 is restricted to somatic cells (**Figure 1B** [↗](#)). Finally, we examined the localization of N-terminal tagged 2xTy1::GFP::ENRI-1, and could not detect any specific localization in either nuclei or cytoplasmic granules (**Figure 1—figure supplement 2C** [↗](#)) (Lewis et al. 2020 [↗](#)). Consequently, we constructed a new strain with C terminal tagged ENRI-1::mCherry::2xHA and confirmed the presence of full-length ENRI-1 protein with Western Blot (**Figure 1—figure supplement 2D** [↗](#)). Nonetheless, we could not detect any ENRI-1 localization in embryos with our newly generated strain (**Figure 1—figure supplement 2C** [↗](#)). These results are consistent with the fact that ENRI-1 does not interact directly with either SIMR-1 or ENRI-2 by immunoprecipitation (**Figure 1A** [↗](#)) (Lewis et al. 2020 [↗](#)). Altogether, these data indicate that SIMR-1 and ENRI-2 colocalize at cytoplasmic granules in the somatic cells of embryos and suggest that ENRI-2 and SIMR-1 may function together at these sites. In contrast, NRDE-3 is spatially separated in the nucleus and no clear expression pattern was observed for ENRI-1.

Unloaded NRDE-3 associates with SIMR-1 in cytoplasmic granules

Next, to determine whether SIMR-1 and ENRI-2 are required for NRDE-3 localization, we introduced the *simr-1* mutant, *enri-2* mutant, *enri-1* mutant, and *enri-1; enri-2* double mutant in the endogenously tagged GFP::3xFLAG::NRDE-3 strain, and examined NRDE-3 localization across embryonic developmental stages. We observed no changes in NRDE-3 expression or nuclear localization in any of the mutants examined at any developmental stage (**Figure 2—figure supplement 1A** [↗](#)).

In previous work, we demonstrated that the germline nuclear Argonaute protein HRDE-1 loses nuclear localization and associates in the cytoplasm with the SIMR compartment of germ granules when it is unable to bind small RNAs (Chen and Phillips 2024a [↗](#)). Additionally, ENRI-2 interacts more strongly with NRDE-3 in an *eri-1* mutant background compared to wild-type (Lewis et al. 2020 [↗](#)), suggesting that the interaction occurs when NRDE-3 does not bind small RNAs. Localization of unloaded NRDE-3 has been examined in the seam cells of L3 stage animals, where, like HRDE-1, it loses nuclear localization and becomes restricted to the cytoplasm (Guang et al. 2008 [↗](#)). Therefore, we next sought to examine the localization of NRDE-3 when it is unbound to small RNA in embryos and germline. First, we aimed to deplete the preferred small RNA binding partners of NRDE-3. NRDE-3 has previously been shown to bind secondary 22G-RNAs downstream of ERGO-class 26G-RNAs, dependent on ERI-1, which is required for 26G-RNA biogenesis (Guang et al. 2008 [↗](#); Han et al. 2009 [↗](#); Seroussi et al. 2023 [↗](#)), and RDE-3/MUT-2, which is a component of the *Mutator* complex and necessary for 22G-RNA production (Chen et al. 2005 [↗](#); Phillips et al. 2012 [↗](#), 2014 [↗](#)) (**Figure 1—figure supplement 1A–B** [↗](#)). Therefore, we introduced an *eri-1* mutant and a *rde-3/mut-2* mutant into the endogenously GFP-tagged NRDE-3 background. We observed that NRDE-3 associates with somatic granules with a similar spatiotemporal pattern to SIMR-1 and ENRI-2, peaking around the 100-cell stage, although the total number of granules per embryo is lower for NRDE-3 granules in the *eri-1* and *rde-3/mut-2* mutant backgrounds compared to SIMR granules (**Figure 2A–B** [↗](#)). Next, to fully abolish the small RNA binding capacity of NRDE-3 and to confirm that the observed granule localization was due to the loss of small RNA loading, we introduced mutations to abolish small RNA binding into the GFP-tagged NRDE-3; specifically,

residues 687H and 691K in the Mid domain were mutated to alanine, hereafter referred to as NRDE-3(HK-AA) (Ma et al. 2005 [\[1\]](#); Guang et al. 2008 [\[2\]](#); Chen and Phillips 2024a [\[3\]](#)). NRDE-3(HK-AA) localizes exclusively to the cytoplasm across embryonic development and in the adult germline, accumulating in somatic granules at 100-cell stage similar to SIMR-1 and ENRI-2 (**Figure 2A** [\[4\]](#)). Quantification of the number of NRDE-3 granules per embryo in the NRDE-3(HK-AA) strain shows that the dynamics of NRDE-3 granule appearance and disappearance are similar to that of SIMR-1, where the number of granules increases from early embryos up until about 100-cell stage and then decreases as the embryos progress to later stages of development (**Figure 2C** [\[5\]](#)). Overall, the total number of NRDE-3(HK-AA) granules quantified per embryo are similar to or modestly higher than SIMR-1 granules (**Figure 1C** [\[6\]](#), **Figure 2C** [\[5\]](#)). Western blot analysis reveals that overall protein level are similar for wild-type NRDE-3 and NRDE-3(HK-AA) (**Figure 2—figure supplement 1B** [\[7\]](#)), indicating that unloaded NRDE-3 is not subject to degradation. This result contrasts with our previous results for unloaded HRDE-1 (HRDE-1(HK-AA), which does show reduced protein expression compared to wild-type HRDE-1 (Chen and Phillips 2024a [\[3\]](#)). Together, these data indicate that NRDE-3 forms granules in the cytoplasm of somatic cells when not associated with a small RNA binding partner.

It is also worth noting that despite the similarity in timing of NRDE-3 granule appearance and disappearance in the *eri-1* and *rde-3* mutants compared to the *nrde-3(HK-AA)* mutant, we observed a striking difference in the NRDE-3 localization in early embryos. Specifically, in *eri-1* and *rde-3* mutants, NRDE-3 localizes to the nucleus in early embryos while NRDE-3(HK-AA) localizes exclusively to the cytoplasm (**Figure 2A** [\[4\]](#)). Similarly, in the Z2 and Z3 primordial germ cells of late embryos, NRDE-3 is still found in the nucleus in *eri-1* and *rde-3* mutants. In contrast, NRDE-3 localizes exclusively to the cytoplasm in the somatic cells of late embryos of all three mutants. This result suggests that there may be a fundamental difference in the small RNAs bound by NRDE-3 in early compared to late embryos, and that the small RNAs bound by NRDE-3 in early embryos are produced independently of ERI-1 and RDE-3.

To determine whether unloaded NRDE-3 localizes to SIMR granules, we examined the localization of SIMR-1 and NRDE-3 together in the *nrde-3(HK-AA)* mutant and *eri-1* mutant backgrounds. We found that SIMR-1 colocalizes with unloaded NRDE-3 in embryonic granules (**Figure 2D** [\[8\]](#), **Figure 2—figure supplement 1C** [\[9\]](#)). Further, the SIMR granules in the *nrde-3(HK-AA)* mutant background exhibit dynamics similar to the wild-type background (**Figure 2—figure supplement 1D** [\[10\]](#)), indicating that *nrde-3(HK-AA)* does not affect the localization of SIMR-1. Interestingly, NRDE-3(HK-AA) does not form granules in germ cells and is instead present exclusively in the cytoplasm, thus it does not colocalize with the SIMR compartment of germ granules (**Figure 2—figure supplement 1E** [\[11\]](#)). These results demonstrate that unloaded NRDE-3 associates with SIMR-1 and ENRI-2 in cytoplasmic granules in the somatic cells of *C. elegans* embryos, indicating a potential role for SIMR-1 in the NRDE-3 nuclear RNAi pathway.

SIMR-1 and ENRI-2 recruit unloaded NRDE-3 to cytoplasmic granules

As previously described, unloaded NRDE-3 localizes to cytoplasmic granules in embryos and colocalizes with SIMR-1. Next, we aimed to determine whether SIMR-1 and ENRI-2 are required for the NRDE-3 granule localization. To this end, we introduced a *simr-1* mutant and an *enri-2* mutant into the GFP-tagged NRDE-3(HK-AA) strain and assessed NRDE-3(HK-AA) localization. Strikingly, we found that NRDE-3(HK-AA) granules disappear completely and NRDE-3(HK-AA) is instead found exclusively in the cytoplasm in all cells across all embryonic stages (**Figure 3A** [\[12\]](#)). Similarly, in a *simr-1*; *eri-1* double mutant, NRDE-3 granules are absent though NRDE-3 remains in the nucleus in early embryos (**Figure 3A** [\[12\]](#)), similar to NRDE-3 expression in the *eri-1* single mutant (**Figure 2A** [\[4\]](#)). However, neither wild-type NRDE-3 nor NRDE-3(HK-AA) protein levels are affected by loss of *simr-1* (**Figure 3—figure supplement 1A** [\[13\]](#)), indicating that loss of granule localization

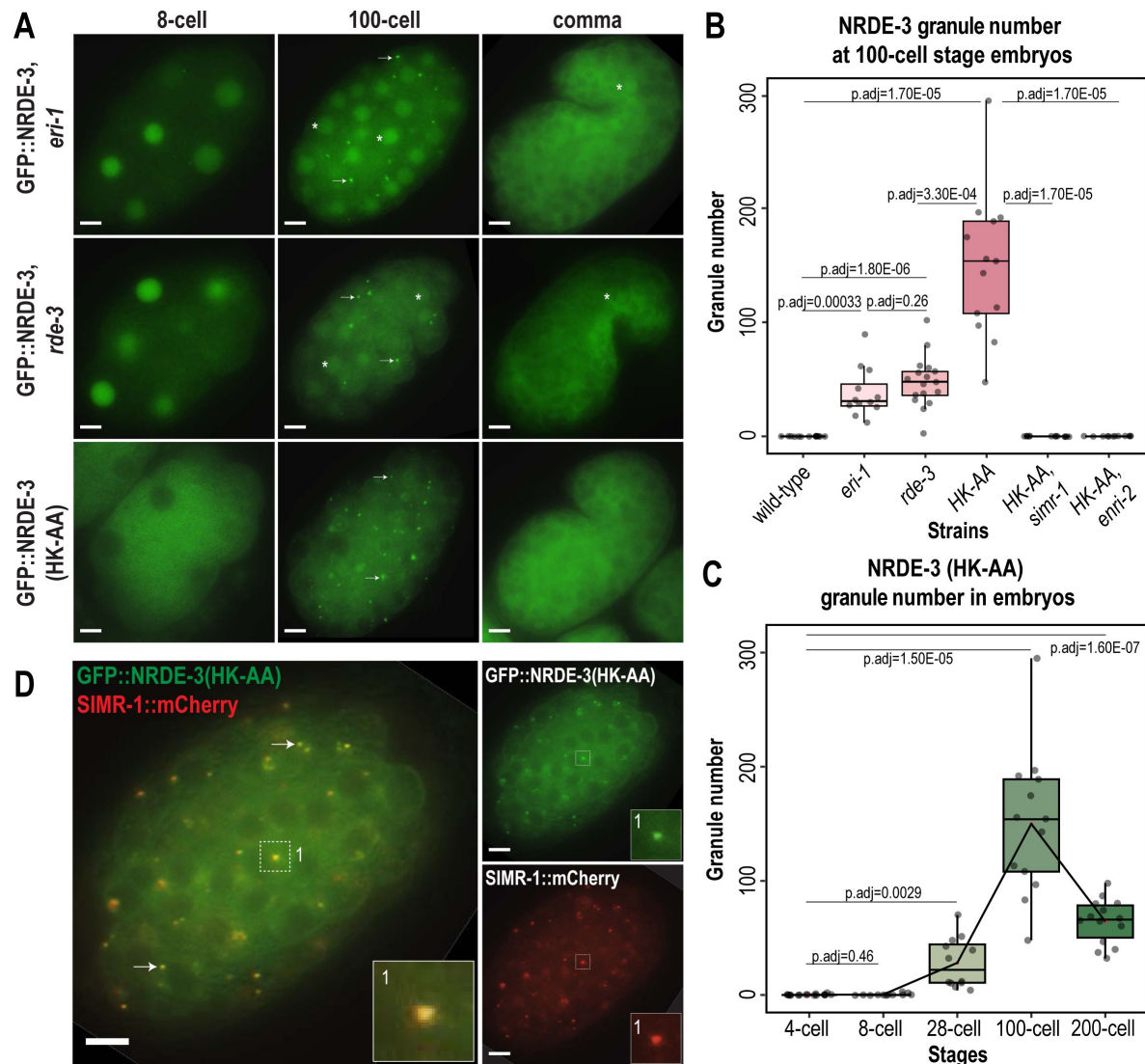


Figure 2.

Unloaded NRDE-3 localizes to cytoplasmic granules with SIMR-1.

A. Live imaging of GFP::3xFLAG::NRDE-3 embryos in *eri-1*, *rde-3*, and *nrde-3*(HK-AA) mutants at 8-cell, 100-cell, and comma stage embryos. At least five individual embryos were imaged for each genotype and stage. Arrows point to granule localization of NRDE-3 in the 100-cell stage. Asterisks highlight the nuclear localization of NRDE-3 in somatic cells of the 100-cell stage embryos and in the primordial germ cells of the comma stage embryos. Scale bars, 5 μ m.

B. Box plot of GFP::3xFLAG::NRDE-3 granule number quantification in different mutants.

C. Box plot of GFP::3xFLAG::NRDE-3(HK-AA) granule number quantification at different embryonic stages. Lines connect the mean granule number (red dots) for each stage, illustrating the change in number of NRDE-3 granules across embryonic development.

D. Live imaging of SIMR-1::mCherry::2xHA; GFP::3xFLAG::NRDE-3(HK-AA) at 100-cell stage. Arrows and insets show examples of colocalization between SIMR-1 and NRDE-3(HK-AA). At least ten individual embryos were imaged. Scale bars, 5 μ m.

For box plots in B and C, at least twelve individual embryos in each mutant were used for quantification. Each dot represents an individual embryo, and all data points are shown. Bolded midline indicates median value, box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range. Two-tailed t-tests were performed to determine statistical significance and p-values were adjusted for multiple comparisons. See Materials and Methods for a detailed description of quantification methods.

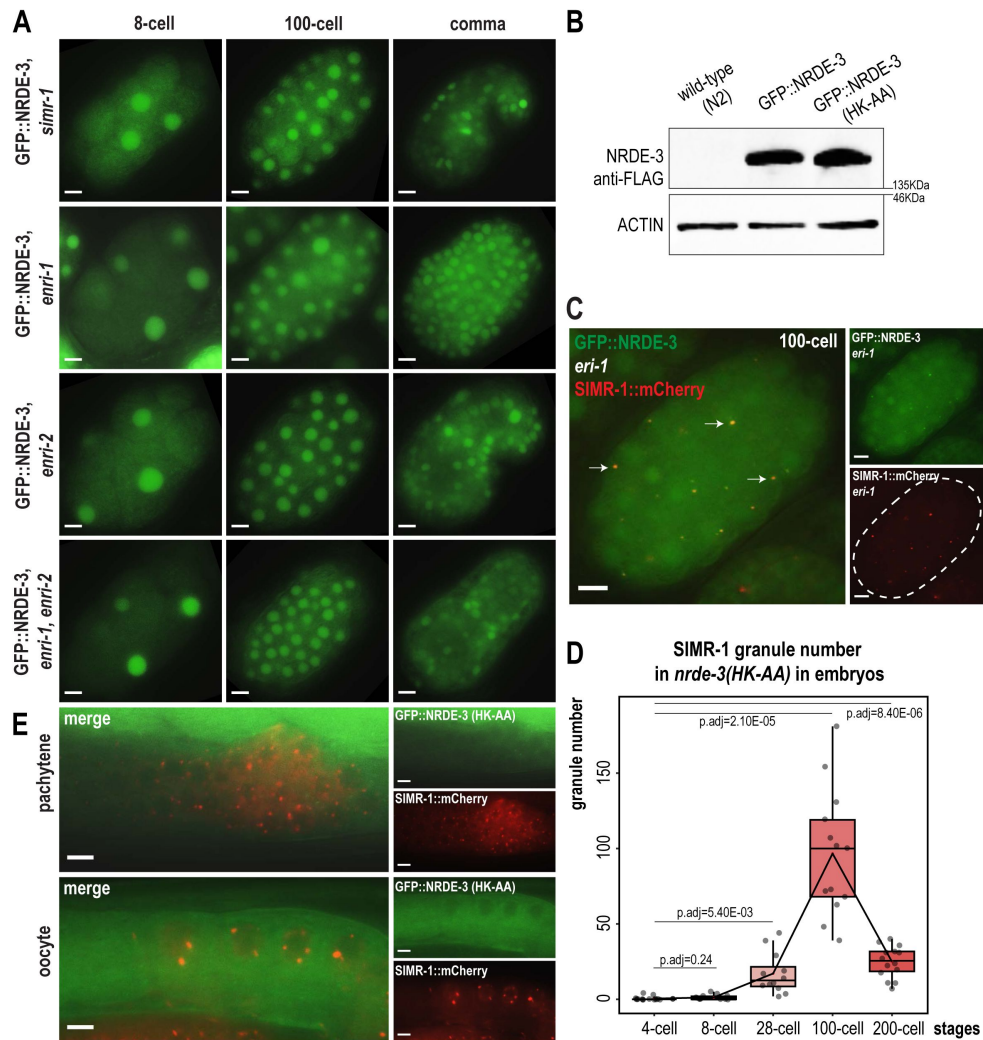


Figure 2—figure supplement 1.

NRDE-3 and SIMR-1 localization and expression in various mutants.

A. Live imaging of GFP::3xFLAG::NRDE-3 embryos in the *simr-1* mutant, *enri-1* mutant, *enri-2* mutant, and *enri-1; enri-2* double mutant in 8-cell, 100-cell, and comma stage embryos. At least five individual embryos were imaged for each genotype and stage.

B. Western blot of wild-type N₂, GFP::NRDE-3, and GFP::NRDE-3(HK-AA) mixed staged embryos, showing that the level of NRDE-3 protein is not significantly changed in the *nrde-3(HK-AA)* mutant compared to GFP::NRDE-3. Anti-FLAG and anti-ACTIN antibodies to detect NRDE-3 and ACTIN, respectively.

C. Live imaging of SIMR-1::mCherry::2xHA; GFP::3xFLAG::NRDE-3 embryos in an *eri-1* mutant at the 100-cell stage. Arrows point to examples of colocalization between SIMR-1 and NRDE-3. At least five individual embryos were imaged. Dotted white line indicates the outline of the embryo.

D. Box plot quantifying the number of SIMR-1::mCherry::2xHA granules in a *nrde-3(HK-AA)* mutant at different embryonic stages. At least twelve individual embryos for each genotype were used for quantification. Each dot represents an individual embryo, and all data points are shown. Bolded midline indicates median value, box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range. Lines connect the mean granule number (red dots) at each stage, illustrating the change in number of SIMR-1 granules across embryonic development. Two-tailed t-tests were performed to determine statistical significance and p-values were adjusted for multiple comparisons. See Materials and Methods for a detailed description of quantification methods.

E. Live imaging of germline regions from SIMR-1::mCherry::2xHA; GFP::3xFLAG::NRDE-3(HK-AA) day-one-old adult animals shows that SIMR-1 and NRDE-3(HK-AA) do not colocalize and NRDE-3(HK-AA) does not form foci in the germline (late pachytene (top) and oocyte (bottom)). At least five individual germlines were imaged. All scale bars, 5 μ m. Source data are provided as a Source Data file.

in *nrde-3(HK-AA)*; *simr-1* is not due to reduced NRDE-3 protein expression. These results demonstrate that both SIMR-1 and ENRI-2 are required for the recruitment of NRDE-3 to cytoplasmic granules.

To investigate the dependence of SIMR-1 and ENRI-2 on one another, we examined ENRI-2 localization in a *simr-1* mutant and SIMR-1 localization in an *enri-2* mutant. We found that ENRI-2 granules are lost in the *simr-1* mutant, while SIMR-1 granules are still present in the *enri-2* mutant, indicating that SIMR-1 functions upstream of ENRI-2 for granule assembly (**Figure 3B** [↗](#)).

To further assess whether ENRI-1 plays a role in the accumulation of unloaded NRDE-3 in cytoplasmic granules, we introduced an *enri-1* mutant into the GFP-tagged NRDE-3(HK-AA) strain and found that NRDE-3 association with cytoplasmic granules was not disrupted (**Figure 3—figure supplement 1B** [↗](#)). We further examined NRDE-3(HK-AA) granule association in the *enri-1*; *enri-2* double mutant and found it to be fully cytoplasmic, indistinguishable from the *enri-2* single mutant (**Figure 3—figure supplement 1B** [↗](#)). While we had already determined that ENRI-1::mCherry did not form visible foci in embryos; to rule out the possibility of partial redundancy between ENRI-1 and ENRI-2, we introduced an *enri-2* mutant into the mCherry-tagged ENRI-1 strain but still unable to detect any distinct ENRI-1 expression (**Figure 3—figure supplement 1C** [↗](#)). Lastly, to determine if NRDE-3 recruitment to granules could alter ENRI-1 localization, we introduced the mCherry-tagged ENRI-1 into the GFP-tagged NRDE-3(HK-AA) strain, and still we could not see any granule localization for ENRI-1 (**Figure 3—figure supplement 1D** [↗](#)). Therefore, we excluded ENRI-1 from further investigation, and conclude that SIMR-1 and ENRI-2, but not ENRI-1, recruit unloaded NRDE-3 to cytoplasmic granules, with SIMR-1 also acting to recruit ENRI-2.

SIMR-1 does not localize to P bodies or other previously identified embryonic granules

A variety of RNA-associated proteins have previously been shown to form granules in *C. elegans* embryos. To determine whether the SIMR-1, ENRI-2, and unloaded NRDE-3 granules that we have observed coincide with a previously identified granule, we examined the colocalization between SIMR-1 and all other embryonic granule-associated proteins that we could identify. It is well known that Processing (P) bodies, the condensates of translationally inactive mRNAs and proteins, localize to cytoplasmic foci of soma in *C. elegans* embryos (Parker and Sheth 2007 [↗](#); Gallo et al. 2008 [↗](#)). To examine if the SIMR-1 cytoplasmic granules are P bodies, we examined the localization of SIMR-1 and CGH-1, a core P body component, using a strain expressing GFP-tagged SIMR-1 and mCherry-tagged CGH-1 (Du et al. 2023 [↗](#)). We found that CGH-1 does not colocalize with SIMR-1 (**Figure 4—figure supplement 1A** [↗](#)). CGH-1 also does not colocalize with NRDE-3 cytoplasmic granules in the *eri-1* mutant (**Figure 4—figure supplement 1B** [↗](#)). Together, these data indicate that the cytoplasmic SIMR granules found in embryos are not P bodies.

Next, we examined two proteins previously shown to colocalize with SIMR foci in the germ cells of adult animals, RSD-2 and HRDE-2 (Manage et al. 2020 [↗](#); Chen and Phillips 2024a [↗](#)). RSD-2 is a small RNA factor required for the response to low doses of exogenously-introduced double-stranded RNA (Sakaguchi et al. 2014 [↗](#); Han et al. 2008 [↗](#); Tijsterman et al. 2004 [↗](#); Zhang et al. 2012 [↗](#)) and HRDE-2 is a factor critical for RNAi inheritance that promotes correct small RNA loading into the nuclear Argonaute HRDE-1 (Chen and Phillips 2024a [↗](#); Spracklin et al. 2017 [↗](#)). However, we did not observe any granule localization for RSD-2 and HRDE-2 in embryos (**Figure 4—figure supplement 1C-D** [↗](#)). In addition, SIMR-1 cytoplasmic granules were not affected by the loss of *hrde-2* (**Figure 4—figure supplement 1E** [↗](#)). These results suggest that HRDE-2 and RSD-2 do not function together with SIMR-1, ENRI-2, and NRDE-3 in embryonic granules and indicate that embryonic SIMR granules and the SIMR compartment of germ granules are distinct, each containing a unique set of proteins.

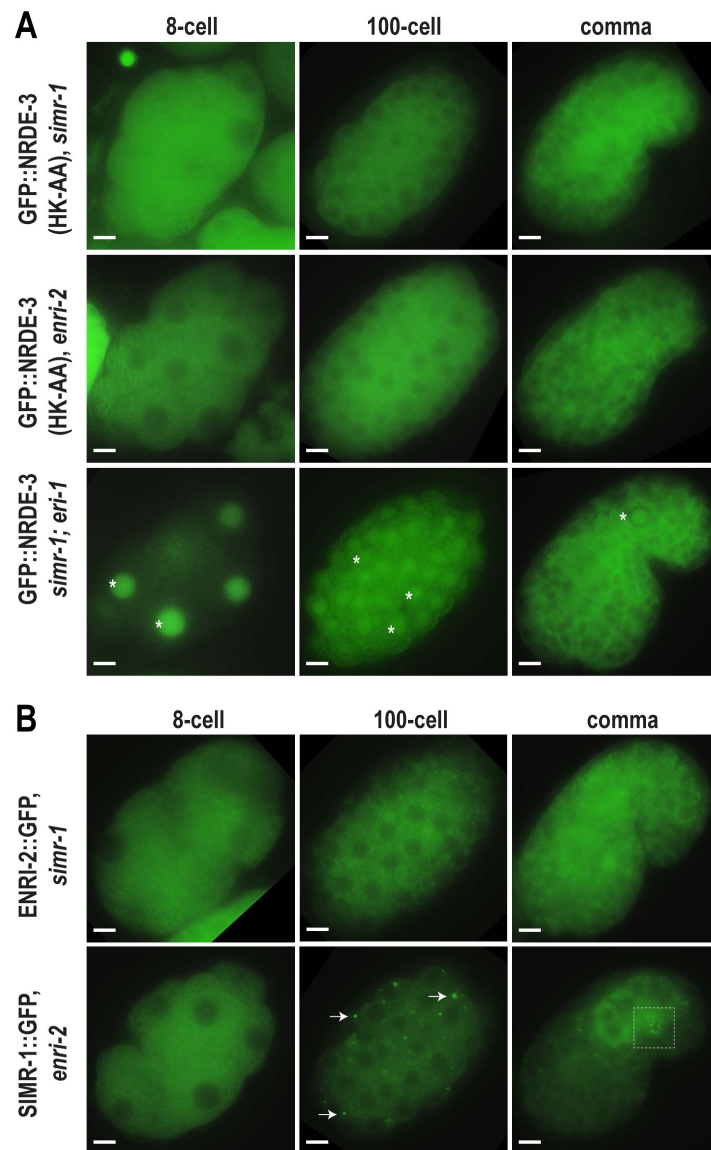


Figure 3.

SIMR-1 recruits ENRI-2 and then NRDE-3 to cytoplasmic granules.

A. Live imaging of GFP::3xFLAG::NRDE-3(HK-AA) embryos in *simr-1* and *eri-2* mutants, and GFP::3xFLAG::NRDE-3 embryos in a *simr-1; eri-1* double mutant at 8-cell, 100-cell, and comma stages. At least five individual embryos were imaged for each genotype and stage. Asterisk marks the nuclear localization of GFP::NRDE-3 in the *simr-1; eri-1* mutant, visible in somatic cells of 8- and 100-cell stage embryos and in a primordial germ cell of the comma stage embryo. Scale bars, 5 μ m.

B. Live imaging of ENRI-2::2xTy1::GFP embryos in a *simr-1* mutant and SIMR-1::GFP::3xFLAG embryos in an *eri-2* mutant. At least five individual embryos were imaged for each genotype and stage. Arrows point to examples of cytoplasmic SIMR granules still visible in the *eri-2* mutant. Box surrounds a primordial germ cell displaying germ granule localization of SIMR-1. Scale bars, 5 μ m.

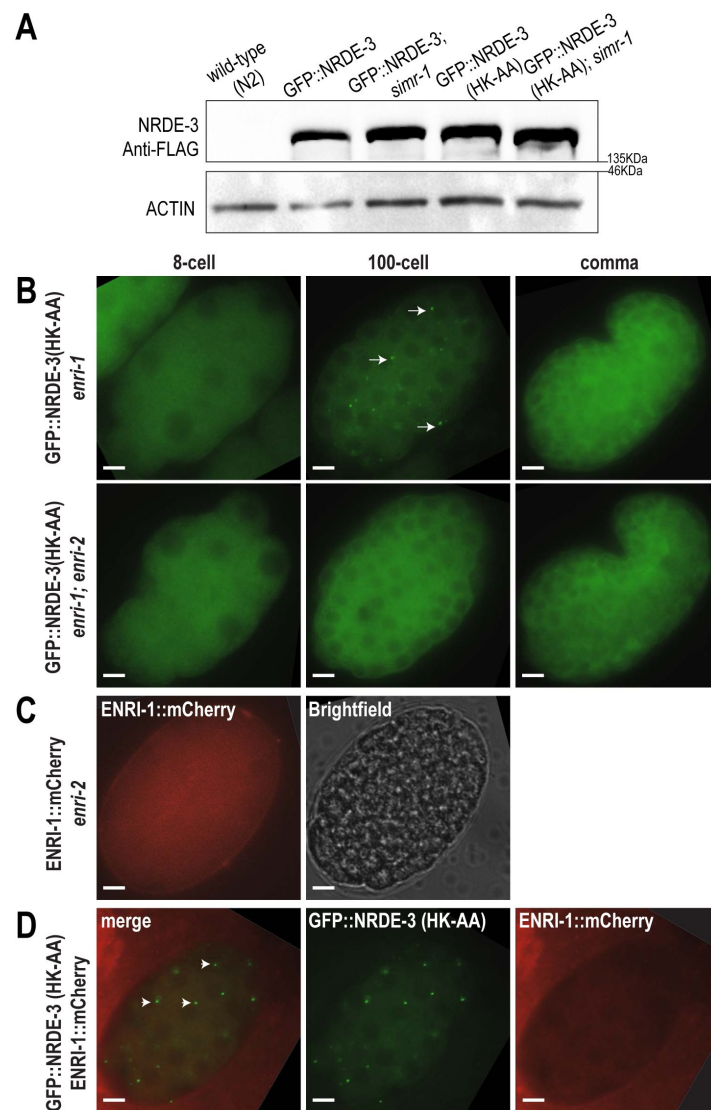


Figure 3—figure supplement 1.

NRDE-3(HK-AA) and ENRI-1 localization in various mutants.

A. Western blot of wild-type (N2), GFP::NRDE-3, GFP::NRDE-3; *simr-1*, GFP::NRDE-3(HK-AA), GFP::NRDE-3(HK-AA); *simr-1* mixed staged embryos, showing that the GFP::NRDE-3 and GFP::NRDE-3(HK-AA) does not exhibit higher expression level in the *simr-1* mutant. Anti-FLAG and anti-ACTIN antibodies were used to detect NRDE-3 and ACTIN, respectively.

B. Live imaging of GFP::3xFLAG::NRDE-3(HK-AA) embryos in an *enri-1* mutant and an *enri-1; enri-2* double mutant. At least five individual embryos were imaged for each genotype and stage. Arrows point to cytoplasmic NRDE-3(HK-AA) granules visible in an *enri-1* mutant. Scale bars, 5 μ m.

C. Live imaging of ENRI-1::mCherry::2xHA embryo in an *enri-2* mutant at 100-cell stage. Both fluorescence (left) and brightfield (right) channels are shown to demonstrate that no specific localization can be observed for ENRI-1 in the *enri-2* mutant. At least five individual embryos were imaged. Scale bars, 5 μ m.

D. Live imaging of ENRI-1::mCherry::2xHA; GFP::3xFLAG::NRDE-3(HK-AA) embryo at 100-cell stage, showing that no specific localization can be observed for ENRI-1 in the *nrde-3(HK-AA)* mutant. Arrows point to cytoplasmic NRDE-3(HK-AA) granules. At least five individual embryos were imaged. Scale bars, 5 μ m.

RDE-12 interacts with Argonaute proteins and RNAi-targeted mRNAs, and has also been shown to localize to cytoplasmic granules in the somatic cells of *C. elegans* embryos (Shirayama et al. 2014 [↗](#); Yang et al. 2014 [↗](#)). We next assessed the localization of mCherry-tagged SIMR-1 relative to GFP-tagged RDE-12 and found that they do not colocalize (**Figure 4—figure supplement 1F** [↗](#)). RSD-6 is a Tudor domain-containing RNAi factor that partially colocalizes with RDE-12 in the R2 bodies in adult germ cells (Yang et al. 2014 [↗](#); Sakaguchi et al. 2014 [↗](#); Zhang et al. 2012 [↗](#)). We examined the expression of GFP-tagged RSD-6 in embryos and, while we did observe RSD-6 at granules in somatic cells, no colocalization with mCherry-tagged SIMR-1 could be detected (**Figure 4—figure supplement 1G** [↗](#)). The RNAi-inheritance factor and defining member of the Z compartment of the germ granule, ZNFX-1, has also been observed in cytoplasmic granules in the somatic cells of embryos (Wan et al. 2018 [↗](#); Ouyang et al. 2019 [↗](#)), however these somatic ZNFX-1 granules also fail to colocalize with SIMR-1 (**Figure 4—figure supplement 1H** [↗](#)). Finally, we compared SIMR-1 localization to peri-centrosomal foci marked tubulin, based on a recent report that NRDE-3 accumulates at these foci (Jin et al. 2024 [↗](#)), but we did not observe any colocalization between SIMR-1 and tubulin in a wild-type background (**Figure 4—figure supplement 1I** [↗](#)). Altogether, we found that SIMR-1 fails to localize to any previously characterized embryonic granules. These results further indicate that there are numerous granule-localized proteins in the somatic cells of embryos, such as RDE-12, RSD-6, and ZNFX-1, which may play important roles in the RNA biology of early embryos.

Multiple *Mutator* complex proteins localize to SIMR granules in embryos

Mutator foci localize adjacent to SIMR foci in the adult germline (Manage et al. 2020 [↗](#); Chen and Phillips 2024a [↗](#)), so we next investigated the localization of *Mutator* components in embryos. We first examined *Mutator* foci component RDE-3/MUT-2, a poly(UG) polymerase required for WAGO-class 22G-RNA production (**Figure 1—figure supplement 1A-B** [↗](#)) (Phillips et al. 2012 [↗](#); Shukla et al. 2020 [↗](#)) and found that GFP-tagged RDE-3 is prominently localized to cytoplasmic granules in embryos that colocalize with SIMR-1 (**Figure 4A-B** [↗](#)). This colocalization led us to the hypothesis that SIMR-1 cytoplasmic granules are sites of WAGO-class 22G-RNA biogenesis. Therefore, we speculated that more small RNA production machinery might be localized with SIMR-1 at these cytoplasmic granules. We next examined the RNA-dependent-RNA-polymerase (RdRP) RRF-1, which synthesizes WAGO-class 22G-RNAs and localizes to *Mutator* foci in the adult germline (**Figure 1—figure supplement 1A-B** [↗](#)) (Sijen et al. 2001 [↗](#); Gent et al. 2010 [↗](#); Vasale et al. 2010 [↗](#); Phillips et al. 2012 [↗](#)). As we predicted, RRF-1 also colocalizes with SIMR-1 in somatic granules (**Figure 4B-C** [↗](#)), and it fails to localize to somatic granules in the *simr-1* mutant (**Figure 4D** [↗](#)).

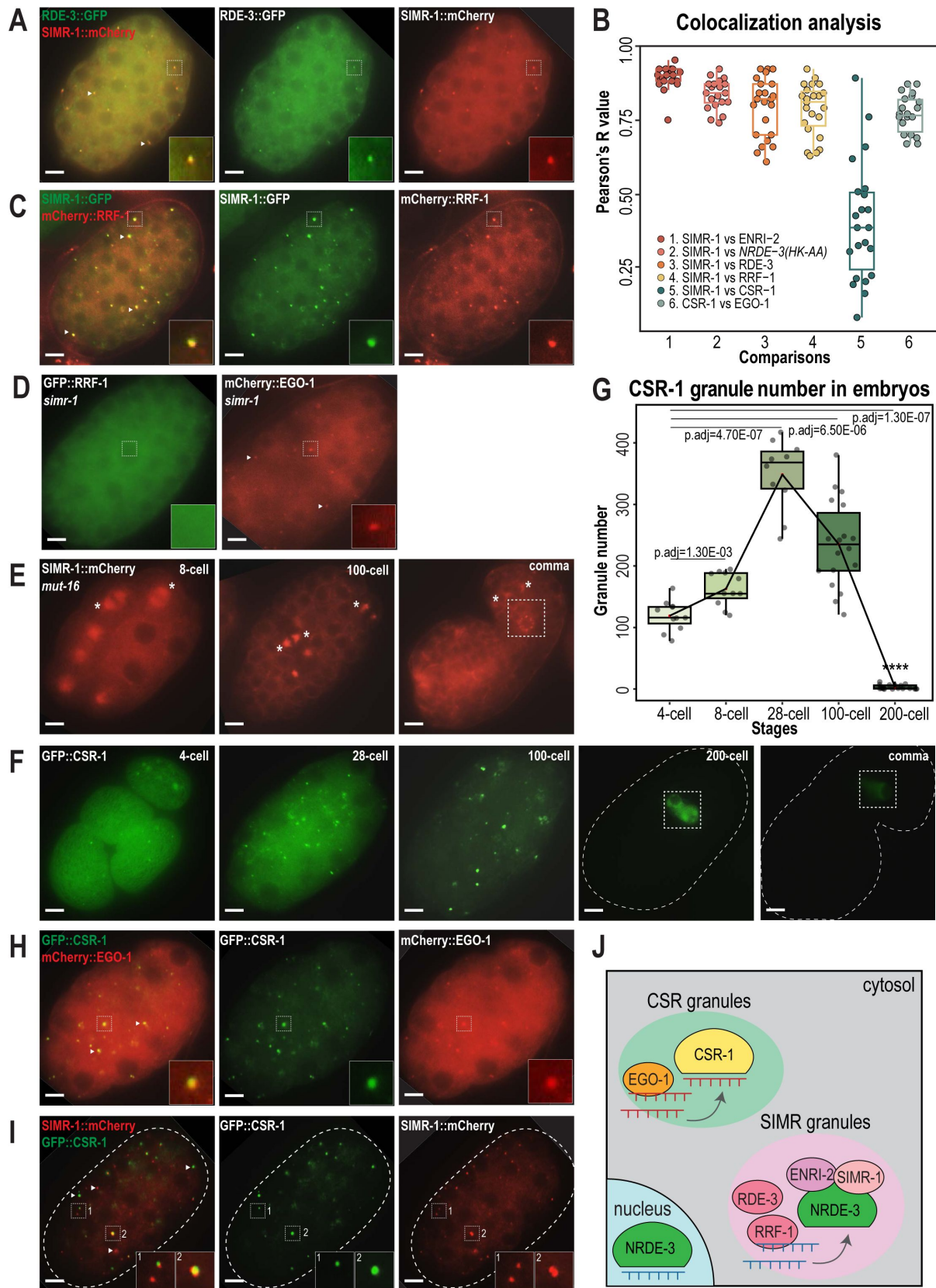


Figure 4.

CSR and WAGO pathway proteins localize to distinct cytoplasmic granules.

A. Live imaging of SIMR-1::mCherry::2xHA; RDE-3::GFP embryo at 100-cell stage, showing that RDE-3 colocalizes with SIMR-1. At least five individual embryos were imaged for each genotype and stage. Arrowheads and insets show examples of colocalization between SIMR-1 and RDE-3 at cytoplasmic granules. Scale bars, 5 μ m.

B. Box plot of Pearson's R value quantifications among different pairs of proteins at 100-cell embryonic stage. At least 20 granules from at least 4 individual embryos were used for quantification. Each dot represents an individual quantification, and all data points are shown. Box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range. See Materials and Methods for a detailed description of quantification methods.

C. Live imaging of SIMR-1::GFP::3xFLAG; HA::EGO-1::mCherry::RRF-1 at 100-cell stage embryo, showing that RRF-1 colocalizes with SIMR-1. At least five individual embryos were imaged for each genotype and stage. Arrowheads and insets show examples of colocalization between SIMR-1 and RRF-1 at cytoplasmic granules. Scale bars, 5 μ m.

D. Live imaging of mCherry::EGO-1::GFP::RRF-1 in a *simr-1* mutant, showing that RRF-1 no longer associates with cytoplasmic granules, while EGO-1 remains associated with granules in the *simr-1* mutant. At least five individual embryos were imaged. Arrowheads point to examples of cytoplasmic EGO-1 granules in a *simr-1* mutant. Insets show examples of cytoplasmic localization of RRF-1 and granule localization of EGO-1 in a *simr-1* mutant. Scale bars, 5 μ m.

E. Live imaging of SIMR-1::mCherry::2xHA embryos in a *mut-16* mutant at 8-cell, 100-cell, and comma stages. At least five individual embryos were imaged. Asterisks indicate spindle localization of SIMR-1 in a *mut-16* mutant. Box highlights germ granule localization of SIMR-1 in a comma-stage, *mut-16* mutant embryo. Scale bars, 5 μ m.

F. Live imaging of GFP::3xFLAG::CSR-1 embryos at different stages (4-cell, 28-cell, 100-cell, 200-cell, and comma), shows that CSR-1 localizes to cytoplasmic granules in early embryos and is restricted to germ granules in late embryos. At least three individual embryos were imaged for each stage. Dotted white line marks perimeter of the embryo. Box marks germ granule localization of CSR-1. Scale bars, 5 μ m.

G. Box plot quantifying GFP::3xFLAG::CSR-1 granules at different embryonic stages. At least ten embryos at each stage were used for quantification. Each dot represents an individual embryo, and all data points are shown. Bolded midline indicates median value, box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range. Lines connect the mean granule number (red dots) at each stage, illustrating the change in number of CSR granules across embryonic development. Two-tailed t-tests were performed to determine statistical significance and p-values were adjusted for multiple comparisons. See Materials and Methods for a detailed description of quantification methods.

H. Live imaging of mCherry::EGO-1; GFP::3xFLAG::CSR-1 embryo at 28-cell stage, showing CSR-1 colocalization with EGO-1. At least ten individual embryos were imaged. Arrowheads and insets show examples of CSR-1 and EGO-1 colocalization at cytoplasmic granules. Scale bars, 5 μ m.

I. Live imaging of SIMR-1::mCherry::2xHA; GFP::3xFLAG::CSR-1 embryo at 28-cell stage, showing the absence of colocalization between SIMR-1 and CSR-1 with occasional adjacent localization. At least ten individual embryos were imaged. Arrowheads point to examples of SIMR and CSR granules that do not colocalize. Insets show examples of SIMR and CSR granules that are found adjacent to each other or fail to colocalize. Dotted white line marks perimeter of embryo. Scale bars, 5 μ m.

J. Model of CSR and SIMR granules in the somatic cells of *C. elegans* embryos. The RdRP EGO-1, which synthesizes CSR-class 22G-RNAs, localizes to CSR granules, where CSR-1 loading may take place. The RdRP RRF-1, along with RDE-3, ENRI-2, and unloaded NRDE-3 localize to SIMR granules. SIMR-1 and ENRI-2 recruits unloaded NRDE-3 to granule

where RRF-1 may synthesize ERGO-dependent 22G-RNAs for loading into NRDE-3. After loading, NRDE-3 translocates to the nucleus and silences genes co-transcriptionally.

MUT-16 is the scaffolding protein for germline *Mutator* foci, thus we next investigated whether MUT-16 similarly scaffolds the cytoplasmic SIMR granules in early embryos (Phillips et al. 2012). We found that MUT-16 can be observed in cytoplasmic granules in the embryonic somatic cells (Figure 4—figure supplement 1J), similar to what has been observed in a previous study (Ouyang et al. 2019), and both SIMR-1 and RDE-3 fail to assemble into cytoplasmic granules in the *mut-16* mutant (Figure 4E, Figure 4—figure supplement 1K). Notably, the germ granule association of SIMR-1 is unaffected, as SIMR-1 still localizes to germ granules at comma stage embryos and in the adult germline (Figure 4E) (Manage et al. 2020). Together, these data indicate that MUT-16 functions upstream of SIMR-1 and mediates the assembly of cytoplasmic granules in embryos. It is curious to note that, in a *mut-16* mutant where SIMR-1 association with cytoplasmic granules is lost in the somatic cells, SIMR-1 instead associates with mitotic spindles (Figure 4E), similar to the localization observed for NRDE-3 in embryos (Jin et al. 2024). To conclude, we have shown that the SIMR granules found in the somatic cells of early embryos contain the biogenesis machinery for WAGO-class 22G-RNAs, including RDE-3 and RRF-1, and depend on the scaffolding protein MUT-16 for assembly (Figure 4J). The differential requirement for MUT-16 on the assembly of somatic and germline SIMR granules highlights a key difference between these two compartments, which have some parallel functions but distinct composition.

CSR-1 and EGO-1 associate with a distinct type of granule in early embryos

The Argonaute protein CSR-1 has also been previously seen at cytoplasmic granules in the soma of early embryos, a time at which CSR-1 is functioning to clear maternal-inherited mRNAs (Quarato et al. 2021; Seroussi et al. 2023; Ouyang et al. 2019). Using a GFP-tagged CSR-1 strain we constructed previously (Nguyen and Phillips 2021), we confirmed that CSR-1 forms prominent cytoplasmic granules in embryos visible prior to the 4-cell stage and present through 100-cell stage embryos, but disappear by the 200-cell stage, at which point only germ granule localization is visible (Figure 4F). Quantification of the total number of “CSR granules” per embryo across development shows that the CSR granules are more abundant than the SIMR-1 and NRDE-3(HK-AA) granules and differ in the timing of their appearance and disappearance relative to SIMR-1 and NRDE-3(HK-AA) granules (Figure 1C, 2C, 4G). Specifically, CSR granules appear earlier and peak at the 28-cell stage, while SIMR-1 and NRDE-3(HK-AA) granules appear between 8- and 28-cell stages and peak at the 100-cell stage (Figure 1C, 2C, 4G). The small RNAs bound by CSR-1 are synthesized by the RdRP, EGO-1, so we next assessed the localization of EGO-1 in early embryos. We found that EGO-1 colocalizes with CSR-1 in the somatic CSR granules (Figure 4B,H) and neither CSR-1 nor EGO-1 fully colocalizes with SIMR-1, although we occasionally observed adjacent localization between SIMR-1 and CSR-1 (Figure 4B,I, Figure 4—figure supplement 1L). Lastly, unlike RRF-1 which requires SIMR-1 to localize to embryonic foci, EGO-1 localizes to cytoplasmic granules in the absence of *simr-1* (Figure 4D). Together, our results show that the RdRPs, RRF-1 and EGO-1, localize to different cytoplasmic granules in the somatic cells of *C. elegans* embryos, where they colocalize with Argonaute proteins, NRDE-3 and CSR-1, respectively. Thus, we postulate that WAGO-class and CSR-class 22G-RNA biogenesis and loading are compartmentalized into cytoplasmic granules, differing from one another both spatially and temporally, in the somatic cells of early embryos (Figure 4J).

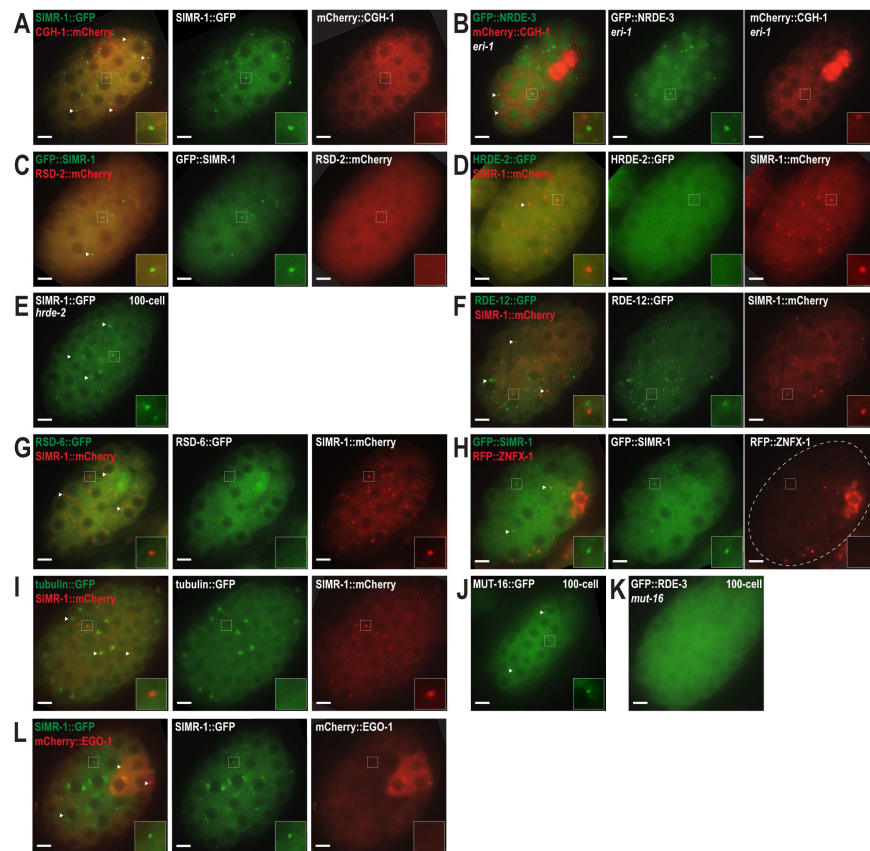


Figure 4—figure supplement 1.

SIMR-1 does not colocalize with any previously identified embryonic granules.

- A. Live imaging of SIMR-1::GFP::3xFLAG; mCherry::CGH-1 embryo at 100-cell stage, showing that SIMR-1 does not colocalize with CGH-1. Arrowheads and insets show examples of SIMR-1 and CGH-1 granules that do not colocalize.
- B. Live imaging of mCherry::CGH-1; *eri-1*; GFP::3xFLAG::NRDE-3 at 100-cell stage embryo, showing that NRDE-3 in the *eri-1* mutant does not colocalize with CGH-1. Arrowheads and insets show examples of NRDE-3 and CGH-1 granules that do not colocalize.
- C. Live imaging of SIMR-1::GFP::3xFLAG; RSD-2::mCherry embryo at 100-cell stage, showing that RSD-2 does not localize to cytoplasmic granules in the embryo. Arrowheads and insets show examples of SIMR granules.
- D. Live imaging of SIMR-1::mCherry::2xHA; HRDE-2::2xTy1::GFP embryo at 100-cell stage, showing that HRDE-2 does not localize to cytoplasmic granules in the embryo. Arrowheads and insets show examples of SIMR granules.
- E. Live imaging of SIMR-1::GFP::3xFLAG; *hrde-2* embryo at 100-cell stage, showing that SIMR-1 still localizes to cytoplasmic granules in the *hrde-2* mutant. Arrowheads and inset show examples of SIMR granules.
- F. Live imaging of SIMR-1::mCherry::2xHA; GFP::3xFLAG::RDE-12 embryo at 100-cell stage, showing that SIMR-1 does not colocalize with RDE-12. Arrowheads and insets show examples of RDE-12 and SIMR granules that do not colocalize.
- G. Live imaging of SIMR-1::mCherry::2xHA RSD-6::GFP::3xFLAG embryo at 100-cell stage, showing that SIMR-1 does not colocalize with RSD-6. Arrowheads and insets show examples of RSD-6 and SIMR granules that do not colocalize.
- H. Live imaging of SIMR-1::GFP::3xFLAG; HA::tagRFP::ZNFX-1 embryo at 100-cell stage, showing that SIMR-1 does not colocalize with ZNFX-1. Arrowheads and insets show examples of SIMR and ZNFX-1 granules that do not colocalize.
- I. Live imaging of SIMR-1::mCherry::2xHA; tubulin::GFP embryo at 100-cell stage, showing that SIMR-1 does not colocalize with tubulin. Arrowheads and insets show examples of tubulin and SIMR granules that do not colocalize.
- J. Live imaging of MUT-16::GFP at 100-cell stage, showing that MUT-16 localizes to somatic granules. Arrowheads and inset show examples of MUT-16 somatic granules.
- K. Live imaging of RDE-3::GFP in a *mut-16* mutant embryo at 100-cell stage, showing that RDE-3 no longer associates with granules in the *mut-16* mutant.
- L. Live imaging of SIMR-1::GFP::3xFLAG; mCherry::EGO-1 embryo at 100-cell stage, showing that SIMR-1 does not colocalize with EGO-1. Arrowheads and insets show examples of SIMR-1 and EGO-1 granules that do not colocalize. At least five individual embryos were imaged for all experiments. All scale bars, 5 μm.

Autophagy regulates the removal of SIMR granules and other cytoplasmic granules during embryogenesis

Both SIMR and CSR granules exhibit dynamic expression patterns across embryonic development, where the granules increase in abundance from early to mid-embryogenesis, and then subsequently disappear from somatic cells, becoming restricted to germ cells by late embryogenesis (**Figure 1B-C**, **Figure 4F-G**). In contrast, P granules become enriched at the posterior half of one-cell embryos and are subsequently partitioned with the germline progenitor cells through the remainder of embryonic development (Strome and Wood 1983). Previous work has shown that autophagic degradation is one mechanism by which P granules are removed from somatic blastomeres (Zhang et al. 2009). Autophagy, often referred to as the ‘self-eating’ pathway, is one of the two major protein degradation systems in eukaryotic cells. Autophagy involves the formation of autophagosomes, which engulf cytoplasmic structures and proteins and deliver the contents to lysosomes for degradation (Klionsky 2005; Mizushima 2007). In contrast, the ubiquitin-proteasome system degrades proteins through ubiquitination and degradation by the 26S proteasome complex, bypassing lysosomes (Nedelsky et al. 2008). In the absence of the autophagy protein LGG-1, the *C. elegans* ortholog of *S. cerevisiae* Atg8, the core P granule proteins PGL-1 and PGL-3 are ectopically expressed in somatic foci during mid to late embryogenesis (Zhang et al. 2009). So we sought to test whether the removal of SIMR and CSR granules from the somatic cells during mid-to-late embryogenesis might similarly depend on the autophagy pathway.

To address this possibility, we first examined the expression of PGL-1 and SIMR-1 at 8-cell, 100-cell, and comma stages of embryogenesis following *lgg-1* or control (L4440) RNAi treatment of the parental animals. Consistent with previous work (Zhang et al. 2009), RNAi of *lgg-1* leads to ectopic expression of PGL-1 in cytoplasmic foci of the somatic cells at mid (100-cell) and late (comma) embryonic stages, while its germ granule localization remains unaffected (**Figure 5A**). Similarly, we found that the number of somatic SIMR granules at the 100-cell stage increases by approximately 2-fold in *lgg-1* RNAi-treated embryos compared to control (L4440) (**Figure 5A-B**). Further, in the comma stage, somatic SIMR granules remain in the soma in *lgg-1* RNAi-treated embryos, whereas SIMR granules are cleared from the somatic cells and restricted to germ cells in control (L4440) RNAi-treated embryos (**Figure 5A**). Examination of other somatic SIMR granule-associated proteins, including ENRI-2, RDE-3, and RRF-1, and unloaded NRDE-3 revealed that they also exhibit ectopic expression in the somatic cells of comma-stage embryos and continue to colocalize with SIMR-1 (**Figure 5—figure supplement 1A-B**). These findings indicate that the removal of SIMR granules from somatic cells during mid and late embryogenesis is regulated by autophagy, similar to the role of autophagy in the degradation of P granules from early somatic blastomeres.

Since both SIMR granules and P granules are regulated by the autophagy pathway during embryogenesis, we also examined the role for autophagy in regulating other granule-associated proteins in early embryos, specifically CSR-1 and ZNFX-1. Similar to PGL-1 and SIMR-1, CSR-1 and ZNFX-1 show increased numbers of somatic granules in 100-cell and comma stage embryos following parental treatment with *lgg-1* RNAi compared to control (L4440) (**Figure 5C**). To further investigate the spatial organization of these somatic granules in the absence of autophagy-mediated degradation, we examined the 100-cell stage embryos following *lgg-1* RNAi treatment using strains expressing PGL-1::BFP; SIMR-1::GFP; RFP::ZNFX-1, and GFP::CSR-1; SIMR-1::mCherry. Interestingly, these proteins display a variety of configurations relative to one another, distinct from their typical organization in the germline (Uebel et al. 2023). These configurations included granules composed of individual protein, multiple proteins adjacent to one another, and large aggregates composed of multiple proteins (**Figure 5D**). We speculate that many of the PGL-1-containing granules, including the larger aggregates, are associated with autophagosomes, as has been shown previously (Zhang et al. 2009). Overall, our findings demonstrate that the

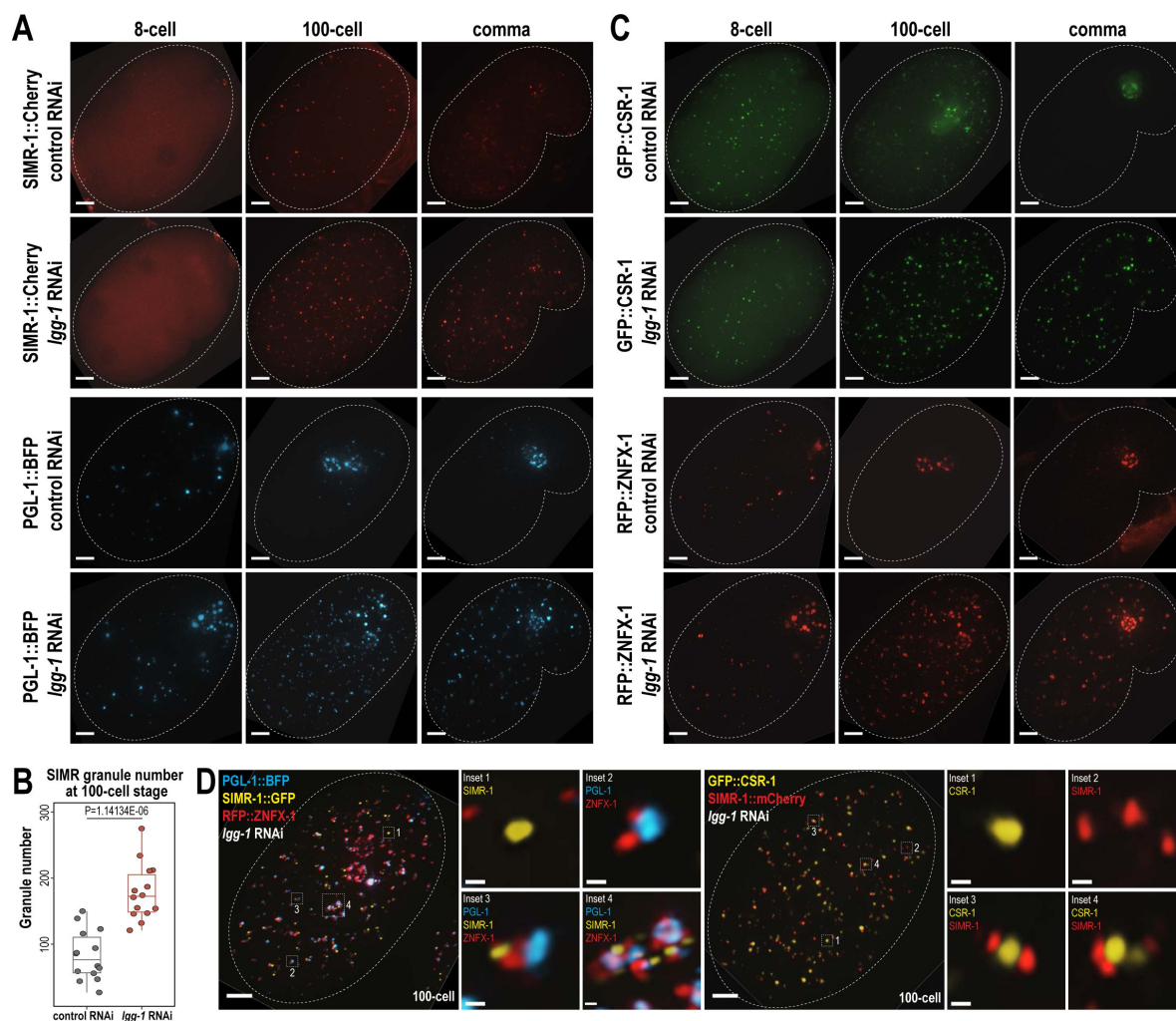


Figure 5.

Autophagy regulates the removal of SIMR granules and other embryonic granules.

A. Live imaging of SIMR-1::mCherry and PGL-1::BFP at 8-cell, 100-cell, and comma stage embryos following treatment of parental animals with control (L4440) and *lgg-1* RNAi. At least ten individual embryos were imaged for each genotype and stage. Dotted white line marks perimeter of embryo. Scale bars, 5 μ m.

B. Box plot quantifying the number of SIMR granules at the 100-cell stage following treatment of parental animals with control (L4440) and *lgg-1* RNAi. Fourteen embryos at each stage were used for quantification. Each dot represents an individual embryo, and all data points are shown. Bolded midline indicates median value, box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range. Two-tailed t-test was performed to determine statistical significance.

C. Live imaging of GFP::CSR-1 and RFP::ZNFX-1 at 8-cell, 100-cell, and comma stage embryos following treatment of parental animals with control (L4440) and *lgg-1* RNAi. At least ten individual embryos were imaged for each genotype and stage. Dotted white line marks perimeter of embryo. Scale bars, 5 μ m.

D. Live imaging of SIMR-1::GFP; RFP::ZNFX-1; PGL-1::BFP embryo and SIMR-1::mCherry; GFP::CSR-1 embryo at 100-cell stage embryos following treatment of parental animals with *lgg-1* RNAi. At least ten individual embryos were imaged for each genotype. Dotted white line marks perimeter of embryo. Insets show examples of granule adjacency. Scale bars for embryos, 5 μ m. Scale bars for insets, 0.2 μ m. Images in A, C-D are maximum projections of deconvolved 12.5 μ m z-stacks (about two-third of embryo depth).

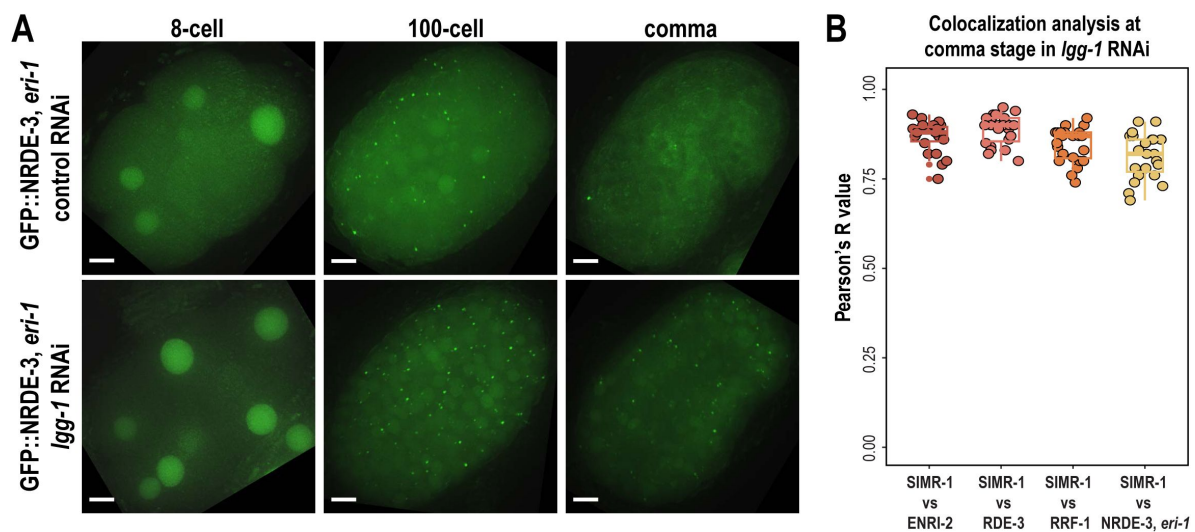


Figure 5—figure supplement 1.

Components of the SIMR granule are regulated by autophagy.

A. Live imaging of GFP::NRDE-3; *eri-1* at 8-cell, 100-cell, and comma stage embryos following treatment of parental animals with control (L4440) and *lgg-1* RNAi. At least ten individual embryos were imaged for each genotype and stage. Scale bars, 5 μ m. Images are maximum projections of deconvolved 12.5 μ m z-stacks (about two-third of embryo depth).

B. Box plot quantifying Pearson's R values of SIMR-1 with ENRI-2, RDE-3/MUT-2, RRF-1, and NRDE-3; *eri-1* at comma stage on *lgg-1* RNAi condition. At least twenty embryos at each stage were used for quantification. Each dot represents an individual embryo, and all data points are shown. Bolded midline indicates median value, box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range.

autophagy pathway is utilized to regulate the spatial-temporal expression of many embryonic granule-associated proteins, including proteins found in P, Z, SIMR, and CSR granules. Further, it is worth noting that P, Z, SIMR, and CSR granules are not all removed at the same developmental timepoint (for example, **Figure 1C**, 4G), suggesting the presence of additional regulatory mechanisms controlling the timing of their degradation.

NRDE-3 switches small RNA partners during embryonic development

The nuclear localization of NRDE-3 in the somatic cells of larvae depends on ERGO-1 and other proteins required for the biogenesis of ERGO-class 26G-RNAs (**Figure 1—figure supplement 1A-B**) (Guang et al. 2008). Sequencing of NRDE-3-bound 22G-RNAs at the L4 to young adult transition identifies a set of endogenous targets that overlaps substantially with those of ERGO-1 (Seroussi et al. 2023). Together, these data have led to the conclusion that NRDE-3 acts downstream of ERGO-1 to transcriptionally silence ERGO-target genes. Yet our data looking at the nuclear localization of NRDE-3 in embryos, demonstrate that this model may be an incomplete picture. Specifically, in *eri-1* and *rde-3* mutants where 26G-RNA or WAGO-class 22G-RNA biogenesis are abolished, respectively, NRDE-3 remains localized to the nucleus in early embryos (**Figure 2A**). The small RNA binding-defective NRDE-3(HK-AA) is localized exclusively to the cytoplasm at the same time point, indicating that small RNA binding is critical for nuclear import at this stage (**Figure 2A**). Accordingly, we must postulate that NRDE-3 binds another class of small RNA to promote nuclear entry in very early embryos. To investigate the identity of NRDE-3-bound small RNAs across embryonic development and to explore the role of the SIMR granules in promoting NRDE-3 small RNA binding, we immunoprecipitated NRDE-3 and sequenced associated small RNAs (IP-sRNA seq) in early embryos (≤ 100 -cell) and late embryos (> 300 -cell) in wild-type, *eri-1* mutant, *simr-1* mutant, and *enri-2* mutant animals (**Figure 6A**).

Prior to analyzing our data, we sought to better define the expected NRDE-3-bound small RNAs. We initially planned to use two previously defined ERGO-target gene lists: the first list (ERGO - Manage) is defined by small RNAs significantly depleted at least two-fold in *ergo-1* mutant compared to wild-type at the gravid adult stage, with at least 10 reads per million (RPM) in wild-type samples and a DESeq2 adjusted p-value of < 0.05 (Manage et al. 2020); the second list (ERGO - Fischer) is defined by genes reduced by 67% in *eri-7* adults or an average of 67% in *ergo-1*, *eri-1*, *eri-6*, and *eri-7* embryos, with at least 10 RPM in wild-type (Fischer et al. 2011). However, there is a poor overlap between these two datasets (**Figure 6—figure supplement 1D**), and small RNAs targeting many of these previously defined ERGO targets were not enriched by NRDE-3 in a published NRDE-3 IP-sRNA seq data on young adult animals that have begun oogenesis but do not yet have embryos (Seroussi et al. 2023) (**Figure 6—figure supplement 1A-B**). To define a more stringent NRDE-3-target gene list at the young adult stage, we chose genes with at least four-fold enrichment ($\log_2FC \geq 2$) and 100 RPM ($RPM \geq 100$) from the NRDE-3 IP-sRNA seq in young adults (Seroussi et al. 2023). This new list contains 119 genes and largely overlaps with the two previously defined ERGO-target gene lists (**Figure 6—figure supplement 1C-D**). To further confirm that this newly defined gene list represents NRDE-3 targets, we analyzed published small RNA and mRNA sequencing data from wild-type and *nrde-3* mutant mixed-stage embryos (before the bean stage) (Padeken et al. 2021). Compared to the Manage and Fischer ERGO-target gene lists, the NRDE-3-target gene list shows more significant small RNA depletion and a greater increase in mRNA expression in the *nrde-3* mutant compared to wild-type (**Figure 6—figure supplement 1A-C,E-F**). Therefore, we use the new NRDE-3-target gene list to represent the ERGO-1 pathway-dependent, NRDE-3-target genes (referred to here as ERGO targets) in the rest of this study.

We next examined the small RNAs bound to NRDE-3 in wild-type early embryos and late embryos, comparing our data to the published NRDE-3 IP-sRNA seq data on young adult animals (Seroussi et al. 2023). Strikingly, we found that in early embryos, the majority of small RNAs bound by

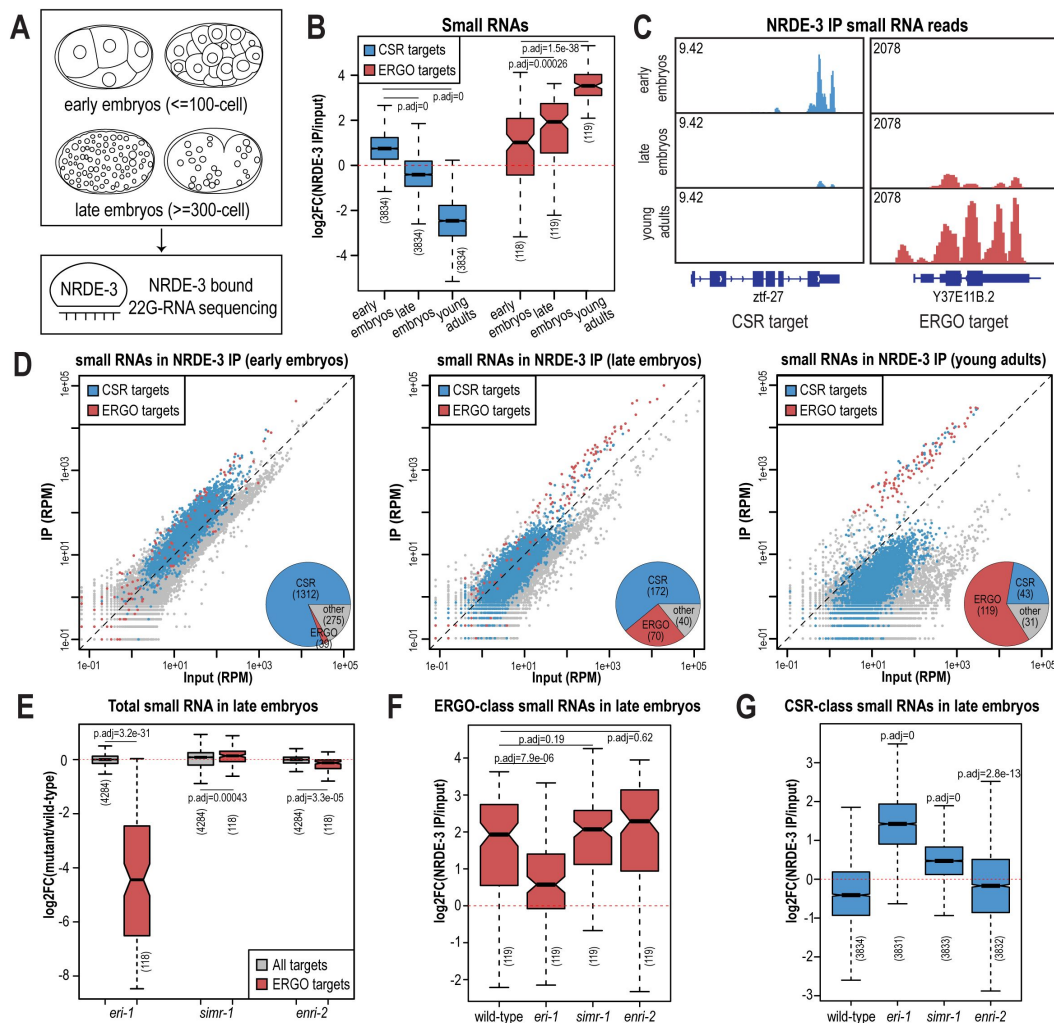


Figure 6.

NRDE-3 switches small RNA targets during development.

A. Diagram of IP-sRNA seq on NRDE-3 early embryos (≤ 100 -cell stage) and late embryos (≥ 300 -cell). GFP::FLAG::NRDE-3 was immunoprecipitated from embryo lysate and its associated small RNAs were isolated for sequencing.

B. Box plots depicting $\log_2$ (fold change small RNA abundance) in NRDE-3 IP compared to input for at least two biological replicates.

C. Normalized NRDE-3-bound small RNA read distribution across a CSR-target gene (*ztf-27*) and an ERGO-target gene (*Y37E11B.2*) in early embryos, late embryos, and young adults. One representative replicate is shown.

D. Normalized NRDE-3 IP compared to input small RNA reads in early embryos, late embryos, and young adults. CSR-target and ERGO-target genes are indicated in blue and red, respectively. One representative replicate is shown. Insets are pie charts describing numbers of CSR targets, ERGO targets, and other targets that are significantly enriched in the NRDE-3 IP. The enriched targets were defined as small RNAs with at least 2-fold enrichment in IP compared to input, average RPM > 10 , and p-values ≤ 0.05 .

E. Box plot depicting $\log_2$ (fold change small RNA abundance) in mutants compared to wild-type in late embryos for two or three biological replicates.

F. Box plots depicting $\log_2$ (fold change of ERGO-class small RNA abundance) in NRDE-3 IP compared to input in wild-type and mutants in late embryos for two or three biological replicates.

G. Box plots depicting $\log_2$ (fold change of CSR-class small RNA abundance) in NRDE-3 IP compared to input in wild-type and mutants in late embryos for two or three biological replicates.

For box plots in B,E-G, bolded midline indicates median value, box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range, excluding outliers. Two-tailed t-tests were performed to determine statistical significance and p-values were adjusted for multiple comparisons.

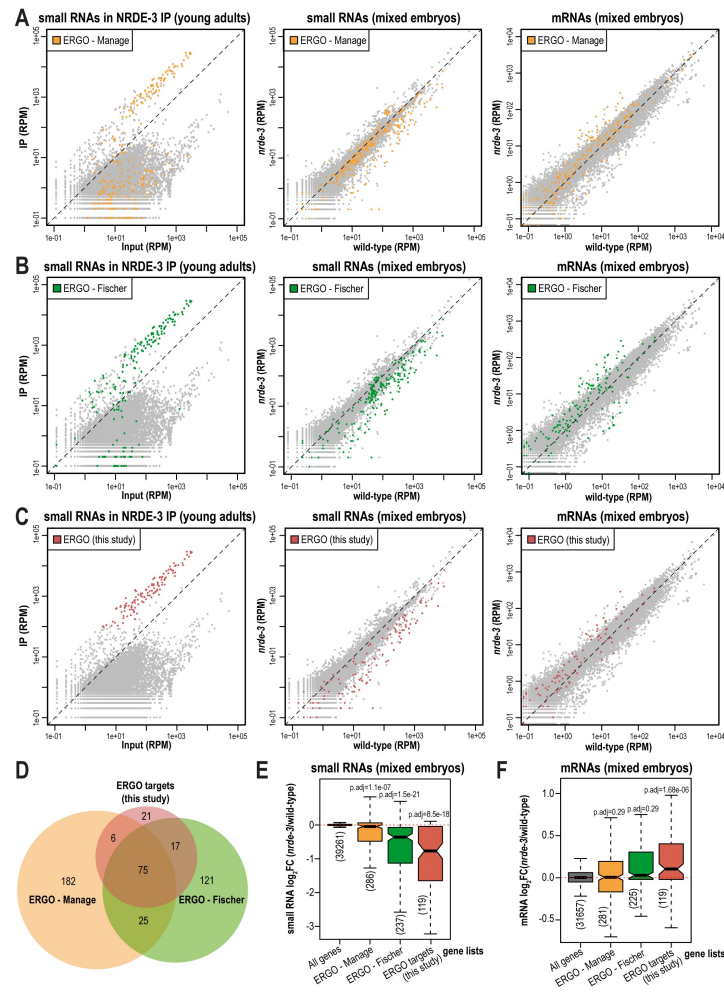


Figure 6—figure supplement 1.

Defining NRDE-3-bound ERGO-target genes.

A. Normalized NRDE-3 IP compared to input small RNA reads in young adults (left), *nrde-3* mutant compared to wild-type small RNA reads in mixed-stage embryos (middle), *nrde-3* mutant compared to wild-type mRNA reads in mixed-stage embryos (right). ERGO-target genes from *Manage et al (2020)* are indicated in yellow. One representative replicate is shown.

B. Normalized NRDE-3 IP compared to input small RNA reads in young adults (left), *nrde-3* mutant compared to wild-type small RNA reads in mixed-stage embryos (middle), *nrde-3* mutant compared to wild-type mRNA reads in mixed-stage embryos (right). ERGO-target genes from *Fischer et al (2011)* are indicated in green. One representative replicate is shown.

C. Normalized NRDE-3 IP compared to input small RNA reads in young adults (left), *nrde-3* mutant compared to wild-type small RNA reads in mixed-stage embryos (middle), *nrde-3* mutant compared to wild-type mRNA reads in mixed-stage embryos (right). NRDE-3-bound, ERGO-target genes are defined as genes with at least four-fold enrichment and an average of 100 RPM from the young adult NRDE-3 IP-sRNA seq libraries, and are indicated in red. One representative replicate is shown.

D. Venn diagrams indicate overlap of ERGO - Manage and ERGO - Fischer targets gene lists with the newly defined NRDE-3-bound, ERGO-target genes.

E. Box plots depicting log2(fold change of small RNA abundance) in a *nrde-3* mutant compared to wild-type in mixed-stage embryos for three biological replicates.

F. Box plots depicting log2(fold change of mRNA abundance) in a *nrde-3* mutant compared to wild-type in mixed-stage embryos for three biological replicates.

For box plots in E,F, bolded midline indicates median value, box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range, excluding outliers. Two-tailed t-tests were performed to determine statistical significance and p-values were adjusted for multiple comparisons. Adjusted p-values are a comparison of the indicated gene list to all genes in the mixed-stage embryos.

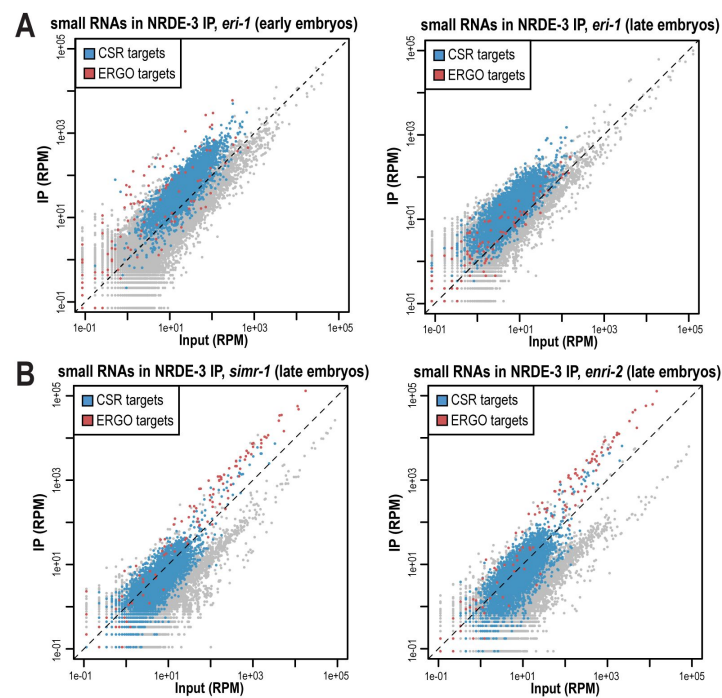


Figure 6—figure supplement 2.

NRDE-3-bound small RNA in different mutants.

A. Normalized NRDE-3 IP compared to input small RNA reads in *eri-1* mutant early (left) and late (right) embryos. CSR-target and ERGO-target genes are indicated in blue and red, respectively. One representative replicate is shown.

B. Normalized NRDE-3 IP compared to input small RNA reads in *simr-1* mutant (left) and *enri-2* mutant (right) late embryos. CSR-target and ERGO-target genes are indicated in blue and red, respectively. One representative replicate is shown.

NRDE-3 are CSR-class 22G-RNAs, which become progressively less enriched as the animals develop into late embryos and then young adults (**Figure 6B-D**). In contrast, enrichment for small RNAs targeting ERGO-target genes increases as *C. elegans* develops, and they become the majority of NRDE-3-bound small RNAs by young adulthood (**Figure 6B-D**). NRDE-3 also binds to CSR-target genes in the early embryos of the *eri-1* mutant, when it is observed to localize to the nucleus, indicating that the production of these NRDE-3-bound CSR-class 22G-RNAs is independent of *eri-1* and that CSR-class 22G-RNAs are likely sufficient to promote nuclear entry of NRDE-3 in the early embryo (**Figure 2A**, **Figure 6—figure supplement 2A**). To conclude, NRDE-3 binds to CSR-class 22G-RNA in early embryos but switches to bind preferentially to ERGO-dependent 22G-RNA in late embryos and young adults, suggesting that NRDE-3 may have two separable functions at distinct developmental time points. It is also curious to note that the change in small RNA preference of NRDE-3 coincides with the appearance and disappearance of the cytoplasmic SIMR granules, suggesting a potential role for SIMR-1 and ENRI-2 in promoting the switch of small RNA loading of NRDE-3.

SIMR-1 and ENRI-2 contributes to an efficient switch of NRDE-3 bound small RNAs

Since ERGO-dependent 22G-RNA loading was mainly observed in late embryos, we focused on NRDE-3-bound small RNAs in the *eri-1* mutant, *simr-1* mutant, and *enri-2* mutant late embryos to determine the role of SIMR granules in promoting NRDE-3 small RNA binding specificity. We first examined the levels of ERGO-dependent small RNAs in the total small RNA samples and observed depletion of small RNAs mapping to ERGO-target genes in the *eri-1* mutant (**Figure 6E**, **Figure 6—figure supplement 2A**). This result is consistent with previous research indicating that ERI-1 is required for ERGO-class 26G RNA production and downstream ERGO-dependent 22G-RNA production (Vasale et al. 2010; Han et al. 2009; Guang et al. 2008). ERGO-dependent small RNAs are not substantially depleted in *simr-1* or *enri-2* mutants, indicating that RRF-1 can still synthesize a similar amount of ERGO-dependent 22G-RNAs when the cytoplasmic SIMR granules are absent (**Figure 6E**). Following NRDE-3 immunoprecipitation in the *eri-1* mutant, we observed a reduction in NRDE-3 binding to ERGO-dependent small RNAs and an increase binding to CSR-class small RNAs (**Figure 6F-G**, **Figure 6—figure supplement 2A**). These data indicate that in the absence of ERGO-dependent small RNAs, some NRDE-3 protein remains associated with CSR-class small RNAs into late embryogenesis. In the *simr-1* and *enri-2* mutants, although we did not observe a significant reduction of NRDE-3-bound ERGO-dependent small RNAs, we saw a significant increase of CSR-class 22G-RNA binding in the *simr-1* mutant and a more modest but still significant increase of CSR-class 22G-RNA binding in the *enri-2* mutant (**Figure 6F-G**, **Figure 6—figure supplement 2B**). These results indicate that SIMR-1 and ENRI-2 are not required for the production of the ERGO-dependent small RNAs during embryogenesis, but may be required for an efficient switch from CSR-class to ERGO-dependent 22G RNAs.

NRDE-3 binds to CSR-class 22G-RNAs but does not silence CSR targets in early embryos

We discovered that NRDE-3 unexpectedly binds to CSR-class 22G-RNAs in early embryos, suggesting a potential new role for NRDE-3 that has not been previously reported. We hypothesized that NRDE-3 may function with CSR-1, perhaps to transcriptionally repress germline-expressed genes in early embryos while CSR-1 utilizes its catalytic activity to clear the same maternally-deposited transcripts (Quarato et al. 2021). To begin to address this hypothesis, we first sought to assess the degree to which NRDE-3-bound 22G-RNAs are similar to CSR-1-bound 22G-RNAs in early embryos. First, we examined the overlap of NRDE-3-targeted genes in early embryos with CSR-1-targeted genes in embryos or young adult animals. We found that the genes targeted by NRDE-3 substantially overlap with CSR-target genes at both stages (**Figure 7A**) (Quarato et al. 2021; Nguyen and Phillips 2021). Furthermore, the CSR-target genes yielding the highest abundance of CSR-1-bound small RNAs in embryos also have the highest abundance of

NRDE-3-bound small RNAs (**Figure 7B** [↗](#)). These CSR-target genes with highly abundant CSR-bound small RNAs are highly enriched by NRDE-3 only in embryos and not in young adults (**Figure 7—figure supplement 1A** [↗](#)). Next, CSR-class 22G-RNAs tend to be enriched at the 3' ends of mRNAs while WAGO-class 22G-RNAs are more evenly distributed across the gene bodies in adult animals (Ishidate et al. 2018 [↗](#); Singh et al. 2021 [↗](#)). Comparing NRDE-3-bound small RNAs from early embryos to a published dataset of CSR-1-bound small RNA from mixed-stage embryos, we found that both NRDE-3 and CSR-1 are heavily enriched for small RNAs derived from the 3' ends of CSR-target genes in embryos (**Figure 7C** [↗](#)). Interestingly, in adult animals, CSR-1-bound 22G-RNAs are still enriched for small RNA derived from the 3' end of CSR-target genes, however there is additionally a much higher enrichment of small RNAs derived from the gene bodies compared to in embryos (**Figure 7C** [↗](#)). It has previously been proposed that two types of CSR-class 22G-RNAs exist, those that depend on CSR-1 catalytic activity for their production and are derived primarily from target gene bodies, and those that are produced independently of CSR-1 catalytic activity and are derived primarily from target 3' UTRs (Singh et al. 2021 [↗](#)). Our data points to both NRDE-3 and CSR-1 binding only the latter, CSR-1 catalytic activity-independent, type of CSR-class 22G-RNA in early embryos. In contrast, NRDE-3 does not show enrichment for small RNAs derived from the 3' ends of ERGO target genes in embryos, and rather the small RNAs are distributed more evenly across the gene bodies (**Figure 7—figure supplement 1B** [↗](#)). CSR-1 utilizes its catalytic activity to slice and clear maternally-inherited mRNAs from early embryos, preferentially binding to transcripts degraded early in embryogenesis (Quarato et al. 2021 [↗](#)). We further demonstrate that NRDE-3 similarly binds preferentially to early-degraded transcripts (**Figure 7—figure supplement 1C** [↗](#)). Lastly, the expression of mRNAs targeted by CSR-1 decreases across embryonic development as CSR-1 actively slices and clears these maternal transcripts (Quarato et al. 2021 [↗](#)). We similarly find that the mRNAs targeted by NRDE-3 in young embryos, which correspond primarily to CSR-target mRNAs, decrease in expression across development, while its targets in young adults, corresponding primarily to ERGO-target mRNAs, increase in expression across development (**Figure 7—figure supplement 1D** [↗](#)). Together, these data reveal that NRDE-3 binds to the same group of small RNAs as CSR-1 in early embryos.

To further investigate whether NRDE-3 and CSR-1 function synergistically, we examined the fertility of the *csr-1::degron* strain and the *csr-1::degron; gfp::nrde-3(HK-AA)* strain upon auxin treatment to deplete CSR-1. As expected, both strains had 100% viable progeny with ethanol control treatment (**Figure 7—figure supplement 1E** [↗](#)). When growing on 4mM auxin plates, the number of embryos laid by the *csr-1::degron; gfp::nrde-3(HK-AA)* double mutant was significantly lower compared to the *csr-1::degron* single mutant and more of the double mutant produced no embryos (11.9%) compared to the *csr-1::degron* single mutant strain (7.5%), indicating a more severe sterility defect in the *csr-1::degron; nrde-3(HK-AA)* double mutant compared to the *csr-1::degron* alone (**Figure 7D** [↗](#), **Figure 7—figure supplement 1E** [↗](#)). Additionally, 5.9% of the auxin-treated *csr-1::degron* animals produced some F1 progeny that hatched, compared to no F1 hatching for any of the auxin-treated *csr-1::degron; gfp::nrde-3(HK-AA)* double mutant animals (**Figure 7—figure supplement 1E** [↗](#)). All together, these results indicate that loss of NRDE-3 enhances the fertility defects of CSR-1.

NRDE-3 is a nuclear Argonaute protein that recruits histone methyltransferases to target genes to deposit histone modifications such as H3K9me3 and H3K27me3 at these loci (Guang et al. 2008 [↗](#); Burton et al. 2011 [↗](#); Mao et al. 2015 [↗](#)). To examine whether NRDE-3 promotes deposition of H3K9me3 at CSR-target genes during embryogenesis, we analyzed the published anti-H3K9me3 ChIP-seq data of wild-type and *nrde-3* mutant mixed-staged embryos (Padeken et al. 2021 [↗](#)). In wild-type embryos, the targets of NRDE-3 in young adults, which correspond to ERGO-target genes, have high H3K9me3 levels, and are significantly decreased in the *nrde-3* mutant (**Figure 7E** [↗](#)). These data are consistent with previous research demonstrating that NRDE-3 deposits H3K9me3 at ERGO target genes (Burton et al. 2011 [↗](#)). However, NRDE-3 targets in early embryos do not show H3K9me3 enrichment in wild-type and do not have a significant change in the *nrde-3* mutant (**Figure 7E** [↗](#)). The same trend is also observed in the early degraded and late degraded targets

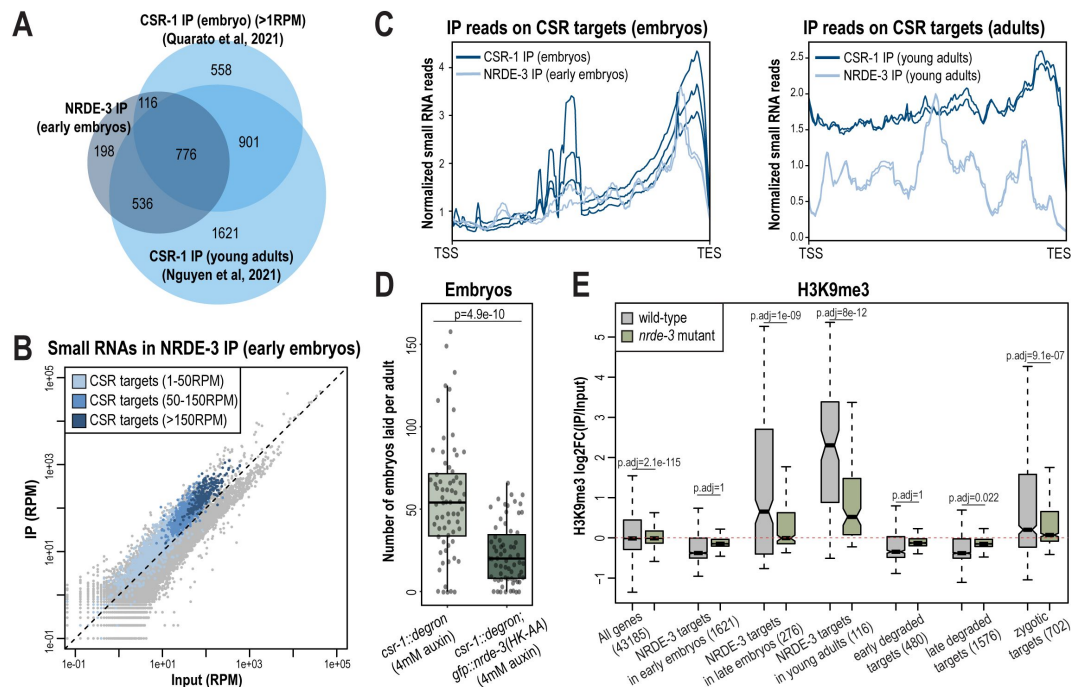


Figure 7.

NRDE-3 associates with CSR-class 22G-RNAs in early embryos.

A. Venn diagrams indicate overlap of NRDE-3 IP enriched targets in early embryos (this work), CSR-1 IP enriched targets in young adults (Nguyen *et al.*, 2021), and CSR-1 IP enriched targets in embryos (Quarato *et al.*, 2021).

B. Normalized NRDE-3 IP compared to input small RNA reads in early embryos. CSR-target genes with 1-50 RPM, with 50-100 RPM, and with more than 150 RPM are indicated in light blue, medium blue, and dark blue, respectively. One representative replicate is shown.

C. Density plot of small RNA enrichment on CSR targets in CSR-1 IP (dark blue), NRDE-3 IP (light blue) in embryos (left) and adults (right). Transcription start site (TSS) to transcription end site (TES) were plotted using normalized small RNA reads. All replicates are shown as individual lines.

D. Box plot quantifying the number of embryos laid per adult *csr-1::degron* or *csr-1::degron; gfp::nrde-3(HK-AA)* animal on 4mM auxin plate. At least 65 individuals from each strain were scored. Each dot represents an individual animal, and all data points are shown.

E. Box plot depicting $\log_2$ (fold change of H3K9me3 level in IP vs input) in wild-type (grey) and *nrde-3* mutant (green) mixed-stage embryos, indicating that the H3K9me3 level of NRDE-3 targets in early embryos are not affected in *nrde-3* mutant. Anti-H3K9me3 ChIP-seq data was obtained from Padeken *et al.* (2021).

For box plots in D-E, bolded midline indicates median value, box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range, excluding outliers. Two-tailed t-tests were performed to determine statistical significance and p-values were adjusted for multiple comparisons.

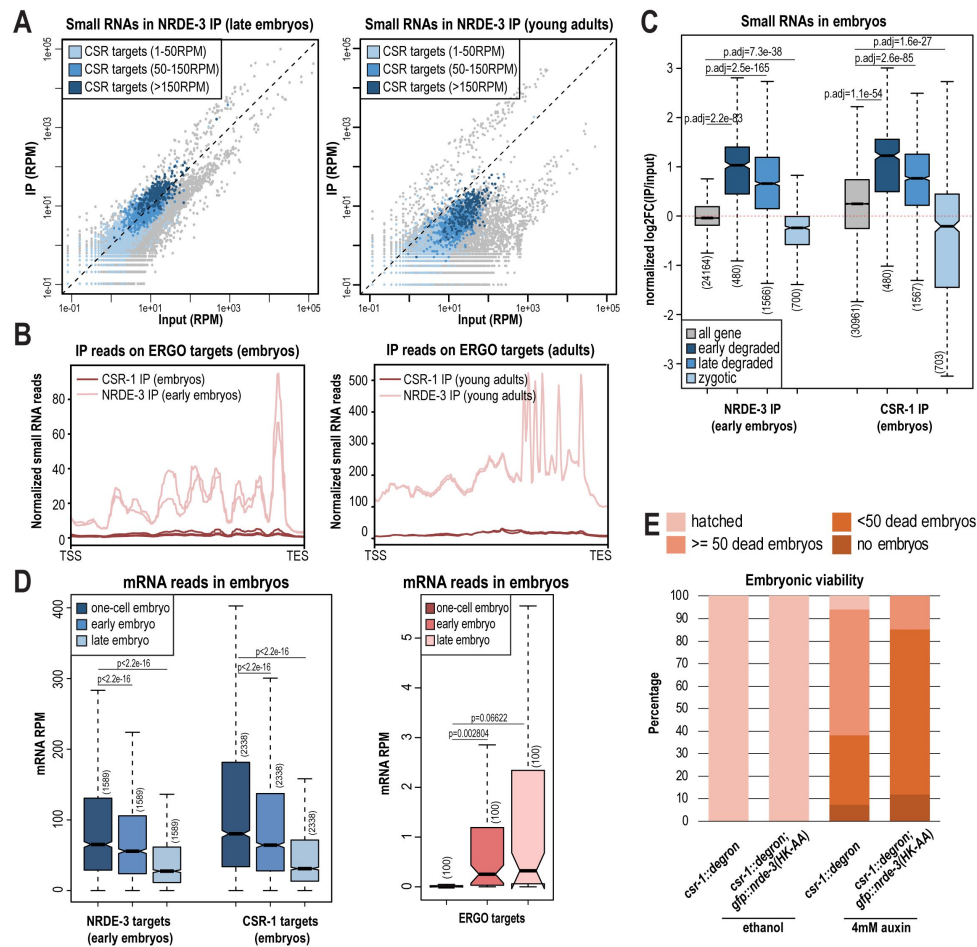


Figure 7—figure supplement 1.

NRDE-3 associates different classes of small RNAs during development.

A. Normalized NRDE-3 IP compared to input small RNA reads in late embryos (left) and young adults (right). CSR-target genes with 1-50 RPM, with 50-100 RPM, and with more than 150 RPM are indicated in light blue, medium blue, and dark blue, respectively. One representative replicate is shown.

B. Density plot of small RNA enrichment on ERGO targets in CSR-1 IP (dark red), NRDE-3 IP (light red) in the embryos (left) and adults (right). Transcription start site (TSS) to transcription end site (TES) were plotted using normalized small RNA reads. All replicates are shown as individual lines.

C. Box plots depicting normalized $\log_2(\text{fold change of small RNA abundance in IP vs input})$ in a NRDE-3 IP in early embryos and CSR IP in embryos for two or three biological replicates. All genes list includes all genes expressed in IP or input. Early degraded mRNAs are maternal mRNAs that show at least twofold reduction in mRNA levels in early embryos (4 to 20 cell-stage) compared to 1-cell embryos (Quarato et al., 2021). Late degraded mRNAs are maternal mRNAs that show stable levels of mRNAs in early embryos and at least twofold reduction in late embryos (more than 20-cell stage) (Quarato et al., 2021). Zygotic mRNAs are mRNAs that are not detectable in 1-cell embryos but accumulate in early and late embryos (Quarato et al., 2021).

D. Box plot depicting mRNA RPM in one-cell embryo (dark blue), early embryo (medium blue), and late embryo (light blue) on NRDE-3 targets in early embryos (this study) and CSR-1 targets in embryos (Quarato et al., 2021) (left), and mRNA RPM in one-cell embryo (dark pink), early embryo (medium pink), and late embryo (light pink) on ERGO targets (this study) (right). All biological replicates (two or three) are included. mRNA-seq data was obtained from Quarato et al. (2021).

E. Quantification of the fertility for *csr-1::degron* and *csr-1::degron, gfp::nrde-3(HK-AA)* animals on ethanol and 4mM auxin plate. The fertility of each animal was categorized as: hatched (some embryos laid by the animal hatched), ≥ 50 dead embryos (animal laid at least 50 unhatched embryos and no hatched embryos), < 50 dead embryos (animal laid less than 50 unhatched embryos and no hatched embryos), no embryos (animal did not lay any embryos). At least 65 individual adult animals of each strain and each condition were used for quantification.

(Figure 7E [↗](#)). These results indicate that the CSR targets are not H3K9 trimethylated in the early embryos. However, we cannot rule out the possibility that NRDE-3 may function to deposit other histone modification targets such as H3K27me3 and H3K23me3 or inhibit RNA Polymerase II (Pol II) on CSR targets to transcriptionally silence these genes in early embryos.

NRDE-3 associates with CSR-class 22G-RNAs in oocytes

In addition to being expressed in early embryos, NRDE-3 is also expressed in germ cells, starting in late pachytene through oogenesis (Seroussi et al. 2023 [↗](#); Chen and Phillips 2024b [↗](#)). Because we were unable to detect a change in H3K9me3 in early embryos in the *nrde-3* mutant, we next sought to determine whether NRDE-3 binds to CSR-class 22G-RNAs in germ cells. Curiously, in late embryos from *eri-1* mutant and *rde-3* mutant, we have observed nuclear localization of NRDE-3 only in the primordial germ cells (Figure 2A [↗](#)), raising an intriguing hypothesis that NRDE-3 might bind to CSR-class 22G-RNAs in germ cells throughout development and inherit NRDE-3-bound CSR-class 22G-RNAs to early embryos.

To determine whether NRDE-3 also binds to CSR-class 22G-RNAs in oocytes, we first asked whether the nuclear localization of NRDE-3 in the adult germline depends on ERI-1 and RDE-3, key components of the ERI and Mutator complexes, respectively (Figure 1—figure supplement 1A–B [↗](#)). Loss of NRDE-3 nuclear localization in these mutants would indicate that NRDE-3 binds to ERGO-dependent 22G-RNAs in oocytes. Instead, we found that NRDE-3 localizes to the nuclei of oocytes in *eri-1* and *rde-3* mutants, similar to wild-type, but is restricted to cytoplasm in the *nrde-3(HK-AA)* small RNA binding mutant (Figure 8A [↗](#)). These data demonstrate that NRDE-3 nuclear localization remains dependent on small RNA binding, but ERGO-dependent 22G-RNAs are not required, consistent with NRDE-3 localization in early embryos. Next, to more directly address whether NRDE-3 binds to CSR-class 22G-RNAs in oocytes, we utilized the auxin-inducible degron (AID) system to deplete the RdRP EGO-1 by growing the worms on 4mM auxin plates starting at the L1 stage (Zhang et al. 2015 [↗](#)). Surprisingly, NRDE-3 still localizes to nuclei in both germ cells and early embryos upon EGO-1 depletion (Figure 8B [↗](#)), indicating that NRDE-3 either does not exclusively bind CSR-class 22G-RNAs in the germline, or NRDE-3 has the capacity to bind other small RNAs when the CSR-class 22G-RNAs are absent. We did observe some cytoplasmic NRDE-3 granules in a subset of 8-cell stage embryos following EGO-1 depletion (Figure 8B [↗](#)), suggesting that a proportion of NRDE-3 might be unloaded. To further probe which small RNAs NRDE-3 binds to in the germline, we introduced a *rde-3* mutation into the GFP::NRDE-3; degron::EGO-1 strain to deplete all WAGO-class 22G-RNAs, with include the ERGO-dependent 22G-RNAs, along with the CSR-class 22G-RNAs. We observed that NRDE-3 no longer localizes to the nucleus in both germline and early embryos in the absence of both WAGO-class and CSR-class 22G-RNAs (Figure 8B [↗](#)). These results, in combination with our sequencing data, indicate that NRDE-3 likely binds CSR-class 22G-RNAs in the germline and early embryos but has the capacity to additionally bind WAGO-class 22G-RNAs when CSR-class 22G-RNAs are depleted. Furthermore, because somatic transcription is not initiated in early embryos until the 4-cell stage (Seydoux and Fire 1994 [↗](#)), we conclude that the NRDE-3 is loaded with CSR-class 22G-RNAs in the parental germline and then transmitted to early embryos.

NRDE-3 represses RNA Pol II and promotes global transcriptional repression in oocytes

Oocytes undergo global transcriptional repression across diverse species (Woodland 1987 [↗](#)). In *C. elegans*, transcriptional shutdown begins during the diakinesis stage of late oocytes to prepare for fertilization, and reactivates at the 4-cell stage embryos to initiate zygotic transcription (Walker et al. 2007 [↗](#)). Previous work has shown that topoisomerase II acting with the condensin II complex, H3K9me3, and the zinc-finger containing protein PIE-1 are critical for global transcriptional

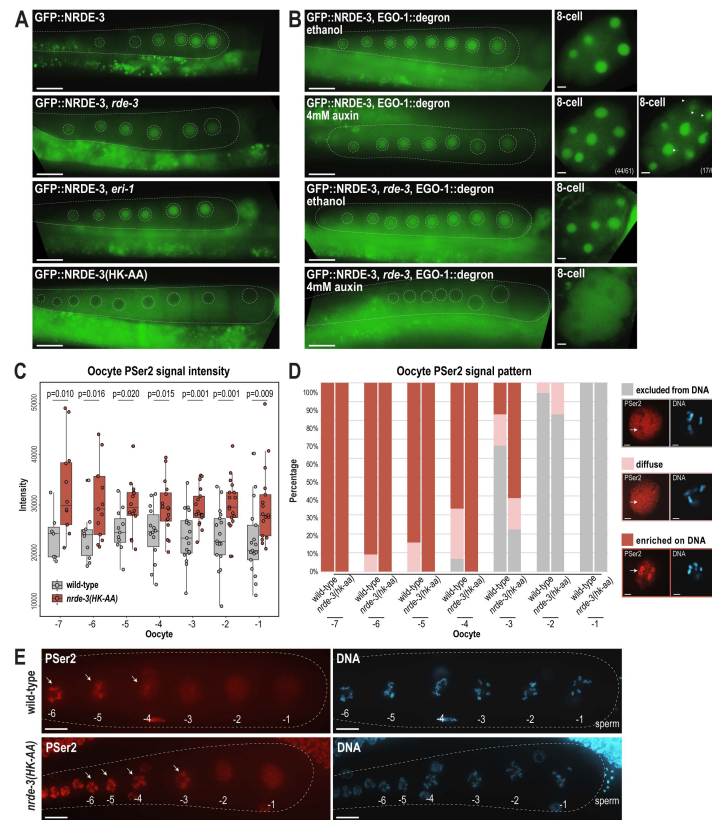


Figure 8.

NRDE-3 associates with CSR-class 22G-RNAs to represses RNA Polymerase II in oocytes.

A. Live imaging of GFP::3xFLAG::NRDE-3 in one-day-adult germlines for wild-type, *eri-1*, *rde-3*, and *nrde-3(HK-AA)* mutants, showing that NRDE-3 localizes to the nuclei of oocytes in wild-type, *eri-1* mutant, and *rde-3* mutants, and to the cytoplasm in the *nrde-3(HK-AA)* mutant. At least five individual gonads were imaged for each genotype. Dotted white line traces the proximal portion of the *C. elegans* gonad and outlines the individual oocytes. Scale bars, 25 μ m.

B. Live imaging of one-day-adult germlines and 8-cell embryos for EGO-1::degron; GFP::3xFLAG::NRDE-3 in a *rde-3* mutant (bottom) with ethanol (control) and 4mM auxin treatment, showing that loss of both WAGO-class and CSR-class 22G-RNAs (*rde-3* mutant and degron-mediated EGO-1 depletion) is necessary to result in cytoplasmic localization of NRDE-3 in both oocytes and early embryos. At least five individual gonads and embryos were imaged for each treatment condition. Dotted white line traces the proximal portion of the *C. elegans* gonad and outlines the individual oocytes. Arrowheads indicate granule localized NRDE-3 in 8-cell stage embryos. Scale bars, 25 μ m in adults and 5 μ m in embryos.

C. Box plot quantifying the RNA Pol II Pser2 signal intensity in oocytes of wild-type (GFP::NRDE-3 strain), and *nrde-3(HK-AA)* mutant (GFP::NRDE-3(HK-AA) strain), showing that Pser2 signal is significantly increased in all oocytes in the *nrde-3(HK-AA)* mutant. Each dot represents an individual oocyte, and all data points are shown. Bolded midline indicates median value, box indicates the first and third quartiles, and whiskers represent the most extreme data points within 1.5 times the interquartile range. Two-tailed t-tests were performed to determine statistical significance. See Materials and Methods for a detailed description of quantification methods.

D. Bar plot quantifying the RNA Pol II Pser2 expression pattern in wild-type (GFP::NRDE-3 strain), and *nrde-3(HK-AA)* mutant (GFP::NRDE-3(HK-AA) strain) oocytes, showing that the Pser2 signal is retained on DNA longer in the *nrde-3(HK-AA)* mutant. At least 10 oocytes were used for quantification for each strain. Examples of three patterns of Pser2 signal are shown on right. Arrows point to a region of DNA to highlight Pser2 enrichment or exclusion. Scale bars, 2 μ m.

E. Immunofluorescence imaging of Pser2 signal and DAPI stained DNA in oocytes of wild-type (GFP::NRDE-3), and *nrde-3(HK-AA)* mutant (GFP::NRDE-3(HK-AA)), showing that the Pser2 signal appears earlier on DNA in the *nrde-3(HK-AA)* mutant. Images are maximum intensity projections of 12.5 μ m z-stack, which allows for optimal visualization of the DNA-associated Pser2 signal, but obscures the 'excluded from DNA' pattern. At least five individual animals for each genotype. Arrows indicate the Pser2 signal on DNA. Scale bars, 25 μ m.

repression (Belew et al. 2023 [↗](#)), but the complete molecular mechanisms underlying the oocyte-to-embryo transition remain not fully understood. We therefore aimed to address whether germline-expressed NRDE-3 could play a role in these pathways.

Our hypothesis that NRDE-3 may mediate transcriptional repression in oocytes is supported by several lines of evidence. First, NRDE-3 is expressed at the correct place and time—in the germline, from late pachytene to diakinetically oocytes (Seroussi et al. 2023 [↗](#))—and is known to co-transcriptionally silencing genes by depositing H3K9me3 and inhibiting Pol II transcription (Guang et al. 2008 [↗](#)). Second, the slicing activity of CSR-1 is required to inhibit Pol II-dependent transcription in maturing oocytes (Fassnacht et al. 2018 [↗](#)). CSR-1 is not known to directly inhibit transcription, but it is required for the production of at least a subset of the CSR-class 22G-RNAs (Singh et al. 2021 [↗](#)); therefore, the CSR-1-dependent Pol II inhibition could be mediated by NRDE-3-bound CSR-class 22G-RNAs. Third, H3K9me3 and the H3K9me3 methyltransferase SET-25 are required for transcription repression in oocytes (Belew et al. 2023 [↗](#)). NRDE-3 can act to recruit SET-25 and establish H3K9me3-containing heterochromatin (Padeken et al. 2021 [↗](#)), making it a strong candidate for initiating H3K9me3-dependent transcriptional silencing in oocytes.

The above evidence connects Pol II repression in oocytes to the co-transcriptional silencing function of NRDE-3 and the requirement for CSR-class 22G-RNAs in oocytes. Phosphorylation of RNA Polymerase II (Pol II) on its large subunit C-terminal domain (CTD) serves as a well-studied marker of transcription activity, with Ser5 phosphorylation indicating transcription initiation and Ser2 phosphorylation marks elongation. Therefore, to monitor Pol II activity in oocytes, we stained wild-type and *nrde-3(HK-AA)* mutant gonads using an antibody specific for phosphorylated serine 2 (PSer2) on the Pol II C-terminal domain (CTD), a marker for transcription elongation (Belew et al. 2023 [↗](#); Seydoux and Dunn 1997 [↗](#)). Consistent with previously reports, PSer2 staining in the most proximal oocytes exhibited distinct patterns of localization: (1) PSer2 excluded from DNA ('excluded from DNA'), (2) PSer2 present both on and off DNA ('diffuse'), and (3) PSer2 enriched on DNA ('enriched on DNA'). The PSer2 signal intensity and the localization pattern serve as indicators of active transcription levels (Belew et al. 2023 [↗](#)). Quantification the PSer2 signal intensity shows a significant increase in PSer2 in the *nrde-3(HK-AA)* mutant across all oocytes (from -1 to -7), indicating a global increase in transcription elongation in the *nrde-3(HK-AA)* mutant (**Figure 8C** [↗](#)). Furthermore, by quantifying the PSer2 localization patterns, we found that elongating RNA Pol II stays associated with DNA longer in the *nrde-3(HK-AA)* mutant, compared to wild-type. This data is consistent with what has been observed previously in the *set-25* mutant (Belew et al. 2023 [↗](#)) (**Figure 8D-E** [↗](#)). Therefore, we conclude that NRDE-3 contributes to global transcriptional repression in oocytes by repressing RNA Pol II.

Discussion

Germ granules are phase-separated condensates that localize to the perinuclear region of germ cells. In *C. elegans*, the known constituents of the germ granule have expanded over the last decades, such that germ granules now comprise multiple domains including P granules, *Mutator* foci, Z granules, SIMR foci, E granules, and D granules. Here we discovered that several components of SIMR foci and *Mutator* foci also localize to cytoplasmic granules during specific stages of embryogenesis, where their temporal expression is regulated by autophagic degradation. We propose that these granules may serve as sites for the synthesis and loading of 22G-RNAs into the nuclear Argonaute NRDE-3. Furthermore, we showed that NRDE-3 switches its small RNA targets during embryogenesis, coincident with the formation of SIMR granules; during oogenesis, NRDE-3 binds to CSR-class 22G-RNAs to promote global transcriptional repression prior to the oocyte-to-embryo transition, and then in the developing soma, NRDE-3 binds to ERGO-dependent 22G-RNAs to silence retrotransposons and recently duplicated genes. Together, our study reveals a

new world of embryonic RNAi factor condensates and uncovers two temporally distinct roles for NRDE-3, underscoring the need for careful examination of localization and targets of RNAi pathways across development (**Figure 9** [↗](#)).

A role for SIMR-1 as a platform for nuclear Argonaute protein loading

Previously we demonstrated that SIMR-1 and HRDE-2 are required to recruit unloaded HRDE-1, the germline nuclear Argonaute protein, to germ granules and to ensure correct 22G-RNA loading (Chen and Phillips 2024a [↗](#)). Here we reveal that SIMR-1 and another HRDE-2 paralog, ENRI-2, are similarly essential to recruit unloaded NRDE-3, the somatic nuclear Argonaute protein, to embryonic SIMR granules. We speculate that SIMR-1 and ENRI-2 are similarly important for NRDE-3 22G-RNA loading; however, we did not observe a significant change in the ERGO-dependent 22G-RNAs loaded by NRDE-3 in *simr-1* or *enri-2* mutant embryos compared to wild-type. While initially surprising based on the results of similar experiments with HRDE-1 in the germline, we envision several possible explanations. First, it is possible that SIMR-1 and ENRI-2 act to bring unloaded NRDE-3 in close proximity to the ERGO-dependent 22G-RNA biogenesis machinery, but that NRDE-3 loading can still occur diffusely in the cytoplasm, albeit with lower efficiency. Both the RdRP RRF-1 and unloaded NRDE-3 diffusely localize to cytoplasm in the *simr-1* mutant (**Figure 3A** [↗](#), **4D** [↗](#)), suggesting that NRDE-3 may load the ERGO-class small RNAs synthesized in the cytoplasm in the absence of SIMR-1. Differences in NRDE-3 loading efficiency would likely not be detected by our NRDE-3 IP-small RNA sequencing experiment. Second, SIMR-1 and ENRI-2 could act to sequester unloaded NRDE-3 away from other small RNAs (i.e. CSR-class 22G-RNAs) to prevent misloading. Misloading should be detectable in our NRDE-3 IP-small RNA sequencing experiment and we do see a modest increase in CSR-class 22G-RNAs loaded by NRDE-3 in late embryos; however, it is unclear the extent to which newly synthesized, and unloaded CSR-class 22G-RNAs are even present in the cytoplasm, as the primary source for CSR-class 22G-RNAs may be the maternal germline. Thus, unlike in the adult germline where HRDE-1 incorrectly loads CSR-class 22G-RNAs in the absence of HRDE-2, there may not be an equivalent source of incorrect small RNAs that NRDE-3 can bind to (i.e. correct length, 5' nucleotide and modifications) in the embryo. To further probe these possibilities, we need to more carefully assess the dynamics of NRDE-3 loading across embryonic development and possibly disrupt the formation of embryonic CSR granules to determine whether compartmentalization of the CSR-class 22G-RNA pathway is also contributing to correct loading of NRDE-3 in the absence of SIMR-1 and ENRI-2.

We do not know the precise functions of SIMR-1 and ENRI-2, however we have previously proposed that SIMR-1 mediates protein-protein interactions through its extended Tudor domain (Manage et al. 2020 [↗](#)). ENRI-2 and its paralog HRDE-2 have structural similarities to a HELICc domain, and SIMR-1, ENRI-2, and HRDE-2 have large unstructured domains (**Figure 9—figure supplement 1A** [↗](#)) (Lewis et al. 2020 [↗](#); Chen and Phillips 2024a [↗](#)). With the advent of protein complex prediction algorithms (Abramson et al. 2024 [↗](#)), we sought to examine the potential physical interactions between ENRI-2 and NRDE-3, as well as their paralogs HRDE-2 and HRDE-1. In both models, the structured HELICc domain of HRDE-2 and ENRI-2 dock on the Mid domain of their respective Argonaute partners. Interestingly, the unstructured C-terminal domains of ENRI-2 and HRDE-2 extend into the small RNA binding pocket of their respective nuclear Argonaute binding partners (**Figure 9—figure supplement 1B** [↗](#)). At this point, we do not know whether these structures are reflective of the actual geometry of the proteins *in vivo*, but it is tempting to speculate that the C-terminal disordered regions of the ENRI-2/HRDE-2 proteins could regulate 22G-RNA loading through interaction with the small RNA binding pocket of NRDE-3 and HRDE-1. Further study will be necessary to determine whether these interactions between disordered regions and the small RNA binding pocket are necessary for correct small RNA loading and whether that mechanism extends to other WAGO proteins.

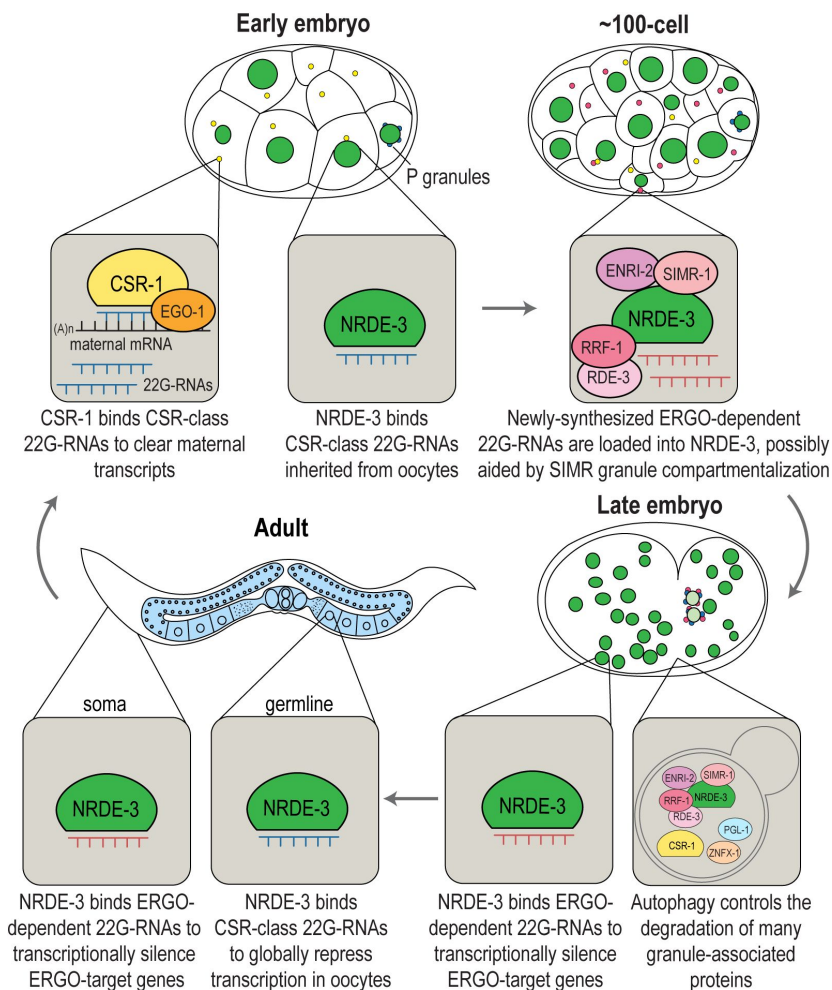


Figure 9.

Model for temporally- and developmentally-regulated NRDE-3 function.

Model of NRDE-3, SIMR-1, and CSR-1 function during *C. elegans* development. In early embryos, CSR-1 and EGO-1 localize to CSR granules and synthesize CSR 22G-RNAs to slice and clear maternal mRNAs. NRDE-3 binds CSR 22G-RNA in the nucleus, which are inherited from the oocytes. During mid-embryogenesis (e.g. around the 100-cell stage), unloaded NRDE-3, ENRI-2, RRF-1, and RDE-3 are localized to SIMR granules in somatic cells dependent on SIMR-1, where we propose that ERGO-dependent 22G-RNAs are produced and loaded into NRDE-3. In late embryos, NRDE-3 binds ERGO-dependent 22G-RNAs and silences ERGO-target genes in the nucleus, while autophagy controls selective degradation of SIMR and other embryonic granules. In adult *C. elegans*, somatic localized NRDE-3 associates with ERGO-dependent 22G-RNAs to transcriptionally silence ERGO-target genes, while germline localized NRDE-3 associates with CSR-class 22G-RNAs to globally repress transcription and promote chromatin compaction in oocytes, ultimately being deposited into early embryos.

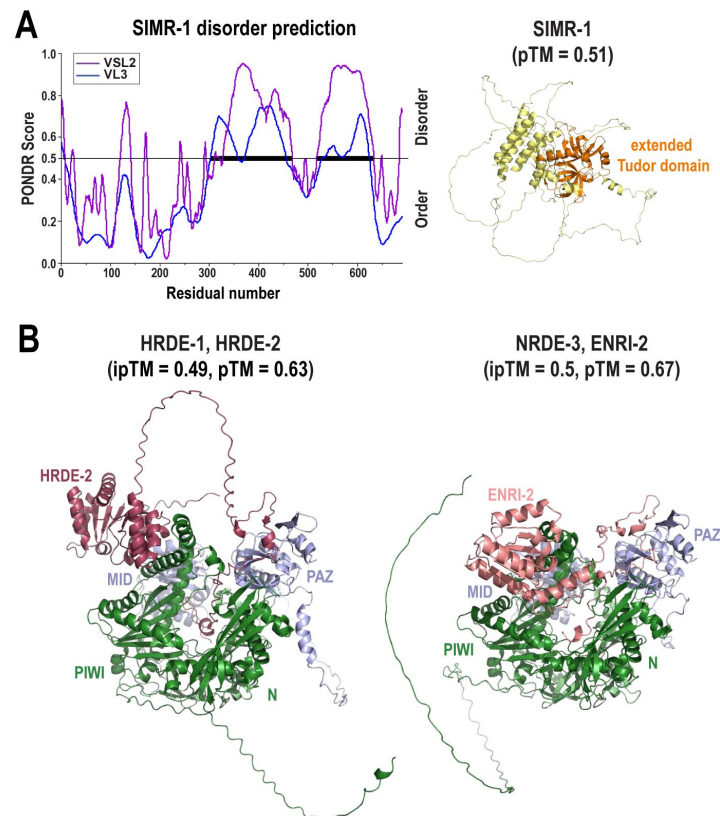


Figure 9—figure supplement 1.

Predicted structures of SIMR-1, nuclear Argonaute proteins, and interactors.

A. Graph displaying disorder tendency for the SIMR-1 protein sequence and AlphaFold3 predicted structures of SIMR-1. The disorder prediction was made using POND (http://www.pondr.com) with VSL2 and VSL3 parameters. Regions with POND scores of greater than 0.5 indicate disorder and regions with POND score less than 0.5 indicate order. The AlphaFold3 predicted confidence score of SIMR-1 is shown. The extended Tudor domain (aa. 89-259) of SIMR-1 is highlighted in orange.

B. AlphaFold3 predicted structures of HRDE-1 - HRDE-2, and NRDE-3 - ENRI-2 complexes, showing that the C-terminus of HRDE-2 and ENRI-2 are inserted into small RNA binding pockets of HRDE-1 and NRDE-3 respectively. Domains of HRDE-1 and NRDE-3 are indicated and AlphaFold3 predicted confidence scores are shown.

Compartmentalization of RNAi pathways

Most of the studies on the organization of *C. elegans* RNAi factors in granules focus on the germline. Here we find that multiple proteins associated with 22G-RNA biogenesis and function, including SIMR-1, RDE-3, RRF-1, ENRI-2, and unloaded NRDE-3, are localized to distinct condensates in *C. elegans* embryos. We speculate that these SIMR granules, which appear and then disappear during the course of embryonic development, play a functional role in the NRDE-3 nuclear RNAi pathway. This idea leads to an intriguing question: what role does organization of the RNAi pathways into condensates play in the soma vs. in the germline?

In germ cells, RNAi factors are visibly segregated into distinct compartments within the germ granule which assemble hierarchically (Uebel et al. 2023 [↗](#)). Germ granules are also intimately linked to nuclear pores, leading to a model where highly concentrated mRNAs, newly exported from and adjacent to the nuclear pore, nucleate assembly of regulatory factors into visible granules. In fact, nuclear pores are clustered beneath germ granules in germ cells, and evidence suggests that most, if not all, nascent mRNAs are exported through pores associated with germ granules (Pitt et al. 2000 [↗](#); Sheth et al. 2010 [↗](#)). In contrast, nuclear pores are distributed more evenly across the nuclear periphery in embryos and, in this work, we find that while some embryonic SIMR granules appear adjacent to the nuclear periphery, many are distributed in the cytoplasm (see **Figure 2A** [↗](#), for example). Thus, unlike in germ granules, there is no obvious trajectory from the nucleus that RNAs would follow to end up in embryonic SIMR granules. Further, in the germline, we have speculated that the adjacent and hierarchical assembly of germ granule compartments could be determined by the order of molecular events required for RNA silencing (Uebel et al. 2023 [↗](#)). While we on occasion see docking between embryonic SIMR granules and CSR granules (**Figure 4I** [↗](#)), we do not see any more complex arrangement of granule compartments in embryos similar to what we have observed in the germline. What that means regarding the functionality of embryonic SIMR granules is unclear. Another possibility worth considering is that SIMR granules are not actually required for ERGO-dependent 22G-RNA biogenesis and NRDE-3 loading in embryos but rather that they reflect a concentration of the small RNA biogenesis machinery beyond the solubility limit of the cytoplasm, resulting in the demixing of some RNP complexes into visible SIMR granules (Putnam et al. 2023 [↗](#)). By this “incidental condensate” model, ERGO-dependent 22G-RNA biogenesis and NRDE-3 loading occur just as efficiently, or perhaps more so, diffusely in the cytoplasm.

Both embryonic and germ granules exhibit dynamic expression patterns, suggesting that expression and function of small RNA factors are critical at discrete developmental time points. In the germline, multiple Argonaute proteins are expressed exclusively during oogenesis (ERGO-1) or spermatogenesis (ALG-3, ALG-4, CSR-1b, WAGO-10) (Billi et al. 2012 [↗](#); Han et al. 2009 [↗](#); Conine et al. 2010 [↗](#); Reinke et al. 2004 [↗](#); Nguyen and Phillips 2021 [↗](#); Charlesworth et al. 2021 [↗](#)) and MUT-16 expression fluctuates across germ cell development, peaking in the mitotic region (Uebel et al. 2020 [↗](#)). Similarly, embryonic SIMR granules appear in early embryos and disappear by late embryogenesis. Regardless as to whether SIMR granules are incidental condensates or functional sites for NRDE-3 loading, these data indicate that the levels or activities of these proteins are developmentally regulated.

It is additionally curious that embryonic and germ granules share many protein components yet possess distinct differences in content and assembly requirements. For instance, several RNAi proteins, such as RRF-1 and RDE-3, are shared between *Mutator* foci and embryonic SIMR granules, while the paralogous ENRI-2/NRDE-3 and HRDE-2/HRDE-1 pairs are found in embryonic SIMR granules and germline SIMR foci, respectively. It is unclear why the *Mutator* and SIMR components are visible as separate compartments in germ granules but are together in embryonic SIMR foci. This difference is highlighted by the requirement for MUT-16 in the assembly of embryonic SIMR granules but not germline SIMR foci (**Figure 4E** [↗](#)) (Manage et al. 2020 [↗](#)).

Further investigation into the assembly and protein components of embryonic and germ granules will be crucial for elucidating the functional differences between embryonic and germ granules and dissecting the mechanisms of 22G-RNA loading into NRDE-3.

Spatial-temporal regulation of SIMR and other embryonic granules by autophagy

Autophagy is a conserved eukaryotic protein degradation pathway involving the formation of double-membrane autophagosomes, which fuse with lysosomes for degradation (Nakatogawa et al. 2009 [\[1\]](#)). Autophagy has been linked to small RNA pathways and germ granules in *C. elegans* and other organisms. In *Arabidopsis*, the turnover of Argonaute protein AGO1 is controlled by autophagy in both antiviral and nonviral contexts (Derrien et al. 2012 [\[2\]](#)). In mammalian cells, AGO2 and Dicer are targeted for degradation via autophagy (Gibbins et al. 2012 [\[3\]](#)). In *C. elegans*, P granule components PGL-1 and PGL-3 are recruited to somatic aggregates during embryogenesis and degraded by autophagy (Zhang et al. 2009 [\[4\]](#)). Interestingly, miRNA pathway components ALG-1, ALG-2 and their interactor AIN-1/GW182 are also degraded by autophagy, while in aggregates distinct from PGL somatic aggregates (Zhang and Zhang 2013 [\[5\]](#)).

Here, we discovered that, similar to P granule components, the expression of many other granule-associated proteins in *C. elegans*, including the core Z granule protein ZNFX-1, the D granule protein CSR-1, and the SIMR granule protein SIMR-1, are also controlled by autophagy during embryogenesis. It is worth noting that the timing of degradation differs amongst the different granules, with P granules and Z granules being cleared from somatic cells within the first few cell divisions of the developing embryo (Strome and Wood 1983 [\[6\]](#); Wan et al. 2018 [\[7\]](#)), while CSR granules are removed between 28- and 100-cell stage (Figure 4G [\[8\]](#)), and SIMR granules disappear between 100- and 200-cell stage of embryonic development (Figure 1C [\[9\]](#), 2C [\[10\]](#)). These results suggest an intriguing hypothesis that different granules might be produced independently at designated developmental stages and cytosolic locations to perform specific functions, and later actively degraded by autophagy when not needed. Future studies will be needed to carefully examine the role and fate of each type of granule during embryogenesis to test this hypothesis.

Lastly, while autophagy pathway plays an important role in regulating expression of many granule components, we cannot rule out the possibility that the ubiquitin-proteasome system may also contribute to protein turnover in the small RNA pathway. In fact, Zhang et al demonstrated that, unlike PGL-1 and PGL-3, the other P granule components GLH-1 and GLH-4 are not degraded via autophagy (Zhang et al. 2009 [\[4\]](#)). Additionally, DeRenzo et al showed that germline-expressed CCH figure proteins, including PIE-1, POS-1, MEX-1, are selectively targeted for degradation in somatic cells by the E3 ubiquitin ligase subunit-interacting protein ZIF-1 through the ubiquitin-proteasome system (DeRenzo et al. 2003 [\[11\]](#)). Furthermore, previous research suggest that the proteasome and autophagy pathways can act as compensatory mechanisms under certain contexts (Ji and Kwon 2017 [\[12\]](#)). Future studies will be needed to interrogate the roles of these two protein degradation pathways in regulating protein turnover in the small RNA pathway.

The small RNA plasticity of NRDE-3

Argonautes are conventionally known to bind small RNAs with high specificity. In this study, we unveil the remarkable versatility of the nuclear Argonaute NRDE-3, demonstrating its ability to bind multiple classes of small RNAs and exhibit distinct functions throughout development. Argonaute proteins with the capacity to bind multiple types or classes of small RNAs have been observed in other organisms. For example, both siRNAs and miRNAs can be loaded into the four human Argonautes (Ago1-4) and both siRNAs and miRNAs can guide Ago2-dependent target cleavage (Meister et al. 2004 [\[13\]](#); Liu et al. 2004 [\[14\]](#)). Our discovery is somewhat different, however, in that NRDE-3 binds its two preferred classes of small RNAs, CSR-class 22G-RNAs and ERGO-dependent 22G-RNAs, at distinct developmental stages, indicating that there must be a switch from one class of small RNA to the other during embryogenesis. Interestingly, a more recent study in the

parasitic nematode *Ascaris* revealed that the *Ascaris* paralog of NRDE-3, AsNRDE-3, exhibits a dramatic change in associated small RNAs during spermatogenesis, targeting repetitive sequences and transposons in early stages of spermatogenesis and mRNAs in late meiosis (Zagoskin et al. 2022 [DOI](#)). Curiously, the mRNAs targeted by AsNRDE-3 in late meiosis largely overlap with the targets of AsCSR-1, the *Ascaris* paralog of CSR-1, and it is proposed that AsNRDE-3 could act in concert with AsCSR-1 at the late stages of meiosis to clear spermatogenic and meiotic mRNAs from the developing spermatids (Zagoskin et al. 2022 [DOI](#)). These data further suggest that the ability of NRDE-3 to target both repetitive sequences and germline-expressed genes at distinct developmental timepoints may be a conserved feature of this protein. It is currently unknown how this small RNA switching is achieved. It is possible that there is an active mechanism to unload the CSR-class 22G-RNAs and replace them with ERGO-dependent 22G-RNAs, or to degrade NRDE-3 loaded with CSR-class 22G-RNAs. However, we prefer the simpler model where NRDE-3 loaded with CSR-class 22G-RNAs, initially deposited into embryo from the maternal germline, are diluted out as the animal develops. Newly synthesized NRDE-3 in the embryo is loaded with ERGO-dependent 22G-RNAs to execute the small RNA “switch”. The idea that Argonaute proteins can be utilized at distinct timepoints with different small RNA partners to create multi-functionality is intriguing, especially in the vein of rapidly clearing transcripts from a cell to engineer a new developmental program. Achieving higher resolution small RNA-Argonaute interactions with tissue- and developmental-specific staging will be crucial to fully elucidate the roles of Argonaute proteins during development in *C. elegans* and other organisms.

In summary, this work investigating the role of SIMR granules in embryos, together with our previous study of SIMR foci in the germline (Chen and Phillips 2024a [DOI](#)), has identified a new mechanism for regulating nuclear Argonaute protein localization in *C. elegans*. The two paralogous proteins, HRDE-2 and ENRI-2, recruit unloaded nuclear Argonautes HRDE-1 and NRDE-3 to small RNA production centers organized by SIMR-1, where we speculate that loading can occur. These small RNA loading sites are essential in the germline to promote small RNA binding specificity, however they may also contribute to efficiency and specificity of small RNA loading in embryos. We further discovered an intriguing repository of cytoplasmic granules during embryogenesis that do not exhibit the same organization or hierarchical assembly as germ granules, but share the same autophagy-mediated degradation as one another. These results highlight the importance of further investigation into the relationship between RNA silencing pathways and RNA granules during embryogenesis. Lastly, we observed a striking phenomenon where the NRDE-3 nuclear Argonaute protein possesses the ability to switch small RNA binding partners, altering mRNA targets and function during development. Together, these findings reveal that the precise regulation of small RNA pathway components through diverse mechanisms, such as spatial-temporal separation and hierarchical physical interactions, is crucial for accurate gene regulation and developmental transitions in *C. elegans*.

Materials and methods

C. elegans stains

C. elegans strains were maintained at 20°C on NGM plates seeded with OP50 *E. coli* according to standard conditions unless otherwise stated (Brenner 1974 [DOI](#)). All strains used in this project are listed in Supplementary File 1.

CRISPR-mediated strain construction

For *nrde-3(cmp324[HK-AA])*, *enri-1(cmp328)*, *enri-2(cmp318)*, and *rde-3/mut-2(cmp337)*, we used an oligo repair template and RNA guide. For *enri-1(cmp320[enri-1::mCherry::2xHA])*, we used an RNA guide and PCR amplified repair template (Supplementary File 2). For injections using a single gene-specific crRNA, the injection mix included 0.25 µg/µl Cas9 protein (IDT), 100 ng/µl tracrRNA (IDT), 14 ng/µl dpy-10 crRNA, 42 ng/µl gene-specific crRNA, and 110 ng/µl of the oligo repair

template. For injections using two gene-specific crRNAs, the injection mix included 0.25 µg/µl Cas9 protein (IDT), 100 ng/µl tracrRNA (IDT), 14 ng/µl dpy-10 crRNA, 21 ng/µl each gene-specific crRNA, and 110 ng/µl of each repair template.

The following strains were used for injection: *enri-2(cmp318)* and *enri-1(cmp320[enri-1::mCherry::2xHA])* into wild-type N2 strain. *nrde-3(cmp324[HK-AA])* and *enri-1(cmp328)* into JMC237: *nrde-3(tor131[GFP::3xFLAG::nrde-3])* X. *rde-3/mut-2(cmp337)* into USC1615: *ego-1(cmp317[ego-1::degron])* I; *ieSi38 [Psun-1::TIR1::mRuby::sun-1 3' UTR]* IV; *nrde-3(tor131[GFP::3xFLAG::nrde-3])* X. Following injection, F1 animals with the Rol phenotype were isolated and genotyped by PCR to identify heterozygous animals with the mutations of interest, then F2 animals were further singled out to identify homozygous mutant animals.

RNAi assays

For RNAi experiment, control L4440 and *lgg-1* RNAi *E. coli* clones were sequenced verified and cultured at 37°C for 16 hours, then RNAi bacteria were seeded on fresh RNAi plates. Two L4 animals were transferred to seeded RNAi plates and raised at 20°C. When the progenies of the L4 animals turn into young adults (about 4 days), the embryos of young adult animals were imaged to assess somatic granule expression.

Immunofluorescence imaging and quantification

For immunofluorescence, *C. elegans* were dissected in egg buffer containing 0.1% Tween-20 and fixed in 1% formaldehyde in egg buffer as described (Phillips et al. 2009 [DOI](#)). Samples were immunostained with anti-RNA Polymerase II CTD (phosphoSer2) at 1:500 (Abcam ab5095). The secondary antibody, anti-Rabbit IgG AlexaFluor 555 was used at 1:1000 (Thermo Fisher A21429). Animals were dissected at the adult stage (24 h post L4). Imaging was performed on a DeltaVision Elite microscope (GE Healthcare) using a 60x N.A. 1.42 oil-immersion objective, data stacks were collected and deconvolution was performed using the SoftWoRx package. 25 optical sections, for a total 12.5µm sample thickness, are presented as a maximum intensity projection. PSer2 signal intensity was calculated in ImageJ (version 1.53a) using a minimum of 10 individual adult gonads.

Live imaging

Live imaging of *C. elegans* embryos was performed in M9 buffer. Young embryos were obtained by dissecting gravid adult *C. elegans*, and old embryos were obtained by manually picking embryos laid on the NGM plate. Live imaging of *C. elegans* adult germline was performed in M9 buffer containing sodium azide to prevent movement. Day-one-adult *C. elegans* were obtained by manually picking L4s and leaving L4s at 20°C for about 24 hours. Imaging was performed on a DeltaVision Elite microscope (GE Healthcare) using a 60x N.A. 1.42 oil-immersion objective. Images were pseudocolored using Adobe Photoshop.

Granule number quantification

Granule number quantification was performed in FIJI/ImageJ2 (version 2.9.0). At least 10 embryos were imaged on a DeltaVision Elite microscope with 37 optical sections of a total 22.20µm sample thickness from the bottom of the sample. Images were deconvolved to eliminate backgrounds. Z stacks were opened using the 3D object counter plugin for FIJI, and the granule counting threshold for each image was manually adjusted to obtain the least background and most granules.

Colocalization analysis

Quantitative colocalization analysis between different granules was performed in FIJI/ImageJ2 (version 2.9.0) using the Coloc2 package and 100-cell staged embryos imaged on a DeltaVision Elite microscope. At least 3 granules from each embryo, and at least 4 individual embryos for a total of at least 20 granules were used to calculate Pearson's R value.

Western blot

Synchronized adult *C. elegans* were harvested (~72 h at 20 °C after L1 arrest) and 200 adults were loaded per lane. Proteins were resolved on 4–12% Bis-Tris polyacrylamide gels (Thermo Fisher, NW04122BOX), transferred to nitrocellulose membranes (Thermo Fisher, LC2001), and probed with rat anti-HA-peroxidase 1:1000 (Roche 12013819001), mouse anti-FLAG 1:1000 (Sigma, F1804), or mouse anti-actin 1:10,000 (Abcam ab3280). Secondary HRP antibodies were purchased from Thermo Fisher. Unedited western blots are provided in the Source Data File.

Small RNA library preparation and sequencing

For *C. elegans* embryo staging and collection, synchronized arrested L1s were grown on enriched peptone plates at 17°C until the young adult stage. Adult *C. elegans* stage was monitored carefully under DeltaVision microscope by live imaging. For early embryo collection (≤ 100 -cell), adult animals were washed off from plates with H₂O and bleached as soon as the first animals had 1–4 eggs (around 68–70 hours depending on the strain and the incubator temperature). For late embryo collection (≥ 300 -cell), adult animals were washed off from plates with H₂O and bleached when about half of the worms had 1–6 eggs (~70–72 hours depending on the strain and the incubator temperature). After bleaching, embryos were washed twice with M9 buffer, and filtered through 40µm cell strainers (Fisherbrand™ Sterile Cell Strainers, 40µm) twice to clear the residual worm body. To reach ≥ 300 -cell stage for late embryo collection, embryos were additionally incubated in M9 buffer at 20°C for 4.5 hours. Then embryos were washed once with IP buffer (50 mM Tris-Cl pH 7.5, 100 mM KCl, 2.5 mM MgCl₂, 0.1% Nonidet P40 substitute) containing Protease Inhibitor (Thermo Fisher A32965). Embryos were kept on ice during washes to prevent further development. 500,000 embryos were collected for each replicate. Following washes, embryos were flash-frozen by placing tubes in a container with ethanol and dry ice. A small aliquot of embryos was examined on the Deltavision microscope to confirm the developmental stage immediately before freezing. Frozen embryos were stored at -80°C until immunoprecipitation.

For immunoprecipitation followed by small RNA sequencing in embryos, ~500,000 synchronized embryos were sonicated with Fisher Sonifier 550 with a microtip (15s on, 45s off, 10% power, total 2 minutes on time). After sonication, insoluble particulate was removed by centrifugation at 21,000g for 30 minutes. Immunoprecipitation was performed using anti-FLAG Affinity Matrix (Sigma Aldrich, A2220). NRDE-3-bound RNAs were isolated using TRIzol reagent (Thermo Fisher, 15596018), followed by chloroform extraction and isopropanol precipitation. Small RNAs (18 to 30-nt) were size selected on homemade 10% Urea-polyacrylamide gels from total RNA samples. Small RNAs were treated with 5' RNA polyphosphatase (Epicenter RP8092H) and ligated to 3' pre-adenylated adapters with Truncated T4 RNA ligase (NEB M0373L). Small RNAs were then hybridized to the reverse transcription primer, ligated to the 5' adapter with T4 RNA ligase (NEB M0204L), and reverse transcribed with Superscript III (Thermo Fisher 18080-051). Small RNA libraries were amplified using Q5 High-Fidelity DNA polymerase (NEB M0491L) and size selected on a homemade 10% polyacrylamide gel. Library concentration was determined using the Qubit 1X dsDNA HS Assay kit (Thermo Fisher Q33231) and quality was assessed using the Agilent BioAnalyzer. Libraries were sequenced on the Illumina NextSeq2000 (SE 75-bp reads) platform. Primer sequences are available in Supplementary File 2. Differentially expressed gene lists and gene lists used in this paper can be found in Supplementary File 3. Sequencing library statistics summary can be found in Supplementary File 4.

Bioinformatic analysis

For small RNA libraries, sequences were parsed from adapters and quality filtered using FASTX-Toolkit (version 0.0.13) (Greg Hannon 2010 [DOI](#)). Filtered reads were mapped to the *C. elegans* genome, WS258, using Bowtie2 (version 2.5.0) (Langmead and Salzberg 2012 [DOI](#)). Mapped reads were assigned to genomic features using featureCounts which is part of the Subread package

(version 2.0.1) (Liao et al. 2014 [↗](#)). Differential expression analysis was performed using edgeR (3.40.2) (Robinson et al. 2010 [↗](#)). To define gene lists from IP experiments, a twofold-change cutoff, an edgeR adjusted p-value of ≤ 0.05 , and at least 10 RPM in the IP libraries were required to identify genes with significant changes in small RNA levels.

Data Availability

The RNA sequencing data generated in this study are available through Gene Expression Omnibus (GEO) under accession code GSE273239. Source data file is provided with this paper.

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Additional information

Author contributions

S.C.: Conceptualization, Investigation, Formal analysis, Writing—original draft, Writing—reviewing and editing, Visualization

C.M.P.: Conceptualization, Formal Analysis, Writing—original draft, Writing—reviewing and editing, Supervision, Funding Acquisition.

Additional files

Supplemental File 1. Reagents, resources, and strains used in this study. [↗](#)

Supplemental File 2. Oligonucleotides sequences used in this study. [↗](#)

Supplemental File 3. Small RNA enrichment in NRDE-3 immunoprecipitations. [↗](#)

Supplemental File 4. Sequencing library statistics. [↗](#)

Source Data. Figure 1—source data 1. This file contains the raw data used to generate the graph of SIMR granule number shown in Figure 1C. Figure 1—figure supplement 2—source data 1. This file contains the original western blots of ENRI-1::mCherry::2xHA used in creating Figure 1—figure supplement 2D. Figure 2—source data 1. This file contains the raw data used to generate the graphs of NRDE-3 granule number shown in Figure 2B-C. Figure 2—figure supplement 1—source data 1. This file contains the original western blots of GFP::NRDE-3 used in creating Figure 2—figure supplement 1B and the raw data used to generate the graphs of NRDE-3 granule number shown in Figure 2—figure supplement 1D. Figure 3—

figure supplement 1—source data 1. This file contains the original western blots of GFP::NRDE-3 used in creating Figure 3—figure supplement 1A. Figure 4—source data 1. This file contains the raw data used to generate the graphs of colocalization analysis and CSR-1 granule number shown in Figure 4B, G. Figure 5—source data 1. This file contains the raw data used to generate the graph of SIMR granule number shown in Figure 5B. Figure 5—figure supplement 1—source data 1. This file contains the raw data used to generate the graphs of colocalization analysis shown in Figure 5—figure supplement 1—source data 1B. Figure 7—source data 1. This file contains the raw data used to generate the graph of number of embryos laid shown in Figure 7D. Figure 8—source data 1. This file contains the raw data used to generate the graphs of oocyte Pser2 signal intensity and pattern number shown in Figure 8C-D. [↗](#)

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Reviewer #1 (Public review):

Summary:

Chen and Phillips describe the dynamic appearance of cytoplasmic granules during embryogenesis analogous to SIMR germ granules, and distinct from CSR-1-containing granules, in the *C. elegans* germline. They show that the nuclear Argonaute NRDE-3, when mutated to abrogate small RNA binding, or in specific genetic mutants, partially colocalizes to these granules along with other RNAi factors, such as SIMR-1, ENRI-2, RDE-3, and RRF-1. Furthermore, NRDE-3 RIP-seq analysis in early vs. late embryos is used to conclude that NRDE-3 binds CSR-1-dependent 22G RNAs in early embryos and ERGO-1-dependent 22G RNAs in late embryos. These data lead to their model that NRDE-3 undergoes small RNA substrate

"switching" that occurs in these embryonic SIMR granules and functions to silence two distinct sets of target transcripts - maternal, CSR-1 targeted mRNAs in early embryos and duplicated genes and repeat elements in late embryos.

Strengths:

The identification and function of small RNA-related granules during embryogenesis is a poorly understood area and this study will provide the impetus for future studies on the identification and potential functional compartmentalization of small RNA pathways and machinery during embryogenesis.

Weaknesses:

(1) The authors acknowledge the following issue that loss of SIMR granules have no significant impact on NRDE-3 small RNA loading weakens the functional relevance of these structures. However, this point is clearly discussed and, as they note in their Discussion, it is entirely possible that these embryonic granules may be "incidental condensates."

<https://doi.org/10.7554/eLife.102226.2.sa3>

Reviewer #2 (Public review):

Summary:

NRDE-3 is a nuclear WAGO-clade Argonaute that, in somatic cells, binds small RNAs amplified in response to the ERGO-class 26G RNAs that target repetitive sequences. This manuscript reports that, in the germline and early embryos, NRDE-3 interacts with a different set of small RNAs that target mRNAs. This class of small RNAs were previously shown to bind to a different WAGO-clade Argonaute called CSR-1, which is cytoplasmic unlike nuclear NRDE-3. The switch in NRDE-3 specificity parallels recent findings in *Ascaris* where the *Ascaris* NRDE homolog was shown to switch from sRNAs that target repetitive sequences to CSR-class sRNAs that target mRNAs.

The manuscript also correlates the change in NRDE-3 specificity with the appearance in embryos of cytoplasmic condensates that accumulate SIMR-1, a scaffolding protein that the authors previously implicated in sRNA loading for a different nuclear Argonaute HRDE-1. By analogy, and through a set of correlative evidence, the authors argue that SIMR foci arise in embryogenesis to facilitate the change in NRDE-3 small RNA repertoire. The paper presents lots of data that beautifully documents the appearance and composition of the embryonic SIMR-1 foci, including evidence that a mutated NRDE-3 that cannot bind sRNAs accumulate in SIMR-1 foci in SIMR-1-dependent fashion.

<https://doi.org/10.7554/eLife.102226.2.sa2>

Reviewer #3 (Public review):

Summary:

Chen and Phillips present intriguing work that extends our view on the *C. elegans* small RNA network significantly. While the precise findings are rather *C. elegans* specific there are also messages for the broader field, most notably the switching of small RNA populations bound to an argonaute, and RNA granules behavior depending on developmental stage. The work also starts to shed more light on the still poorly understood role of the CSR-1 argonaute protein and supports its role in the decay of maternal transcripts. Overall, the work is of excellent quality, and the messages have a significant impact.

Strengths:

Compelling evidence for major shift in activities of an argonaute protein during development, and implications for how small RNAs affect early development. Very balanced and thoughtful discussion.

Weaknesses:

The switch between maternal and zygotic NRDE-3 remains unaddressed

<https://doi.org/10.7554/eLife.102226.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public review):

Summary:

*Chen and Phillips describe the dynamic appearance of cytoplasmic granules during embryogenesis analogous to SIMR germ granules, and distinct from CSR-1-containing granules, in the *C. elegans* germline. They show that the nuclear Argonaute NRDE-3, when mutated to abrogate small RNA binding, or in specific genetic mutants, partially colocalizes to these granules along with other RNAi factors, such as SIMR-1, ENRI-2, RDE-3, and RRF-1. Furthermore, NRDE-3 RIP-seq analysis in early vs. late embryos is used to conclude that NRDE-3 binds CSR-1-dependent 22G RNAs in early embryos and ERGO-1 dependent 22G RNAs in late embryos. These data lead to their model that NRDE-3 undergoes small RNA substrate "switching" that occurs in these embryonic SIMR granules and functions to silence two distinct sets of target transcripts - maternal, CSR-1 targeted mRNAs in early embryos and duplicated genes and repeat elements in late embryos.*

Strengths:

The identification and function of small RNA-related granules during embryogenesis is a poorly understood area and this study will provide the impetus for future studies on the identification and potential functional compartmentalization of small RNA pathways and machinery during embryogenesis.

Weaknesses:

(1) While the authors acknowledge the following issue, their finding that loss of SIMR granules has no apparent impact on NRDE-3 small RNA loading puts the functional relevance of these structures into question. As they note in their Discussion, it is entirely possible that these embryonic granules may be "incidental condensates." It would be very welcomed if the authors could include some evidence that these SIMR granules have some function; for example, does the loss of these SIMR granules have an effect on CSR-1 targets in early embryos and ERGO-1-dependent targets in late embryos?

We appreciate reviewer 1's concern that we do not provide enough evidence for the function of the SIMR granules. As suggested, we examined the NRDE-3 bound small RNAs more deeply, and we do observe a slight but significant increased CSR-class 22G-RNAs binding to NRDE-3 in late embryos of *simr-1* and *enri-2* mutants (see below, right). We hypothesize that this result could be due to a slower switch from CSR to ERGO 22G-RNAs in the absence of SIMR granules. We added these data to Figure 6G.

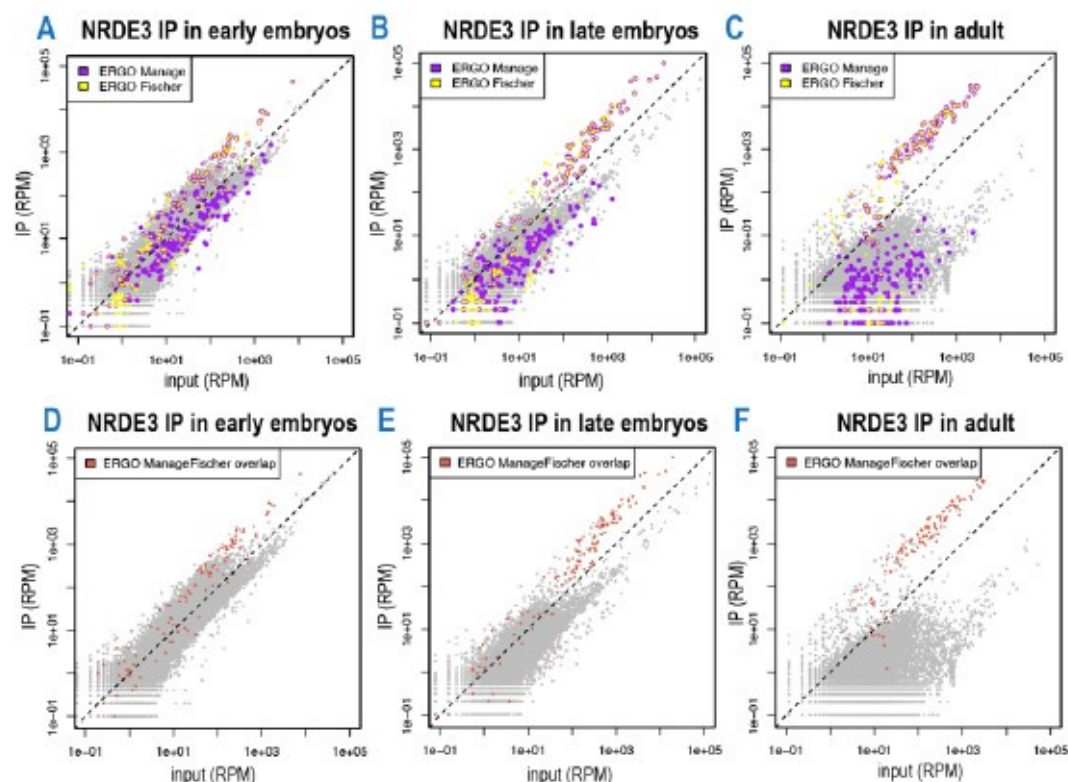
(2) The analysis of small RNA class "switching" requires some clarification. The authors re-define ERGO1-dependent targets in this study to arrive at a very limited set of genes and their justification for doing this is not convincing. What happens if the published set of ERGO-1 targets is used?

As we mentioned in the manuscript, we initially attempted to use the previously defined ERGO targets. However, the major concern is fewer than half the genes classified as ERGO targets by Manage *et al.* and Fischer *et al.* overlap with one another (Figure 6—figure supplement 1D and below). We reason this might be because the gene sets were defined as genes that lose small RNAs in various ERGO pathway mutants and because different criteria were used to define the lists as discussed in the manuscript (lines 471-476). As a result, some of the previously defined ERGO target genes may actually be indirect targets of the pathway. Here we focus on genes targeted by small RNAs enriched in an ERGO pathway Argonaute IP, which should be more specific.

In this manuscript, we are interested specifically in the ERGO targets bound by NRDE-3, thus we utilized the IP-small RNA sequencing data from young adult animals (Seroussi *et al.*, 2023), to define a new ERGO list. We are confident about this list because 1) Most of our new ERGO genes overlap with the overlap between ERGO-Manage and ERGO-Fischer list (see Figure 6—figure supplement 1D in our manuscript and below). 2) We observed the most significant decrease of small RNA levels and increase of mRNA levels in the *nrde-3* mutants using our newly defined list (see Figure 6—figure supplement 1E-F in our manuscript).

To further address reviewer 1's concern about whether the data would look significantly different when using the ERGO-Manage and ERGO-Fischer lists, we made new scatter plots shown in Author response image 1 panels A-C below (ERGO-Manage – purple, ERGO-Fischer-yellow, and the overlap - yellow with purple ring). We found that the small switching pattern of NRDE-3 is consistent with our newly defined list, particularly if we look at the overlap of ERGO-Manage and ERGO-Fischer list (Author response image 1 panels D-F below, red).

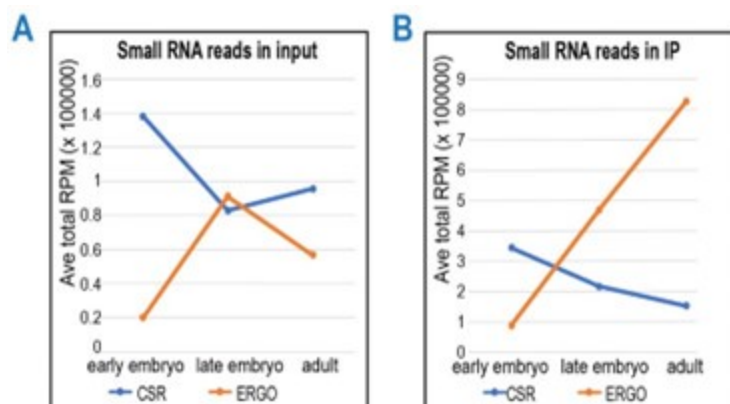
Author response image 1.



Further, the NRDE-3 RIP-seq data is used to conclude that NRDE-3 predominantly binds CSR-1 class 22G RNAs in early embryos, while ERGO-1-dependent 22G RNAs are enriched in late embryos. a) The relative ratios of each class of small RNAs are given in terms of unique targets. What is the total abundance of sequenced reads of each class in the NRDE-3 IPs?

To address the reviewer's question about the total abundance of sequenced reads of each class in the NRDE-3 IPs: Author response image 2 panel A-B below show the total RPM of CSR and ERGO class sRNAs in inputs and IPs at different stages. Focusing on late embryos, the total abundance of ERGO-dependent sRNAs is similar to CSR-class sRNAs in input, while much higher in IP, indicating an enrichment of ERGO-dependent 22G-RNAs in NRDE-3 consistent with our log2FC (IP vs input) in Figure 6B. This data supports our conclusion that NRDE-3 preferentially binds to ERGO targets in late embryos.

Author response image 2.



b) The "switching" model is problematic given that even in late embryos, the majority of 22G RNAs bound by NRDE-3 is the CSR-1 class (Figure 5D).

It is important to keep in mind the difference in the total number of CSR target genes (3834) and ERGO target genes (119). The pie charts shown in Figure 6D are looking at the total proportion of the genes enriched in the NRDE-3 IP that are CSR or ERGO targets. For the NRDE-3 IP in late embryos, that would be 70/119 (58.8%) of ERGO targets are enriched, while 172/3834 (4.5%) of CSR targets are enriched. These data are also supported by the RPM graphs shown in Author response image 2 panels A-B above, which show that the majority of the small RNA bound by NRDE-3 in late embryos are ERGO targets. Nonetheless, NRDE-3 still binds to some CSR targets shown as Figure 6D and panel B, which may be because the amount of CSR-class 22G-RNAs is reduced gradually across embryonic development as the maternally-deposited NRDE-3 loaded with CSR-class 22G-RNAs is diluted by newly transcribed NRDE-3 loaded with ERGO-dependent 22G-RNAs (lines 857-862).

c) A major difference between NRDE-3 small RNA binding in *eri-1* and *simr-1* mutants appears to be that NRDE-3 robustly binds CSR-1 22G RNAs in *eri-1* but not in *simr-1* in late embryos. This result should be better discussed.

In the *eri-1* mutant, we hypothesize that NRDE-3 robustly binds CSR-class 22G-RNAs because ERGOclass 22G-RNAs are not synthesized during mid-embryogenesis, so either NRDE-3 is unloaded (in granule at 100-cell stage in Figure 2A) or mis-loaded with CSR-class 22G-RNAs (in the nucleus at 100cell stage in Figure 2A). We don't have a robust method to address the proportion of loaded vs. unloaded NRDE-3 so it is difficult to address the degree to which NRDE-3 is misloaded in the *eri-1* mutant. In the *simr-1* mutant, both classes of small RNAs are present and NRDE-3 is still preferentially loaded with ERGO-dependent 22G-RNAs, though we do see a subtle increase in association with CSR-class 22GRNAs. These data could suggest a less efficient loading of NRDE-3 with ERGO-dependent 22G-RNAs, but we would need more precise methods to address the loading dynamics in the *simr-1* mutant.

(3) Ultimately, if the switching is functionally important, then its impact should be observed in the expression of their targets. RNA-seq or RT-qPCR of select CSR-1 and ERGO-1 targets should be assessed in *nrde-3* mutants during early vs late embryogenesis.

The function of NRDE-3 at ERGO targets has been well studied (Guang *et al*, 2008) and is also assessed in our H3K9me3 ChIP-seq analysis in Figure 7E where, in mixed staged embryos,

H3K9me3 level on ERGO targets (labeled as ‘NRDE-3 targets in young adults’) is reduced significantly in the *nrde-3* mutant.

To understand the function of NRDE-3 binding on CSR targets in early embryos, we attempted to do RTqPCR, smFISH, and anti-H3K9me3 CUT&Tag-seq on early embryos, and we either failed to obtain enough signal or failed to detect any significant difference (data not shown). We additionally tested the possibility that NRDE-3 functions with CSR-class 22G-RNAs in oocytes. We present new data showing that NRDE-3 represses RNA Pol II in oocytes to promote global transcriptional repression at the oocyte-to-embryo transition, we now included these data in Figure 8.

Reviewer #2 (Public review):

Summary:

*NRDE-3 is a nuclear WAGO-clade Argonaute that, in somatic cells, binds small RNAs amplified in response to the ERGO-class 26G RNAs that target repetitive sequences. This manuscript reports that, in the germline and early embryos, NRDE-3 interacts with a different set of small RNAs that target mRNAs. This class of small RNAs was previously shown to bind to a different WAGO-clade Argonaute called CSR1, which is cytoplasmic, unlike nuclear NRDE-3. The switch in NRDE-3 specificity parallels recent findings in *Ascaris* where the *Ascaris* NRDE homolog was shown to switch from sRNAs that target repetitive sequences to CSR-class sRNAs that target mRNAs.*

The manuscript also correlates the change in NRDE-3 specificity with the appearance in embryos of cytoplasmic condensates that accumulate SIMR-1, a scaffolding protein that the authors previously implicated in sRNA loading for a different nuclear Argonaute HRDE-1. By analogy, and through a set of correlative evidence, the authors argue that SIMR foci arise in embryogenesis to facilitate the change in NRDE-3 small RNA repertoire. The paper presents lots of data that beautifully documents the appearance and composition of the embryonic SIMR-1 foci, including evidence that a mutated NRDE-3 that cannot bind sRNAs accumulates in SIMR-1 foci in a SIMR-1-dependent fashion.

Weaknesses:

*The genetic evidence, however, does not support a requirement for SIMR-1 foci: the authors detected no defect in NRDE-3 sRNA loading in *simr-1* mutants. Although the authors acknowledge this negative result in the discussion, they still argue for a model (Figure 7) that is not supported by genetic data. My main suggestion is that the authors give equal consideration to other models - see below for specifics.*

We appreciate reviewer 2's comments on the genetic evidence for the function of SIMR foci. A similar concern was also brought up by reviewer 1. By re-examining our sequencing data, we found that there is a modest but significant increase in NRDE-3 association with CSR-class sRNAs in *simr-1* and *enri-2* mutants in late embryos. We believe that this data supports our model that SIMR-1 and ENRI-2 are required for an efficient switch of NRDE-3 bound small RNAs. Please refer our response to the reviewer 1 - point (1), and Figure 6G in the updated manuscript.

Reviewer #3 (Public review):

Summary:

*Chen and Phillips present intriguing work that extends our view on the *C. elegans* small RNA network significantly. While the precise findings are rather *C. elegans* specific there are also messages for the broader field, most notably the switching of small RNA populations bound to an argonaute, and RNA granules behavior depending on*

developmental stage. The work also starts to shed more light on the still poorly understood role of the CSR-1 argonaute protein and supports its role in the decay of maternal transcripts. Overall, the work is of excellent quality, and the messages have a significant impact.

Strengths:

Compelling evidence for major shift in activities of an argonaute protein during development, and implications for how small RNAs affect early development. Very balanced and thoughtful discussion.

Weaknesses:

Claims on col-localization of specific 'granules' are not well supported by quantitative data

We have now included zoomed images of individual granules to better show the colocalization in Figure 4 and Figure 4—figure supplement 1, and performed Pearson's colocalization analysis between different sets of proteins in Figure 4B.

Reviewer #2 (Recommendations for the authors):

- The manuscript is very dense and the gene names are not helpful. For example, the authors mention ERGO-1 without clarifying the type of protein, etc. I suggest the authors include a figure to go with the introduction that describes the different classes of primary and secondary sRNAs, associated Argonautes, and other accessory proteins. Also include a table listing relevant gene names, protein classes, main localizations, and proposed functions for easy reference by the readers.

We agree that the genes names in different small RNA pathways are easily confused. We added a diagram and table in Figure 1—figure supplement 1 depicting the ERGO/NRDE and CSR pathways and added clarification about the ERGO/NRDE-3 pathway in the text from line 126-128.

- Line 424 - the wording here and elsewhere seems to imply that SIMR-1 and ENRI-2, although not essential, contribute to NRDE-3 sRNA loading. The sequencing data, however, do not support this - the authors should be clearer on this. If the authors believe there are subtle but significant differences, they should show them perhaps by adding a panel in Figure 5 that directly compares the NRDE-3 IPs in wildtype versus simr-1 mutants. Figure 5H however does not support such a requirement.

As brought up by reviewer 1, we do not see difference in binding of ERGO-dependent sRNA in *simr-1* mutant in late embryos. We do, however, see a modest, but significant, increase of CSR-sRNAs bound by NRDE-3 in *simr-1* and *enri-2* mutants, which we hypothesize could be due to a less efficient loading of ERGO-dependent 22G-RNAs by NRDE-3. The updated data are now in Figure 6G. We have also edited the text and model figure to soften these conclusions.

*- Condensates of PGL proteins appear at a similar time and place (somatic cells of early embryos) as the embryonic SIMR-1 foci. The PGL foci correspond to autophagy bodies that degrade PGL proteins. Is it possible that SIMR-1 foci also correspond to degradative structures? The possibility that SIMR-1 foci are targeted for autophagy and not functional would fit with the finding that *simr-1* mutants do not affect NRDE-3 loading in embryos.*

We appreciate reviewer 2's comments on possibility of SIMR granules acting as sites for degradation of SIMR-1 and NRDE-3. We think this is not the case for the following reasons: 1) if SIMR granules are sites of autophagic degradation, then we would expect that embryonic

SIMR granules in somatic cells, like PGL granules, should only be observed in autophagy mutants; however we see them in wild-type embryos 2) we would not expect a functional Tudor domain to be required for granule localization; however in Figure 1—figure supplement 2B, we show that a point mutation in the Tudor domain of SIMR-1 abrogates SIMR granule formation, and 3) if NRDE-3(HK-AA) is recruited to SIMR granules for degradation while wild-type NRDE-3 is cytoplasmic, then NRDE-3(HK-AA) should show a significantly reduced protein level comparing to wild-type NRDE-3. In the western blot in Figure 2—figure supplement 1B, NRDE-3 and NRDE-3(HK-AA) protein levels are similar, indicating that NRDE-3(HK-AA) is not degraded despite being unloaded. This is in contrast to what we have observed previously for HRDE-1, which is degraded in its unloaded state. If SIMR-1 played a role directly in promoting degradation of NRDE-3(HK-AA), we would similarly expect to see a change in NRDE-3 or NRDE-3(HK-AA) expression in a *simr-1* mutant. We performed western blot and did not observe a significant change in protein expression for NRDE-3 (Figure 3—figure supplement 1A).

Although under wild-type conditions, SIMR granules do not appear to be sites of autophagic degradation, upon treatment with *lgg-1* (an autophagy protein) RNAi, we found that SIMR-1, as well as many other germ granule and embryonic granule-localized proteins, increase in abundance in late embryos. This data demonstrates that ZNFX-1, CSR-1, SIMR-1, MUT-2/RDE-3, RRF-1, and unloaded NRDE-3 are removed by autophagic degradation similar to what have been shown previously for PGL-1 proteins (Zhang et al, 2009, Cell). We added these data to Figure 5. It is important to emphasize, however, that the timing of degradation differs for each granule assayed (Lines 447-450), indicating that there must be multiple waves of autophagy to selectively degrade subsets of proteins when they are no longer needed by the embryo.

- The observation that an NRDE-3 mutant that cannot load sRNAs localizes to SIMR-1 foci does not necessarily imply that wild-type unloaded NRDE-3 would also localize there. Unless the authors have additional data to support this idea, the authors should acknowledge that this hypothesis is speculative. In fact, why does cytoplasmic NRDE-3 not localize to granules in the *rde-3;ego-1* degon strain shown in Figure 6B?? Is it possible that the NRDE-3 mutant accumulates in SIMR-1 foci because it is unfolded and needs to be degraded?

We believe that wild-type NRDE-3 also localizes to SIMR foci when unloaded. This is supported by the localization of wild-type NRDE-3 in *eri-1* and *rde-3* mutants, where a subset of small RNAs are depleted. Wild-type NRDE-3 localizes to both somatic SIMR-1 granules and the nucleus, depending on embryo stage (Figure 2A, Figure 2—figure supplement 1C). The granule numbers in *eri-1* and *rde-3* mutants are less than the *nrde-3(HK-AA)* mutant, consistent with the imaging data that NRDE-3 only partially localizes to somatic granule (Figure 2A – 100-cell stage).

In the *rde-3; ego-1* double mutant, the embryos have severe developmental defects: they cannot divide properly after 4-8 cell stage and exhibit morphology defects after that stage. In wild-type, SIMR foci do not appear until around 8-28-cell stage (shown in Figure 1C), so we believe that cytoplasmic NRDE-3 does not localize to foci in the double mutant because of the timing.

- The authors propose that NRDE-3 functions in nuclei to target mRNAs also targeted in the cytoplasm by CSR-1. If so, how do they propose that NRDE-3 might do this since little transcription occurs in oocytes/early embryos?? Are the authors suggesting that NRDE-3 targets germline genes for silencing specifically at the times that zygotic transcription comes back on, or already in maturing oocytes? Is the transcription of most CSR-1 targets silenced in early embryos??

We appreciate the suggestions to check the function of NRDE-3 in oocytes. We tested this possibility and found it to be correct. NRDE-3 functions in oocytes for transcriptional repression by inhibiting RNA Pol II elongation. We added these data to Figure 8. We also attempted to do RT-qPCR, smFISH, and antiH3K9me3 Cut&Tag-seq on early embryos to further test the hypothesis that NRDE-3 acts with CSR-class 22G-RNAs in early embryos, but we either failed to obtain enough signal or failed to detect any significant difference (data not shown). Therefore, we think that the primary role for NRDE-3 bound to CSR-class 22G-RNAs may be for global transcriptional repression of oocytes prior to fertilization.

- Line 684-686: "In summary, this work investigating the role of SIMR granules in embryos, together with our previous study of SIMR foci in the germline (Chen and Phillips 2024), has identified a new mechanism for small RNA loading of nuclear Argonaute proteins in *C. elegans*". This statement appears overstated/incorrect since there is no evidence that SIMR-1 foci are required for sRNA loading of NRDE3. The authors should emphasize other models, as suggested above.

We have revised the text on line 869-871 to emphasize that SIMR granule regulate the localization of nuclear Argonaute proteins, rather than suggesting a direct role on controlling small RNA loading. We also edit the title, text, and legend for our model in Figure 9.

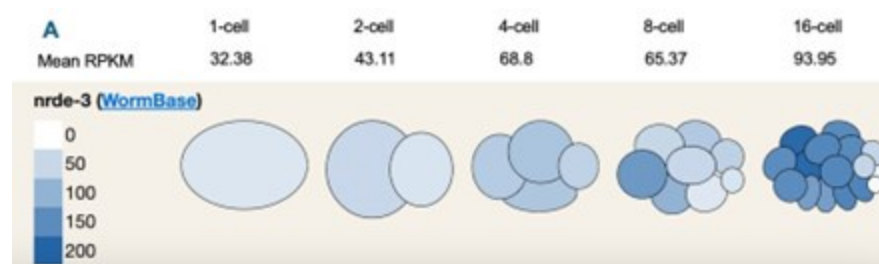
Reviewer #3 (Recommendations for the authors):

Issues to be addressed:

- The authors show a switch in 22G RNA binding by NRDE-3 during embryogenesis. While the data is convincing, it would be great if it could be tested if the preferred NRDE-3 replacement model is indeed correct. This could be done relatively easily by giving NRDE-3 a Dendra tag, allowing one to colour-switch the maternal WAGO-3 pool before the zygotic pool comes up. Such data would significantly enhance the manuscript, as this would allow the authors to follow the fate of maternal NRDE-3 more precisely, perhaps identifying a period of sharp decline of maternal NRDE-3.

We think the NRDE-3 Dendra tag experiment suggested by the reviewer is a clever approach and we will consider generating this strain in the future. However, we feel that optimization of the color-switching tag between the maternal germline and the developing embryos is beyond the scope of this manuscript. To partially address the question about NRDE-3 fate during embryogenesis, we examined the single-cell sequencing data of *C. elegans* embryos from 1-cell to 16-cell stage (Tintori *et al*, 2016, Dev Cell; Visualization tool from John I Murray lab), as shown in Author response image 3 Panel A below, NRDE-3 transcript level increases as embryo develops, indicating that zygotic NRDE-3 is being actively expressed starting very early in development. We hypothesize that maternal NRDE-3 will either be diluted as the embryo develops or actively degraded during early embryogenesis.

Author response image 3.



- Figure 3A: * should mark PGCs, but this seems incorrect. At the 8-cell stage there still is only one PGC (P4), not two, and at 100 cells there are only two, not three germ cells. Also, the identification of PGCs with a marker (PGL for instance) would be much more convincing.

We apologize for the confusion in Figure 3A. We changed the figure legend to clarify that the * indicate nuclear NRDE-3 localization in somatic cells for 8- and 100-cell stage embryos rather than the germ cells.

- Overall, the authors should address colocalization more robustly. In the current manuscript, just one image is provided, and often rather zoomed-out. How robust are the claims on colocalization, or lack thereof? With the current data, this cannot be assessed. Pearson correlation, combined with line-scans through a multitude of granules in different embryos will be required to make strong claims on colocalization. This applies to all figures (main and supplement) where claims on different granules are derived from.

We thank reviewer 3 for this important suggestion. To better address the colocalization, we included insets of individual granules in Figure 2D and Figure 4. We also performed colocalization analysis by calculating the Pearson's R value between different groups of proteins in Figure 4B, to highlight that SIMR-1 colocalizes with ENRI-2, NRDE-3(HK-AA), RDE-3, and RRF-1, while CSR-1 colocalizes with EGO-1.

For the proteins that lack colocalization in Figure 4—figure supplement 1, we also added insets of individual granules. Additionally, we included a new set of panels showing SIMR-1 localization compared to tubulin::GFP (Figure 4—figure supplement 1I) in response to a recent preprint (Jin et al, 2024, BioRxiv), which finds NRDE-3 (expressed under a *mex-5* promoter) associating with pericentrosomal foci and the spindle in early embryos. We do not see SIMR-1 (or NRDE-3, data not shown) at centrosomes or spindles in wild-type conditions but made a similar observation for SIMR-1 in a *mut-16* mutant (Figure 4E). All of the localization patterns were examined on at least 5 individual 100-cell staged embryos with same localization pattern.

- Figure 7: Its title is: Function of cytoplasmic granules. This is a much stronger statement than provided in the nicely balanced discussion. The role of the granules remains unclear, and they may well be just a reflection of activity, not a driver. While this is nicely discussed in the text, figure 7 misses this nuance. For instance, the title suggests function, and also the legend uses phrases like 'recruited to granule X'. If granules are the results of activity, 'recruitment' is really not the right way to express the findings. The nuance that is so nicely worded in the discussion should come out fully in this figure and its legend as well.

We have changed the title of Figure 7 (now Figure 9) to “Model for temporally- and developmentally regulated NRDE-3 function” to deemphasize the role of the granules and to highlight the different functions of NRDE-3. Similarly, we have rephrased the text in the figure and legend and add a some details about our new results.

Minor:

Typo: line 663 *Acaris*

We corrected the typo.

<https://doi.org/10.7554/eLife.102226.2.sa0>