The conserved helicase ZNFX-1 memorializes silenced RNAs in perinuclear condensates

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RNA-mediated interference (RNAi) is a conserved mechanism that uses small RNAs (sRNAs) to silence gene expression. In the Caenorhabditis elegans germline, transcripts targeted by sRNAs are used as templates for sRNA amplification to propagate silencing into the next generation. Here we show that RNAi leads to heritable changes in the distribution of nascent and mature transcripts that correlate with two parallel sRNA amplification loops. The first loop, dependent on the nuclear Argonaute HRDE-1, targets nascent transcripts and reduces but does not eliminate productive transcription at the locus. The second loop, dependent on the conserved helicase ZNFX-1, targets mature transcripts and concentrates them in perinuclear condensates. ZNFX-1 interacts with sRNA-targeted transcripts that have acquired poly(UG) tails and is required to sustain pUGylation and robust sRNA amplification in the inheriting generation. By maintaining a pool of transcripts for amplification, ZNFX-1 prevents premature extinction of the RNAi response and extends silencing into the next generation.
is transcribed in nuclei in the late pachytene region\(^2\) and accumulates in the shared cytoplasm (rachis) that supplies the growing oocytes\(^3\). As expected, we detected \textit{mex-6} transcripts diffuse in the rachis and cytoplasm of growing oocytes and concentrated in bright nuclear puncta in pachytene nuclei (but not oocyte nuclei; Fig. 1b,c). At high magnification, the nuclear puncta overlapped with 4,6-diamidino-2-phenylindole (DAPI) staining and occasionally resolved into twin or triplet dots (Fig. 1c), consistent with tight pairings of replicated and tightly synapsed homologous chromosomes in pachytene nuclei\(^2\). The nuclear puncta represent nascent transcripts at the \textit{mex-6} locus given that: (1) two-colour FISH targeting \textit{mex-6} and a linked locus (\textit{puf-5}, <1 Mb distance from \textit{mex-6}) revealed closely linked puncta (Extended Data Fig. 1b), (2) two-colour FISH targeting \textit{mex-6} and its non-linked homologue \textit{mex-5} revealed well-separated puncta with no cross-hybridization (Extended Data Fig. 1c) and (3) \textit{mex-6} nuclear puncta were only detected in the pachytene region and were not detected using a sense probe (Extended Data Fig. 1d).

To silence the \textit{mex-6} gene, we exposed synchronized first-day adult hermaphrodites to a 600-base pair (bp) dsRNA trigger targeting the 3’ end of the \textit{mex-6} transcript (Extended Data Fig. 2a and Methods). RNA sequencing (RNAseq) and two-colour FISH experiments confirmed that the RNAi treatment was specific for \textit{mex-6} and did not affect \textit{mex-5} levels (Extended Data Fig. 2b,c). We first detected a reduction in \textit{mex-6} signal in the cytoplasm of diplotene oocytes after 4 h of RNAi treatment, culminating in >90% decrease by 24 h (Fig. 2a,b). We also detected a transient increase in the intensity distribution of \textit{mex-6} pachytene nuclear puncta at 6 and 8 h (Fig. 2c,d). We still observed near co-localization of \textit{mex-6} and \textit{puf-5} nuclear puncta under \textit{mex-6} RNAi conditions, confirming that the puncta identify nascent transcripts at the \textit{mex-6} locus (Extended Data Fig. 2d). We conclude that in the first 24 h of exposure to the dsRNA trigger, RNAi induces a transient increase in the accumulation of nascent transcripts at the locus and a steady decrease in cytoplasmic transcripts.

**Targeted transcripts accumulate in nuage.** From 4 h after the start of RNAi treatment, we also noticed accumulation of \textit{mex-6} transcripts in micrometre-sized clusters in the cytoplasm of growing oocytes (Fig. 2a,e,f). The clusters overlapped with the P-granule marker PRG-1 and the Z granule-marker ZNFX-1, and overlapped partially with the Mutator foci-marker MUT-16 (Fig. 2e and Extended Data Fig. 2e,f). At 6 and 8 h, we also detected \textit{mex-6} accumulation in perinuclear nuage in the diplotene and pachytene regions (Fig. 2a). At 24 h, \textit{mex-6} accumulation in nuage in diplotene and growing oocytes was strongly diminished (Fig. 2f), mirroring the strong depletion of \textit{mex-6} transcripts in the cytoplasm (Fig. 2b). However, \textit{mex-6} signal could still be detected in nuage in the pachytene region where the \textit{mex-6} locus is transcribed (Extended Data Fig. 2g). We conclude that \textit{mex-6} transcripts accumulate in nuage throughout the RNAi response. The resolution of the in situ experiments was not sufficient to determine whether \textit{mex-6} RNA accumulates in a specific nuage subcompartment(s).

**RNAi-induced changes depend on the RNAi machinery and are heritable.** RDE-1 is the Argonaute that recognizes primary sRNAs derived from exogenous triggers\(^{11,27}\) and MUT-16 is required for...
amplification of secondary sRNAs\textsuperscript{28}. We found that \textit{rde-1} and \textit{mut-16} mutants were completely defective in the RNAi response (Extended Data Fig. 2h,i), confirming that the observed changes require synthesis of secondary sRNAs initiated by primary siRNAs. To examine inheritance of the response, we isolated embryos (F\textsubscript{1} generation) from gravid hermaphrodites (P\textsubscript{0} generation) fed the \textit{mex-6} trigger and raised the F\textsubscript{1} worms to the adult stage in the absence of the trigger. Cytoplasmic and nuclear \textit{mex-6} RNA was strongly reduced in the F\textsubscript{1} worms compared with the F\textsubscript{1} controls (derived from P\textsubscript{0} worms exposed to control RNAi; Fig. 3a,b and Extended Data Fig. 3a). Despite this strong reduction, we still detected transcripts in perinuclear dots overlapping with the nuage...
markers GFP::PRG-1 and GFP::ZNFX-1 in the pachytene region (Fig. 3c and Extended Data Fig. 3b). In contrast, little to no nuage accumulation was evident in oocytes (Fig. 3a). A similar pattern was detected in F2 animals (Extended Data Fig. 3c). These observations suggest that, despite a reduction in nascent transcripts, some mex-6 transcripts are still exported from the nucleus and allowed to accumulate at least transiently in nuage in the pachytene region in F1 and F2 animals.

Fig. 3 | Levels of mex-6 RNA in adult progeny (F1 generation) of animals exposed to mex-6 dsRNA. a, Maximum projection photomicrographs of germlines showing mex-6 RNA (magenta) in adult F1 generation of animals exposed to control or mex-6 RNAi (Methods). Scale bar, 10 µm. Images are representative of eight worms examined. b, Comparison of the maximum F1 mex-6 nuclear signal (pachytene region) in the F1 progeny of animals exposed to control or mex-6 RNAi. Each dot represents one nucleus. Nuclei were quantified across three worms (the exact number of nuclei quantified for each condition are provided in the Source Data). Values (arbitrary units, a.u.) were normalized to puf-5 RNA FISH signals visualized in the same nuclei (Methods). The central black dot and error bars represent the mean and s.d., respectively. The P value was calculated using an unpaired two-tailed Wilcoxon rank-sum test. c, Maximum projection photomicrographs of pachytene nuclei showing the nuage marker GFP::PRG-1 (green) and mex-6 RNA (magenta) in F1 generation of animals exposed to control or mex-6 RNAi. High-resolution images of a single pachytene nucleus (outlined by red boxes) are provided (bottom). The arrows point to mex-6 RNA signal at the locus and the arrowheads point to mex-6 RNA foci overlapping with perinuclear nuage. Scale bar, 2.5 µm. Images are representative of three worms examined. Note that the mex-6 RNA signal overlaps but is not perfectly coincident with the P granule-marker PRG-1; mex-6 RNA also partially overlaps with the Z granule-marker ZNFX-1, as shown in Extended Data Fig. 3b. Z granules are immediately adjacent to and/or overlap with P granules (within the diffraction limit) in pachytene and merge with P granules in embryos22,63. a, c, Results were consistent across three independent FISH experiments.
**hrde-1** is required for nuclear RNAi response in **P**<sub>0</sub> and **F**<sub>1</sub> animals. HRDE-1 (also known as WAGO-9) is a germine-specific nuclear Argonaute required for inheritance of the RNAi-induced silenced state<sup>12–14</sup>. A normal RNAi response, including rapid loss of **mex-6** RNA in the cytoplasm and accumulation in nuage, was observed in the oocytes of **hrde-1** mutant **P**<sub>0</sub> hermaphrodites (Fig. 4a andExtended Data Fig. 4a). However, the **hrde-1** mutants showed no change in the intensity distribution of nuclear puncta in the pachytene region (Fig. 4a–c). To explore the possibility that **hrde-1** mutants do not silence the **mex-6** locus, we examined the accumulation of **mex-6** transcripts in the rachis, the shared cytoplasm adjacent to pachytene nuclei. At 24 h, the levels of **mex-6** mRNA in the rachis declined by >90% in the wild-type worms compared with only about 50% in the **hrde-1** mutants (Fig. 4d,e). These observations suggest that **hrde-1** mutants fail to interfere with the production of **mex-6** transcripts in **P**<sub>0</sub> hermaphrodites. We obtained similar results in a strain mutated for another component of the nuclear RNAi machinery, **hrde-2** (Extended Data Fig. 4b).

Failure to silence the **mex-6** locus was also observed in **hrde-1** **F**<sub>1</sub> progeny. The intensities of nuclear puncta were similar in the **hrde-1** **F**<sub>1</sub>, **mex-6** RNAi and **F**<sub>1</sub> control RNAi animals (Fig. 4a,c). As in the wild type, however, **hrde-1** **F**<sub>1</sub> progeny accumulated **mex-6** transcripts in nuage in the pachytene region (Extended Data Fig. 4c). The levels of **mex-6** RNA in the pachytene rachis were higher in the **hrde-1** **F**<sub>1</sub> than wild-type **F**<sub>1</sub> animals from the **mex-6** RNAi condition but averaged only 50% of that observed in the **hrde-1** **F**<sub>1</sub> control condition (Extended Data Fig. 4d). We conclude that **hrde-1** is required for silencing of the locus in **P**<sub>0</sub> and **F**<sub>1</sub> animals (nuclear response) but is not essential for RNA degradation in the cytoplasm and accumulation in nuage in **P**<sub>0</sub> and **F**<sub>1</sub> animals (cytoplasmic response).

**Accumulation in nuage requires **znfx-1** in **P**<sub>0</sub> and **F**<sub>1</sub> animals.** ZNFX-1 is an SFI helicase domain-containing zinc finger protein that, like HRDE-1, is required for inheritance of the RNAi-induced silenced state<sup>11,22</sup>. Unlike HRDE-1, which is primarily nuclear, ZNFX-1 localizes to nuage (Z granules)<sup>22</sup>. We found that the **mex-6** cytoplasmic transcripts in **znfx-1** **P**<sub>0</sub> animals were rapidly degraded upon **mex-6** RNAi as in the wild type (Extended Data Fig. 5a). However, **mex-6** transcripts failed to accumulate in nuage (Fig. 5a,b).

We detected an increase in the intensity distribution of **mex-6** pachytene nuclear puncta in **znfx-1** mutants at the 4 h time point, 4 h earlier than the wild-type group (Extended Data Fig. 5a,b). This premature peak was followed by a decrease to levels lower than the non-RNAi condition by 24 h (Extended Data Fig. 5a,b). No changes in nuclear signal were observed in the **znfx-1**;**hrde-1** double-mutant animals, indicating that the nuclear response in the **znfx-1** mutants was dependent on **hrde-1**, as in the wild-type animals (Extended Data Fig. 5c,d). We conclude that **znfx-1** is required for robust recruitment of **mex-6** transcripts to nuage but not for RNA degradation in the cytoplasm or for engagement of the nuclear RNAi machinery in **P**<sub>0</sub> animals. Despite a failure to silence the **mex-6** locus and enrich **mex-6** transcripts in nuage, **znfx-1**;**hrde-1** **P**<sub>0</sub> animals still showed a rapid loss of cytoplasmic **mex-6** RNA throughout the germine, confirming that neither ZNFX-1 nor HRDE-1 is required for RNA turnover in the cytoplasm of **P**<sub>0</sub> animals (Extended Data Fig. 5c).

In **znfx-1** **F**<sub>1</sub> animals derived from **mex-6** RNAi fed **P**<sub>0</sub>s, we observed a partial reduction (approximately 50%) in cytoplasmic accumulation of **mex-6** transcripts in the pachytene rachis and no accumulation in nuage in the pachytene region (Fig. 5c,d and Extended Data Fig. 5e). The intensity distribution of nuclear puncta was reduced just as it was observed for the wild-type **F**<sub>1</sub> group (Extended Data Fig. 5b). This reduction was dependent on **hrde-1**, as the nuclear puncta intensities of the **znfx-1**;**hrde-1** **F**<sub>1</sub> animals matched that of the **znfx-1**;**hrde-1** **F**<sub>1</sub> control RNAi animals (Extended Data Fig. 5c,d). We conclude that **znfx-1** is not required for silencing of the locus in **P**<sub>0</sub> and **F**<sub>1</sub> animals (nuclear response) but is required for the accumulation of targeted transcripts in nuage in **P**<sub>0</sub> and **F**<sub>1</sub> animals (cytoplasmic response).

**hrde-1 and **znfx-1** contribute additively to silencing in **F**<sub>1</sub> animals.** Unlike in **P**<sub>0</sub> animals, the cytoplasmic **mex-6** RNA levels in **znfx-1**;**hrde-1** **F**<sub>1</sub> animals were indistinguishable from the control condition, indicating that **znfx-1** and **hrde-1** are both required for maximal silencing in **F**<sub>1</sub> animals (Fig. 5c,d). To examine this further, we compared the **mex-6** RNA levels in the wild-type as well as **znfx-1**, **hrde-1** and **znfx-1**;**hrde-1** mutant **F**<sub>1</sub> animals using quantitative PCR with reverse transcription (Extended Data Fig. 6a). These experiments confirmed partial silencing of **mex-6** transcripts in the single mutants and complete loss of silencing in the **F**<sub>1</sub> double mutants (Extended Data Fig. 6a). We obtained similar results when we targeted two other germine-expressed genes by RNAi (**oma-1** and **puf-5**; Extended Data Fig. 6b,c). We conclude that **hrde-1** and **znfx-1** contribute independently to silencing in **F**<sub>1</sub> worms and are required additively for maximal silencing.

**hrde-1 and **znfx-1** are responsible for distinct sRNA populations.** The additive phenotype of the **znfx-1**;**hrde-1** double mutant suggested that **hrde-1** and **znfx-1** function in separate mechanisms to maintain nuclear and cytoplasmic silenced states. To examine this possibility, we sequenced sRNAs in **znfx-1**;**znfx-1** and **znfx-1**;**hrde-1** mutant as well as wild-type **F**<sub>1</sub> animals. As expected, the wild-type **F**<sub>1</sub> worms exhibited a 23-fold increase in sRNAs mapping to the **mex-6** locus compared with the control **F**<sub>1</sub> animals, with a dominant peak corresponding to the location targeted by the dsRNA trigger (Fig. 6a,b). We also detected an increase in sRNAs at the **mex-6** locus.
Figure a: Wild type and hrde-1 mutant embryos treated with Control RNAi or mex-6 RNAi. Normalized mex-6 nuclear signal at 24 h and 48 h.

Figure b: Wild type and hrde-1 mutant embryos treated with Control RNAi or mex-6 RNAi. Normalized mex-6 cytoplasmic signal at 24 h and 48 h.

Figure c: Graph showing normalized mex-6 nuclear signal (a.u.) at 4 h, 8 h, 24 h, and F1 for WT and hrde-1 embryos.

Figure d: WT and hrde-1 embryos treated with pul-5 RNAi or mex-6 RNAi. Normalized mex-6 nuclear signal at 48 h.

Figure e: Comparison of normalized mex-6 cytoplasmic signal (a.u.) for Oocyte and Pachytene stages in WT and hrde-1 mutant embryos.
locus in the hrde-1 and znfx-1 mutant F1 animals, but to different extents. The increase in sRNAs reached 83% of the wild type in the hrde-1 mutants but only 6% of the wild type in the znfx-1 mutants (Fig. 6a,b). Wan et al. also reported low levels of sRNAs in znfx-1 mutant F1 animals after 4 h of RNAi treatment. Each dot represents an individual nuage granule. Nuage granules were quantified across five worms (the exact number of nuage granules quantified for each condition are provided in Source Data). c, Maximum projection photomicrographs of germlines showing mex-6 RNA (magenta) in F1 wild-type, znfx-1 mutant and znfx-1; hrde-1-double-mutant animals derived from P0 animals exposed to the indicated RNAi treatment. Scale bar, 10 µm. Images are representative of eight worms examined for each condition. a,c, Results were consistent across three independent FISH experiments. d, Comparison of the mean cytoplasmic mex-6 RNA FISH signal in the pachytene region of znfx-1 and znfx-1; hrde-1 mutant F1 progeny derived from animals exposed to the indicated RNAi treatment. Each dot represents one animal (n = 5 worms). P values were calculated using an unpaired two-tailed Student’s t-test. b,d, The central black dot and error bars represent the mean and s.d., respectively. Values (arbitrary units, a.u.) were normalized to puf-5 RNA FISH signals visualized in the same nuage granules (b) or germlines (d; Methods).

Fig. 5 | Enrichment of RNAi-targeted transcripts in nuage requires znfx-1. a, Single z-plane photomicrographs of oocytes in wild-type and znfx-1 mutant P0 animals showing staining for the nuage marker GFP::PRG-1 (green), DNA (stained with DAPI; blue) and mex-6 RNA (magenta) after 4 h of RNAi treatment. Scale bar, 2.5 µm. Images are representative of five worms examined for each condition. b, Comparison of the mean mex-6 RNA FISH signal in oocyte nuage in wild-type and znfx-1 mutant P0 animals after 4 h of RNAi treatment. Each dot represents an individual nuage granule. Nuage granules were quantified across five worms (the exact number of nuage granules quantified for each condition are provided in Source Data). c, Maximum projection photomicrographs of germlines showing mex-6 RNA (magenta) in F1 wild-type, znfx-1 mutant and znfx-1; hrde-1-double-mutant animals derived from P0 animals exposed to the indicated RNAi treatment. Scale bar, 10 µm. Images are representative of eight worms examined for each condition. a,c, Results were consistent across three independent FISH experiments. d, Comparison of the mean cytoplasmic mex-6 RNA FISH signal in the pachytene region of znfx-1 and znfx-1; hrde-1 mutant F1 progeny derived from animals exposed to the indicated RNAi treatment. Each dot represents one animal (n = 5 worms). P values were calculated using an unpaired two-tailed Student’s t-test. b,d, The central black dot and error bars represent the mean and s.d., respectively. Values (arbitrary units, a.u.) were normalized to puf-5 RNA FISH signals visualized in the same nuage granules (b) or germlines (d; Methods).
generation, especially around the sequence targeted by the original trigger. We noticed that the number of sRNA reads mapping to the \textit{mex-6} locus in \textit{znfx-1} and \textit{hrde-1} single mutants added up to 89% of the reads observed in wild-type F1 animals (Extended Data Fig. 6e; see Methods). This observation confirms that the ZNFX-1 and HRDE-1 amplification cycles function mostly independently, with possibly some synergy between the two cycles accounting for approximately 10% of sRNAs observed in the wild types.

To determine whether \textit{znfx-1} is also required for sRNA amplification in P0 animals, we sequenced sRNAs in wild-type and \textit{znfx-1} mutant hermaphrodites at different time points following feeding onset. We observed an increase of approximately 200-fold in sRNA accumulation at the \textit{mex-6} locus in the wild-type and \textit{znfx-1} P0 animals compared with control conditions (Extended Data Fig. 6f,g). The increase in sRNAs was slightly lower in the \textit{znfx-1} mutants compared with the wild types (reduction of approximately 16%),
znfx-1 is required to sustain pUGylation in F₁, progeny. A minority of sRNAs mapping to the trigger region correspond to primary sRNAs, with the majority corresponding to secondary sRNAs templated from pUGylated transcripts. To determine whether hrde-1 or znfx-1 are required for pUGylation, we amplified pUGylated mexc-6 transcripts from wild-type and mutant F₁ animals (position of primers shown in Extended Data Fig. 2a). As expected, we detected (see mexc-6 pUGylated transcripts) in wild-type F₁ animals from mexc-6 RNAi fed P₀, but not in the F₁ controls (Fig. 6c) or in worms mutated for the pUGylase RDE-3 (ref. 9; Extended Data Fig. 7a). We detected pUGylated mexc-6 transcripts in hrde-1 mutant F₁ adults but not in znfx-1 or znfx-1;hrde-1 F₁ mutant adults (Fig. 6c). Similar results were obtained in experiments with puf-5 and oma-1 mutants (Extended Data Fig. 7b,c). In contrast to F₁ adults, we detected pUGylated transcripts in znfx-1 P₀ adults (Fig. 6d) and F₁ embryos (Fig. 6e). We conclude that znfx-1 is not required for the initial production of pUGylated transcripts in the P₀ generation but is required to sustain production and/or maintenance in adult F₁ animals.

Discussion

Together with previous studies, our findings suggest the following model for silencing by an exogenous dsRNA trigger (Fig. 7c). Primary sRNAs loaded on Argonaute RDE-1 recognize complementary transcripts and mark them for cleavage, pUGylation and synthesis of secondary sRNAs by RdRP₈-₁₀,₁₂-₁₆. Secondary sRNAs load on HRDE-1 and other Argonautes₁₄-₁₅,₁₇ to activate three parallel silencing pathways. In the first pathway (Pathway I; red in Fig. 7c), WAGO proteins tag transcripts in the cytoplasm for rapid degradation by an unknown mechanism. In the second pathway (II; yellow in Fig. 7c), HRDE-1 shuttles into the nucleus to initiate 'nuclear RNAi', a silencing programme that suppresses, but does not eliminate, productive transcription of the locus. In the third pathway (III; blue in Fig. 7c), WAGO proteins that associate with ZNFX-1 recruit a subset of targeted transcripts to nuage and initiate a new cycle of pUGylation and sRNA amplification. Only the HRDE-1 and ZNFX-1 cycles generate tertiary sRNAs that feed back into their respective cycles to generate parallel self-reinforcing sRNA amplification loops. The HRDE-1 and ZNFX-1 amplification loops are transmitted to the next generation independently of each other and both are required for maximum silencing in F₁ progeny. In the following sections, we summarize evidence supporting the three silencing pathways and discuss remaining open questions.

Pathway I: secondary sRNAs induce RNA degradation in the cytoplasm. Under our RNAi conditions, we detected a reduction in transcript levels in the cytoplasm after 4h of feeding, eventually reaching 95% reduction by 24h. As expected, mRNA degradation was dependent on the primary Argonaute RDE-1 and MUT-16, a scaffolding protein required for amplification of secondary sRNAs. Messenger RNAs targeted by microRNAs for degradation have been reported to enrich in P bodies, cytoplasmic RNA granules that enrich components of the RNA degradation machinery. In our feeding experiments, we observed enrichment of targeted mRNAs in nuage condensates, but this enrichment was not linked to RNA degradation. Most strikingly, no nuage enrichment was observed in znfx-1 mutants, despite normal RNA degradation in these animals. We conclude that, unlike microRNA-induced RNA degradation, RNAi-induced RNA degradation does not require visible RNA enrichment in cytoplasmic granules. The robust sRNA amplification observed in znfx-1 mutant P₀ animals also suggests that secondary sRNA amplification initiated by primary sRNAs occurs in bulk cytoplasm or, at a minimum, does not require accumulation of targeted transcripts in granules. We cannot exclude that transit through nuage or some other RNA granules, in the absence of visible accumulation, is required for secondary sRNA amplification and/or RNA degradation. The RDE-1-initiated cycle of pUGylation and sRNA amplification is sufficient to eliminate most cytoplasmic transcripts in animals exposed to the dsRNA trigger. However, this cycle is not self-perpetuating and on its own eventually self-extinguishes, leaving no memory of the RNAi response.

Pathway II: HRDE-1 reduces but does not eliminate transcription. We detected a transient increase in nascent transcripts after 6h of RNAi in P₀ animals. This response requires the nuclear Argonaute HRDE-1 and may reflect stalling of RNA polymerase II and/or pre-mRNA processing, causing nascent transcripts to accumulate at the locus. Stalling of RNA polymerase has previously been implicated in nuclear RNAi, and several lines of evidence have linked RNAi and splicing, including apparent co-evolution of the RNAi and splicing machineries, splicing factors identified as HRDE-1 interactors, sRNA defects associated with mutations or knock down of spliceosome components and insensitivity to nuclear RNAi of an endogenous transcript whose introns were removed by genome editing. At 24h post feeding and even more acutely in F₁ animals, we observed a decrease in nascent transcripts, which may reflect a reduction in transcription initiation at the locus. The nuclear RNAi machinery deposits chromatin marks at the locus predicted to decrease transcription. Despite this apparent decrease in transcriptional output, we continued to observe transcripts in peri-nuclear nuage even in F₁ animals, indicating that a baseline level of transcription and export is maintained at the silenced locus. In S. pombe, transcription is maintained at the silent locus but export is blocked and replaced by rapid degradation of nuclear transcripts. This difference may reflect a C. elegans-specific adaptation that allows mature transcripts to be used as templates for sRNA amplification in perinuclear condensates (see Pathway III).

The HRDE-1 cycle generates sRNAs that map throughout the locus without preference for the trigger area and with a slight preference for the 5' end of the transcript. A similar pattern was described previously in the context of transgenes and endogenous transcripts targeted by endogenous sRNA pathways, and was found suggesting that znfx-1, although not essential, contributes modestly to sRNA amplification in P₀ animals. Unlike in the F₁ generation, znfx-1 P₀ animals accumulated sRNAs predominantly in the trigger region, as observed in the wild type (Extended Data Fig. 6g). These observations indicate that znfx-1 is not essential for sRNA amplification in P₀ animals exposed to the trigger but is required in the F₁ generation (Fig. 6a,b).
**Fig. 7 | ZNFX-1 immunoprecipitates with pUGylated RNAs and is required for localization of pUGylated RNAs to nuage.**

**a.** Gel showing amplification of pUGylated *mex-6* RNA from input or FLAG immunoprecipitates (IP) of lysates collected from adult worms grown for 8 h on either *mex-6* (+) or *puf-5* (−) RNAi. PGL-3 is a nuage protein that serves here as a negative control. Refer to Extended Data Fig. 8a for western blots demonstrating efficient immunoprecipitation of 3xFLAG-tagged ZNFX-1 and PGL-3. Gels are representative of three independent pull downs.

**b.** Photomicrographs of pachytene nuclei in dissected germlines from wild-type (top) and *znfx-1* mutant (bottom) animals showing staining for endogenous pUGylated RNAs (magenta), control *tbb-2* RNA (red) and DNA (stained with DAPI; blue). The contrast of the *znfx-1* images labelled with an asterisk were adjusted to match the intensity of the *tbb-2* signal in the wild type. See Extended Data Fig. 8c for unadjusted photomicrographs. Scale bar, 2.5 µm. Images are representative of six worms examined for each condition. Results were consistent across three independent FISH experiments.

**c.** Working model for exogenous RNAi. The dsRNA trigger is processed into primary sRNAs that load with RDE-1 to target complementary transcripts for pUGylation. The pUGylated transcripts recruit RdRP to generate secondary sRNAs in the cytoplasm (grey). Secondary sRNAs bind to secondary Argonautes (WAGO proteins), which initiate three distinct pathways. The first pathway (red) leads to degradation of cytoplasmic transcripts with no further sRNA amplification. On its own, this pathway is sufficient to silence gene expression in *P₀* animals exposed to dsRNA triggers but is insufficient to propagate the RNAi response across generations. A second pathway (yellow), dependent on the nuclear Argonaute HRDE-1, partially silences the locus and uses nascent transcript as templates for the production of tertiary sRNAs. A third pathway (blue), dependent on the nuage helicase ZNFX-1, enriches targeted transcripts in nuage, where they are pUGylated and used as templates for further tertiary sRNA amplification. Tertiary sRNAs feed back into their respective cycles, ensuring inheritance of the silenced state. The *hrde-1* and *znfx-1* sRNA amplification pathways are not essential for silencing in *P₀* animals but are required additively for full silencing in *F₁* animals. Possible crosstalk between the HRDE-1 and ZNFX-1 cycles is not shown. See Discussion for further considerations.
to be dependent on the nuclear RNAi machinery\textsuperscript{15}. One hypothesis is that the 5' bias is due to RdRPs that use nascent transcripts as templates for sRNA synthesis as described in \textit{S. pombe}. Consistent with this hypothesis, the nuclear RNAi machinery has been shown to interact with pre-mRNAs at the locus, which naturally exhibit a 5' bias\textsuperscript{36,48}. We suggest that HRDE-1, initially loaded with secondary sRNAs templated in the cytoplasm, initiates a nuclear cycle of sRNA amplification by recruiting an RdRP to nascent transcripts. The HRDE-1 cycle generates tertiary sRNAs, which in turn become complexed with HRDE-1 to perpetuate the cycle. The RdRP EGO-1 has been reported to localize in nuclei\textsuperscript{49} but a specific molecular interaction between EGO-1 and HRDE-1 has not been reported. However, analyses of silencing in operons have provided indirect evidence for an RdRP activity in nuclei\textsuperscript{15,30,32}. Although we favour a model where HRDE-1 and associated machinery use nascent transcripts to direct sRNA synthesis (Fig. 7c, yellow), we cannot exclude the possibility that HRDE-1-dependent sRNA amplification occurs outside the nucleus after export into the cytoplasm. Investigation into the factors that support HRDE-1-dependent sRNA production is an important future goal.

Pathway III: ZNFX-1 memorializes targeted RNAs in nuage. The HRDE-1 amplification cycle is insufficient for maximum silencing in \textit{F1} progeny. A second cycle dependent on the nuage protein ZNFX-1 is also required. The ZNFX-1 cycle generates sRNAs focused primarily on the area targeted by the original trigger and is responsible for the bulk of sRNA production in \textit{F1} animals. ZNFX-1 is required for the production and/or maintenance of pUGylated transcripts in \textit{F1} adults, for enrichment of RNAi-targeted transcripts and pUGylated RNAs in nuage, and can be immunoprecipitated with pUGylated transcripts. Together, these observations suggest that ZNFX-1 maintains a pool of silenced transcripts in nuage to enable their use as templates for sRNA amplification. Compartmentalization in nuage may serve to protect transcripts from RNA degradation enzymes in the cytoplasm and facilitate recognition by the pUGylase MUT-3 and RdRPs for synthesis of tertiary sRNAs (Fig. 7). Consistent with this model, ZNFX-1 has been reported to immunoprecipitate with the secondary Argonautes WAGO-1 and WAGO-4, and the RdRP EGO-1 (refs. \textsuperscript{31,32,38}). Presumably, tertiary sRNAs generated in the ZNFX-1 loop feed back into additional cycles of pUGylation and sRNA amplification to ensure propagation of sRNA amplification across generations. Because this self-perpetuating cycle is initiated by secondary sRNAs that target the trigger region, the ZNFX-1 cycle preferentially amplifies sRNAs near the trigger. ZNFX-1 has been proposed to help maintain uniform distribution of RdRPs on silenced transcripts, based on the observation that endogenous sRNAs exhibit a 5' bias in \textit{znfx-1} mutants\textsuperscript{31}. We suggest another possible explanation: in \textit{znfx-1} mutants, the only sRNAs remaining are those created by nuclear RNAi pathways, which are naturally 5'-biased given that they are templated from nascent transcripts.

It has been suggested that, in \textit{C. elegans}, initiation of sRNA amplification by non-primary sRNA–Argonaute complexes is limited in vivo to prevent dangerous runaway loops\textsuperscript{20}. We speculate that enrichment of ZNFX-1 in nuage places the ZNFX-1 amplification loop under tight regulation by competing sRNA pathways (for example, piRNAs) that protect transcripts from permanent silencing\textsuperscript{19}.

A role for ZNFX-1 in promoting sRNA amplification is consistent with the role of Hrr1, the \textit{S. pombe} orthologue of ZNFX-1, which functions with an RdRP\textsuperscript{34} and the predicted poly-A polymerase Cid12, which may be relevant to the role we propose here for ZNFX-1 in promoting the synthesis and/or stabilization of pUGylated sRNAs. However, unlike ZNFX-1, Hrr1 is nuclear and targets nascent transcripts\textsuperscript{44}. ZNFX-1 homologues in mice and humans function in the primary immune response against RNA viruses and bacteria\textsuperscript{35–39}. Mammalian ZNFX1 recognizes viral RNAs and localizes to the surface of mitochondria\textsuperscript{35}. Nuage-like compartments have been observed on the surface of mitochondria in several germ cell types, including mouse sperm\textsuperscript{39}. A common function for ZNFX1 orthologues in higher eukaryotes may therefore be to recognize and isolate transcripts in perinuclear or perimitochondrial nuage-like compartments for long-term silencing.

The HRDE-1 and ZNFX-1 sRNA amplification loops function in parallel. In contrast to RDE-1-initiated sRNA amplification, the HRDE-1 and ZNFX-1 programmes are self-sustaining cycles that maintain a pool of targeted transcripts for use as templates for sRNA amplification. In our RNAi feeding paradigm, the HRDE-1 and ZNFX-1 programmes were both required for full silencing in \textit{F1} animals. However, it is possible that reliance on the HRDE-1 or ZNFX-1 programmes will vary between loci and in response to other silencing triggers, such as endogenous sRNAs. Different genetic requirements for RNAi inheritance in different contexts have been previously documented\textsuperscript{50–52}.

Although our analyses suggest that the HRDE-1 and ZNFX-1 pathways function primarily independently of each other, two lines of evidence hint at possible crosstalk. First, the sum of mex-6 sRNAs induced by RNAi in \textit{hrde-1} and \textit{znfx-1} \textit{F1} animals added up to only 89% of what is observed in the wild type. Although this observation needs to be repeated in different contexts to ensure reproducibility, it suggests that sRNAs produced by one amplification cycle extend sRNA production in the other cycle. Second, the nuclear RNAi response was accelerated in \textit{znfx-1} \textit{F1} animals compared with the wild type, raising the possibility that the two cycles compete for secondary sRNAs and/or RdRPs in the early stages of the RNAi response. Alternatively, ZNFX-1 may antagonize HRDE-1-initiated transcriptional silencing to ensure sufficient production of mature mRNAs for use in the ZNFX-1 cycle. More complex interplays involving Argonautes that participate in multiple sRNA amplification mechanisms are also possible. How the RDE-1, HRDE-1 and ZNFX-1 sRNA amplification mechanisms coordinate in cells and across generations will be an important focus for future investigations.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41556-022-00940-w.

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Methods

Strains and maintenance. Strains were cultured at 20°C on OP50 bacteria plated on NNGM medium or NA22 bacteria plated on Enriched Peptone medium. The following strains were used in this study: N2 (H11), nts-1(gg61) II (Y1996)
bdr-1(tm200) II; nts-1(gg61) II (Y4054), prg-1(ne4523) II; ldr-1(t)}) (H4055, this study), prg-1(ne4523)[gfp;tag:flag-prg-1] I (WM527)
zzl-10(gg544) [3xflag; gfp;tag:zzl-10] I (YY996) pgg-1(ne4523) I; ldr-1(t) (H4053, this study).

RNA extraction and purification. Up to 100 µg of worms frozen in liquid nitrogen and stored at −80°C were resuspended in 1 ml TRIzol® (Thermo Fisher, cat no. 15596026), subjected to three freeze–thaw cycles and incubated at room temperature for 5 min with shaking at 1,500 r.p.m. (Benchmark Scientific, model M5000-HC) and an additional 5 min without shaking. After the addition of 200 µl chloroform, the samples were shaken by hand for 15 s, followed by an incubation of 2–3 min at room temperature and 12,000 centrifugation at 4°C for 15 min. The upper aqueous phase was removed and an equal volume of 95–100% ethanol was added. The samples were concentrated and purified using the Zymo RNA clean & concentrator kit columns (Zymo, cat no. R1017). On-column DNAse I digestions with MgCl₂ buffer (Thermo Fisher, cat no. EN0521) were used to remove contaminating DNA. The samples were eluted in water.

Plasmid construction. RNA plasmids were constructed using the L4440 vector and In-Fusion HD cloning kit (Takara Bio, cat no. 639650) transformed into Stellar competent cells (Takara Bio, cat no. 636766) and isolated using Qiaquick mini-prep kits (cat no. 27104). Primers (Supplementary Table 1) were designed using the Takara Bio In-Fusion online design tool. NEB Phusion PCRs (NEB, cat no. M7123) were used for plasmid construction.

RNAi. The empty L4440 vector was used as a control RNAi construct. NEB Phusion PCRs (NEB, cat no. M7123) were used for plasmid construction. NEB Phusion PCRs (NEB, cat no. M7123) were used for plasmid construction. NEB Phusion PCRs (NEB, cat no. M7123) were used for plasmid construction.

Embryos were hatched in M9 overnight at 20°C with shaking at 110 r.p.m. and plated onto NA22 bacteria cultured on Enriched Peptone medium. The adult worms were washed off the plates 60 h after plating, collected using a filter, re-plated onto either control (empty L4440 vector) or gene-specific RNAi plates for specified time periods and fixed (see the ‘FISH protocol’ section), used for RNA extraction (see RNA collection) or used to collect F₁ embryos for RNAi inheritance assays.

For the RNAi inheritance assays, F₁ embryos (isolated from P₀ adults by bleaching) were synchronized by shaking overnight (110 r.p.m.) at 20°C, plated onto NA22 plates and collected for 24 h for RNAi and for 24 h for RNAi and for 24 h for RNAi and for 24 h for RNAi and for 24 h for RNAi. The embryos were plated on plates that were allowed to dry before use.

For experiments examining late and early F₁ embryos, adult worms were fed RNAi for 24 h and bleached to isolate ‘early embryos’. Embryos laid on the plate were also collected and considered ‘late embryos’. Late embryo samples were also bleached to eliminate potential contamination by hatched animals in the first larval stage.

FISH protocol. Stellaris Probe Designer (v4.2) was used to design smFISH probes (Supplementary Table 2), which were purchased with Quasar670 and Quasar705 dyes. For FISH of the whole worm (undigested), 100 µl of live worms were fixed in 1,000 µl fixation buffer (1xPBS and 3.7% formaldehyde) on a rotating shaker at room temperature for 45 min. spun down at 3,000 g in a table-top centrifuge and washed twice with 1,000 µl 1xPBS. The worms were pelleted and stored at 4°C for at least 4 h in 1,000 µl of 75% ethanol. The samples were washed once with 1,000 µl of freshly prepared Stellaris Buffer A Mix (10% deionized formamide, 20% Stellaris RNA FISH Wash Buffer A (Biosearch Technologies, cat no. SRF-3B) and 70% RNAi-free water) and resuspended in 100 µl of freshly prepared Hybe Buffer Mix (two-colour in situ hybridization, 85.5 µl Stellaris RNA FISH Hybrization Buffer (Biosearch Technologies; cat no. SRF-HB1–10), 9.5 µl deionized formamide, 2.5 µl of 5 µM probe 1 suspended in TE and 2.5 µl of 5 µM probe 2 suspended in TE) before overnight incubation at 37°C. After the addition of 1,000 µl of freshly prepared Stellaris Buffer A Mix at 37°C for 30 min, the samples were resuspended in 1,000 µl Stellaris Buffer A Mix with 5 µM DAPI at 37°C for 30 min, resuspended in Stellaris RNA FISH Wash Buffer B (Biosearch Technologies, cat no. SRF-WB1–20) for 5 min at room temperature and finally resuspended in Vectashield Antifade Mounting Medium with DAPI (VWR, cat no. H-1200-10) before placing on slides for microscopy.

For FISH of dissected germlines, worms in M9 with 5% FISH in the wash buffer were dissected to release germlines, freeze-cracked on dry ice, placed into cold (~20°C) methanol, washed three times in PBS + 0.1% Tween 20 and fixed in 4% parformaldehyde for 1 h at room temperature. The samples were washed on slides with Stellaris Buffer A Mix, which was replaced with 100 µl of freshly prepared Hybe Buffer Mix and incubated at 37°C overnight. The slides were washed in 500 µl of freshly prepared Stellaris Buffer A Mix, incubated in the same for 30 min at 37°C, washed in 500 µl Stellaris Buffer A Mix containing 5 ng/ml DAPI, incubated in the same for 30 min at 37°C, washed with 500 µl Stellaris RNA FISH Wash Buffer B (Biosearch Technologies, cat no. SRF-WB1–20) and incubated in the same for 5 min at room temperature before replacing the buffer with Vectashield and sealing under a coverslip.

RNAseq. For sRNAseq, 5 µg of total RNA was treated with 5' polyphosphate (20 µg µl⁻¹ RNA) for 30 min at 37°C and purified using Zymo RNA clean & concentrator kit columns (Zymo, cat no. R1017). The treated RNA (1 µg) was isolated into an Illumina TrueSeq RNA Library Prep Kit (Illumina, cat no. RS-200-0012) with 11 cycles of PCR amplification. The libraries were run on either a 6% Novex TBE gel or a 5% Criterion TBE gel and size-selected according to the Illumina protocol. Purified samples were sequenced on an Illumina HiSeq2500 system at the Johns Hopkins University School of Medicine Genetic Resources Core Facility.

For mRNAseq, 1 µg of total RNA isolated using TruSeq stranded total RNA library prep gold (Illumina, cat no. 20020598) was mixed with 2 µl of a 1:10 dilution of the ERCC RNA spike-in mix (1 (Thermo Fisher, cat no. 4456740). TruSeq RNA UD indices were used for indexing (Illumina, cat no. 20022371) and libraries were pooled for sequencing on a NovaSeq6000 system at the Johns Hopkins University School of Medicine Genetic Resources Core Facility.

High-throughput sequencing analyses. For the sRNAseq libraries, 5’ Illumina adaptor sequences were removed using the default settings of Cutadapt and reads that were longer than 30 nucleotides or shorter than 18 nucleotides were discarded. The libraries were aligned to the UCSC cel10 reference genome using HISAT2 (ref. 9). For assessing the number of reads mapping to the mex-6 gene, the total number of reads aligning to mex-6 were counted for two technical replicates and normalized to the number of singly aligned reads mapping to the genome (that is, library size) per million reads (RPM).

The number of Ash reads were comparable between the wild-type and multiple libraries (Extended Data Fig. 9), suggesting that there are no global changes in sRNA levels that could skew comparisons between genotypes. Comparisons of replicates confirmed the quality of each library (Extended Data Fig. 9).

For RNA read–coverage analysis, mapped sRNA reads across the mex-6 gene were placed into 5-bp bins. The number of nucleotides per bin was normalized to library size and averaged across two technical replicates. sRNA present in the control mRNA condition (L4440 RNA vector) were then subtracted from the RNAi condition. Scripts are available on request.

For the mRNAseq libraries, reads were aligned to the UCSC cel10 reference genome using HISAT2 (ref. 9). To assess the number of reads mapping to the mex-6 genes, the total number of reads mapping to these loci were counted and normalized to the number of singly aligned reads mapping to the entire genome (that is, library size) per million reads (RPM).

All high-throughput sequencing data were analysed on an Ubuntu 16.04 LTS (GNU/Linux 4.15.0-14-generic x86_64) computer.

pUGlylation assays. We synthesized pUG complementary DNA using the SuperScript III first-strand synthesis system (Thermo Fisher, cat. no. 18080051) according to the manufacturer’s instructions and stored it at −20°C.

The pUG DNA (1 µl) was inputted into a 20 µl GoTaq PCR reaction (Promega, cat no. M7123) with the first adaptor-specific primer (Shukla et al.; OIP3098 in Supplementary Table 1) and the first gene-specific primer (2’ primers in Supplementary Table 1). The samples were diluted 1:100 and 1 µl was added to a second 20 µl GoTaq PCR reaction with the second adaptor-specific primer (Shukla et al.; OIP3099 in Supplementary Table 1) and the second gene-specific primer (2’ primers in Supplementary Table 1). The reactions were run on a 1% agarose gel and imaged using a Typhoon imager for analysis of transcripts 500–1,000 bp in length.
For puGylation assays in immunoprecipitated samples, 8 µl of 20 µl RNA eluted from the immunoprecipitation RNA extraction was used for the puG cDNA synthesis (representative of approximately 40% of the immunoprecipitated RNA). RNA (5 µl) was used for the immunoprecipitation puG cDNA synthesis (representative of approximately 0.25% of the input RNA). Reverse transcription reactions were subjected to two rounds of PCR as described earlier. Immunoprecipitation. Filtered adult worms were washed in sonication buffer (20 mM Tris–HCl pH 7.5, 200 mM NaCl, 2.5 mM MgCl₂, 10% glycerol, 0.9% NP-40 and 1 mM dithiothreitol) with Complete, Mini, EDDA-free protease inhibitor cocktail (Millipore Sigma, cat. no. 1183617001; one tablet per 10 ml) and stored at −80 °C. The samples were thawed on ice with SUPERase•In RNase inhibitor (Thermo Fisher, cat. no. AM2694; final concentration of 80 U ml⁻¹), sonicated using a Branson Digital Sonifier SFX 250 with a microtip (15 s on, 45 s off, 20% power, 3 min in total), cleared through centrifugation at 18,400g and 4 °C for 15 min and the protein concentrations were determined using a Pierce BCA assay (Thermo Fisher, cat. no. 23225). For the immunoprecipitation, 400 µl of immunoprecipulated, 400 µl of 500 µg/ml lysate was added to 20 µl anti-FLAG M2 magnetic beads slurry (Millipore Sigma, cat. no. MR823-1ML) that had been washed three times in 200 µl of sonication buffer + 80 U ml⁻¹ SUPERase•In RNase inhibitor. An equivalent of 1% input lysate was used for analysis of the immunoprecipitation by western blotting (see the ‘Western blotting’ section). An additional equivalent of 50% of input lysate was saved for RNA extraction (see ‘RNA extraction and purification’). The samples were rotated at 4 °C for 2 h, cleared with a magnetic stand and 4.2 µl of the supernatant (approximately 1%) was saved for western analysis (see ‘Western blotting’). The magnetic beads were washed with 500 µl of sonication buffer + 80 U ml⁻¹ SUPERase•In RNase inhibitor and resuspended in 100 µl of sonication buffer + 80 U ml⁻¹ SUPERase•In RNase inhibitor. A 1-µl volume of bead slurry (1%) was removed for western analysis of the immunoprecipitates (elution occurred through the addition of sample buffer and boiling; see: ‘Western blotting’). TRIzol was added to the remaining elution/bead solution for RNA extraction (see ‘RNA extraction and purification’).

Western blotting. Samples were resuspended in 200 µM dithiothreitol and 1X-Tris-Glyc SDS sample buffer (Thermo Fisher, cat. no. LC2676), flash-frozen and stored at −80 °C. The samples were heated to 95 °C for 10 min and run in a Novex Tris–glycine SDS running buffer (Thermo Fisher, cat. no. LC2675) on a Novex. Wells were 6%, 10%, 15%, or 20% gels, 0.1 mm, 1X, 2X, 3X, 4X, and 5X Tris–glycine, 1.0 mm, mini protein 12-well gel (Thermo Fisher, cat. no. XP00062BOX) with a Spectra multicolor high range protein ladder (Thermo Fisher, cat. no. XP00062BOX) with a Spectra multicolor high range protein ladder (Thermo Fisher, cat. no. XP00062BOX) with a Spectra multicolor high range protein ladder (Thermo Fisher, cat. no. XP00062BOX) with a Spectra multicolor high range protein ladder (Thermo Fisher, cat. no. XP00062BOX).

Image analysis and quantification. All FISH experimental values were normalized across experiments using control RNA (typically puf-5) visualized by FISH in a second colour. Images (Zeiss Axios Observer) were processed in Fiji (https://imagej.net/software/fijidownload). All quantification was processed using Fiji (version 1.4.0.1) and RStudio version 1.4.1717.

For quantification of the cytoplasmic signals, five worms were used for each condition. Regions of interest (ROIs) were drawn in single z planes, mean intensity values were calculated for each channel (mex-6 (experimental) and puf-5 (control)) and background mean intensity values measured in adjacent soma tissues were subtracted. The background-subtracted mean mex-6 germine measurements were normalized to the background-subtracted mean puf-5 germine measurement.

For quantification of the pachybyte nuclear signals, maximum projections were taken from half of the C. elegans germline (in the z-direction) and individual ROIs were drawn around ten rows of pachyteine nuclei, starting in the centre of the z-stack projection. The maximum, mean and median value for each ROI was measured in each channel. The median mex-6 value for each nucleus was subtracted from its respective mex-6 maximum value. The mex-6 maximum value was then normalized by dividing it by the mean puf-5 value measured for the respective ROI. The final equation was as follows: 

\[
y = \frac{\text{maximum mex-6 value of ROI} - \text{median mex-6 signal of ROI}}{\text{mean puf-5 signal ROI}}
\]

Values were plotted for three individual worms for each condition. Values across nuclei from different worms overlap (Extended Data Fig. 9d).

For quantification of the granule signals, ROIs for individual granules were drawn by masking in Fiji, and the mean mex-6 and puf-5 values were measured for each granule. Background mean intensity values were measured in adjacent soma tissues for both channels and subtracted from the measured values in the background-subtracted mean mex-6 germine measurement. 

The background-subtracted mean mex-6 germine measurement was then normalized to the background-subtracted mean puf-5 germine measurement and the values were plotted. Five individual worms were used for each condition.

To normalize the image display shown in Fig. 7b, the pixel-intensity distribution mean of the ibf-2 RNA channel (used as a control for normalization) of the zip-f1 mutant was adjusted to match that of the wild-type using ImageJ. The pUG RNA pixel-intensity distribution was then proportionally adjusted. Equally adjusted channels are also shown in Extended Data Fig. 8c.

Statistics and reproducibility. For information regarding the statistical analysis used, see the figure legends for each graph. P values are indicated in each figure. Sample sizes were chosen based on an estimated number of samples that seemed to reflect the variability of the population. No statistical methods were used to pre-determine sample size. Data were only excluded from the analysis if the control feature (that is, puf-5 RNA) used for normalizing between samples and conditions seemed to be aberrant (such instances were infrequent). The investigators were not blinded to allocation during experiments and outcome assessment. All in situ experiments were performed three or more times, with the exception of Extended Data Figs. 1c, 2b, e, h, i, 3b, 5c, which were all performed twice. The in situ experiments in Extended Data Figs. 3c and 4b were performed once. The mRNaseq from Extended Data Fig. 2c and quantitative-PCR data from Extended Data Fig. 6a–c were prepared from single biological replicates for each condition. The sRNAseq experiments were conducted in duplicate from a single biological source of RNA (Fig. 6a, b and Extended Data Fig. 6d–g). The puGylation immunoprecipitation experiments were performed three independent times.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. RNASeq datasets have been deposited onto the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA915556. Source data are provided with this paper. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

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Author contributions
Experiments were conducted by J.P.T.O. and W.L.Z. Experimental design and analysis were conducted by J.P.T.O., W.L.Z. and G.S. J.P.T.O. and G.S. wrote the manuscript.

Competing interests
G.S. serves on the Scientific Advisory Board of Dewpoint Therapeutics, Inc. The remaining authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to Geraldine Seydoux.
Peer review information Nature Cell Biology thanks the anonymous reviewers for their contribution to the peer review of this work.
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Extended Data Fig. 1 | Characterization of the mex-6 transcript. a) IGV genome browser views of sRNAseq reads (wild-type adult hermaphrodites) mapping to the mex-6 locus. rde-11 is a locus that is highly targeted by endogenous sRNAs52, shown here for comparison. Genome views are representative of two independent sequencing libraries. b) Maximum projection photomicrographs of pachytene nuclei showing DNA (blue, stained with DAPI), mex-6 RNA (magenta), and puf-5 RNA (green). The mex-6 and puf-5 loci are linked on Chromosome II and, as expected, exhibit closely linked nuclear puncta (arrows). Scale bar is 2.5 µm. Images are representative of 3 worms examined. Results were consistent across four independent FISH experiments. c) Same as in B but showing mex-6 (magenta) and mex-5 (green) RNAs. mex-6 and mex-5 are homologous loci on different chromosomes. Scale bar is 2.5 µm. Images are representative of 3 worms examined. Results were consistent across two independent FISH experiments. The mex-6 and mex-5 puncta do not co-localize, as expected, confirming the specificity of the FISH probes. d) Same as in B and C but using a mex-6 sense probe (magenta) and a puf-5 antisense probe (green). Scale bar is 2.5 µm. Images are representative of 3 worms examined. Results were consistent across two independent FISH experiments. As expected, the mex-6 sense probe does not detect any signal, confirming that the signals detected by the mex-6 antisense probe in panels B and C correspond to RNA and not DNA. Note also the lack of signal under RNAi conditions, suggesting that our FISH protocol does not detect sRNAs.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | *mex-6* RNAi induces cytoplasmic and nuclear changes to the *mex-6* transcript, dependent upon the RNAi machinery.

**a**) Schematic of the *mex-6* transcript. The regions targeted by smFISH probes and the dsRNA trigger are indicated. Red arrows denote primers used for amplification of pUGylated transcripts shown in Fig. 6. **b**) FISH experiment showing that *mex-6* RNAi conditions reduce *mex-6* but not *mex-5* levels in oocytes. Scale bar is 2.5µm. Number of worms examined is indicated. Results were consistent across two independent FISH experiments. **c**) Control RNAseq experiment on adult hermaphrodites showing that *mex-6* RNAi conditions reduce *mex-6* but not *mex-5* transcripts. *mex-6* RPM counts exclude the trigger region. Each bar represents the *mex-6/mex-5* RPM of a single biological replicate. **d**) Control FISH experiment showing near co-localization of *mex-6* and *puf-5* signals under control and *mex-6* RNAi conditions. Scale bar is 2.5µm. Images are representative of 6 worms examined. Results were consistent across four independent FISH experiments. **e**) Photomicrographs of oocytes showing the Z granule marker GFP::ZNFX-1, DNA, and *mex-6* RNA 4 h post *mex-6* RNAi treatment. Scale bar is 2.5µm. Images are representative of 3 worms examined. Results were consistent across two independent FISH experiments. **f**) Photomicrographs of oocytes showing the Mutator foci marker MUT-16::GFP and *mex-6* RNA 4 h post *mex-6* RNAi treatment. Scale bar is 2.5µm. Images are representative of 3 worms examined. Results were consistent across three independent FISH experiments. **g**) Photomicrographs of pachytene nuclei showing GFP::PRG-1 and *mex-6* RNA 24 h post either control or *mex-6* RNAi treatment. Scale bar is 2.5µm. Images are representative of 5 worms examined. Results were consistent across four independent FISH experiments. **h**) Photomicrographs of *rde-1* mutant germlines showing *mex-6* RNA in either control or *mex-6* RNAi conditions at 8 and 24 h of RNAi treatment. Scale bar is 10µm. Images are representative of 8 worms examined. Results were consistent across two independent FISH experiments. **i**) Photomicrographs of *mut-16* mutant germlines showing *mex-6* RNA in either control or *mex-6* RNAi conditions at 8 and 24 h of RNAi treatment. Scale bar is 10µm. Images are representative of 8 worms examined. Results were consistent across two independent FISH experiments.
Extended Data Fig. 3 | Transgenerational analysis of the mex-6 transcript upon RNAi. **a)** Graph comparing the mean mex-6 RNA FISH signal from the pachytene rachis in wild-type F1 progeny of P0s administered either control (red) or mex-6 (blue) RNAi. Each dot represents a single worm (n = 5 worms). Central black dot and error bars represent the mean and standard deviation, respectively. Values (arbitrary units) were normalized to puf-5 RNA FISH signals visualized in same region (Methods). **b)** Maximum projection photomicrographs of pachytene nuclei showing the nuage marker GFP::ZNFX-1 (green) and mex-6 RNA (magenta) in F1 progeny of animals exposed to mex-6 RNAi. Bottom rows shows high-resolution images of a single pachytene nucleus. Scale bar is 2.5 µm. Images are representative of 7 worms examined in the control condition and 8 worms examined in the mex-6 RNAi condition. Results were consistent across two independent FISH experiments. **c)** Maximum projection photomicrographs showing mex-6 RNA in germlines from F1 and F2 progeny derived from P0 animals exposed to mex-6 or control RNAi conditions. Scale bar is 10 µm. Images are representative of 6 worms examined in each condition. This experiment was performed once.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | The nuclear RNAi pathway affects *mex-6* nascent transcripts upon RNAi. **a)** Photomicrographs of P0 wild-type and *hrde-1* mutant oocytes showing the nuage marker GFP::PRG-1 (green), DNA (blue, stained with DAPI), and *mex-6* RNA (magenta) following 4 h of *mex-6* RNAi treatment. Scale bar is 2.5 µm. Images are representative of 3 wild-type worms examined and 6 *hrde-1* worms examined. Results were consistent across three independent FISH experiments. **b)** Single z-plane photomicrographs of P0 wild-type and *nrde-2* mutant germlines (pachytene region and oocytes) at 24 h after control or *mex-6* RNAi co-stained for *mex-6* (magenta) and *puf-5* (red, negative control) RNAs. Scale bar is 10 µm. Images are representative of 8 worms examined in each condition. This experiment was performed once. **c)** Maximum projection photomicrographs of F1 wild-type or *hrde-1* mutant pachytene nuclei showing the nuage marker GFP::PRG-1 (green) and *mex-6* RNA (magenta) under control or *mex-6* RNAi in the P0 generation. Scale bar is 2.5 µm. Images are representative of 3 worms examined for each condition. Results were consistent across three independent FISH experiments. **d)** Graph comparing the mean *mex-6* RNA FISH signal from the pachytene rachis in wild-type and *hrde-1* mutant F1 progeny of P0s administered either control (red) or *mex-6* (blue) RNAi. Each dot represents a single worm (n = 5 worms). Central black dot and error bars represent the mean and standard deviation, respectively. Values (arbitrary units) were normalized to *puf-5* RNA FISH signals visualized in same nuclei (Methods). WT values are the same as shown in Fig. S3A.
Extended Data Fig. 5 | mex-6 RNA patterning in znfx-1 and znfx-1; hrde-1 mutants upon RNAi. a) Maximum projection photomicrographs of znfx-1 P0 mutant germlines showing mex-6 RNA at the indicated time points following either control or mex-6 RNAi. Scale bar is 10 µm. Images are representative of 8 wild-type worms examined for each condition. Results were consistent across three independent FISH experiments. b) Graph comparing the maximum mex-6 nuclear FISH signal in control (red) vs mex-6 (blue) RNAi at the indicated time points following RNAi in znfx-1 P0 animals. Each dot of the violin plot represents one nucleus. Nuclei were quantified across 3 worms (see Extended Data Fig. 5 source data for the exact number of nuclei quantified for each condition). Values (arbitrary units) were normalized to puf-5 RNA FISH signals visualized in same nuclei (Methods). Central black dot and error bars represent the mean and standard deviation, respectively. P values were calculated using an unpaired two-tailed Wilcoxon rank-sum test. Refer to Fig. 2d for a comparison to wildtype. c) Maximum projection photomicrographs of znfx-1; hrde-1 P0 germlines showing mex-6 RNA at the indicated time points following either control or mex-6 RNAi. Scale bar is 10 µm. Images are representative of 8 wild-type worms examined for each condition. Results were consistent across two independent FISH experiments. d) Graph comparing the maximum mex-6 nuclear FISH signal in control (red) vs mex-6 (blue) RNAi at the indicated time points following RNAi in znfx-1; hrde-1 P0 animals. Each dot of the violin plot represents one nucleus. Nuclei were quantified across 3 worms (see Extended Data Fig. 5 source data for the exact number of nuclei quantified for each condition). Values (arbitrary units) were normalized to puf-5 RNA FISH signals visualized in same nuclei (Methods). Central black dot and error bars represent the mean and standard deviation, respectively. P values were calculated using an unpaired two-tailed Wilcoxon rank-sum test. e) Maximum projection photomicrographs of F1 wild-type and znfx-1 mutant pachytene nuclei showing the nuage marker GFP::PRG-1 (green) and mex-6 RNA (magenta) following administration of mex-6 RNAi in the P0 generation. Scale bar is 2.5 µm. Images are representative of 3 wild-type worms examined for each condition. Results were consistent across three independent FISH experiments.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | RNAi inheritance is dependent upon both znfx-1 and hrde-1. a–c) Graphs showing relative mRNA levels comparing control (−; set to 1) and RNAi conditions (+) in PO worms of the indicated genotypes. RT-qPCR Ct values in each sample were normalized to tbb-2 RT-qPCR Ct values in the same sample and to the control RNAi condition (see Methods). Points indicate three technical replicates for each condition from a single source of biological RNA. d) Genome browser view of sRNA seq reads mapping to the mex-6 locus in the genotypes and RNAi conditions indicated. Same data as Fig. 6b but values for the control and mex-6 RNAi conditions are shown separately. Reads were averaged between two technical sequencing replicates from a single source of biological RNA for each genotype/condition. e) Graph comparing mex-6 sRNA reads induced by RNAi in WT compared to the sum of the sRNA reads induced by RNAi in the hrde-1 and znfx-1 single mutants. See Methods for calculations. Reads were averaged between two technical sequencing replicates from a single source of biological RNA for each genotype. f) Graph showing the fold increase in sRNAseq reads mapping to the mex-6 transcript in wild-type and znfx-1 POs at the indicated time points following either control (red) or mex-6 (blue) RNAi. Each bar represents the mex-6 RPM of a single biological replicate. g) sRNAs mapping to the mex-6 locus in the PO generation in WT and znfx-1 mutants under control and mex-6 RNAi conditions. Unlike F1 znfx-1 animals (Fig. S6D), PO znfx-1 animals accumulate sRNAs in the trigger region. Each genome browser view is derived from sequencing of a single biological replicate.
Extended Data Fig. 7 | pUGylation in the F1 generation is dependent upon ZNFX-1, but not HRDE-1 following RNAi. a) Gel showing PCR amplification of pUGylated mex-6 RNAs from lysates derived from WT and rde-3(ne298) P0 animals treated with control (\(\sim\)) or mex-6 (\(\sim\)) RNAi (top panel). rde-3 mutants lack the pUGylase and do not produce pUGylated transcripts. gsa-1 is the pUG amplification control (bottom panels). b-c) Gels showing PCR amplification of puf-5 or oma-1 pUGylated RNAs from lysates derived from P0 animals of the indicated genotypes treated with control (\(\sim\)) or experimental (\(\sim\)) RNAi as indicated. Red dots indicate position of non-specific bands. The pUGylation experiments in a-c have been performed once.
Extended Data Fig. 8 | ZNFX-1 interacts/colocalizes with pUGylated transcripts. a) Anti-FLAG western blots to control for the efficiency of immunoprecipitation experiments shown in Fig. 7. 'Input' represents 1% of the input sample prior to immunoprecipitation; 'Sup' represents 1% of the supernatant following immunoprecipitation; 'IP' represents 1% of the immunoprecipitation sample following FLAG elution. Blots are representative of three independent pull downs.

b) Gel showing PCR amplification of pUGylated put-5 RNA from input (top panel) or FLAG immunoprecipitates (bottom panel) from animals where the znfx-1 or pgl-3 locus is untagged or tagged with 3xFLAG. Lysates were collected from adult worms grown for 8 h on either put-5 (‘+’) or mex-6 (‘-’) RNAi and are the same lysates used in Fig. 7a. Gels are representative of three independent pull downs. c) Same images as in Fig. 7b without contrast adjustment. See Fig. 7b for further details. d) Maximum projection photomicrographs of dissected wild-type and znfx-1 mutant germlines showing pUG RNA FISH (magenta) and control tbb-2 RNA. The pachytene region and oocytes are indicated with arrows. In this example, the dissected germline is extended rather than bent as shown in Fig. 1. Scale bar is 10 µm. Images are representative of 7 wild-type worms examined and 6 znfx-1 worms examined. Results were consistent across three independent FISH experiments.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | sRNAseq quality control analysis. a) Scatter plots comparing miRNA RPMs in wild-type (X-axis) and mutants (Y-axis) as indicated under control RNAi conditions. Linear regression is used to fit the data. miRNA counts in each condition are averaged across two replicates. b) Scatter plots comparing sRNA RPMs in the F1 progeny of control and *mex-6* RNAi fed P0 worms for the indicated genotypes. Each dot corresponds to a locus in the C. elegans genome. The red dot corresponds to the *mex-6* locus. Linear regression is calculated without *mex-6* sRNA counts. sRNA counts in each condition were averaged across two replicates. c) Linear regression statistics modelling the relationship between the two sRNAseq replicates for each genotype in both control and *mex-6* RNAi conditions. d) Super plot showing data from Fig. 2d with nuclei colour-coded to indicate worm origin. Central black dot and error bars represent the mean and standard deviation respectively. The distribution of values from each worm overlap. See legend for Fig. 2d for further description.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection  Microscopy data were collected using 3i SlideBook6 and ZEISS ZEN 3.4 (blue edition).

Data analysis  Microscopy data were analyzed using Fiji software (v2.1.0/1.53f; Build 5723140693), R (version 4.1.0), RStudio (version 1.4.1717) using custom scripts. Statistics and graphs were calculated/constructed in R. High-throughput sequencing data were analyzed on an Ubuntu 16.04.6 LTS (GNU/Linux 4.15.0-142-generic x86_64) computer using Cutadapt (version 2.10) and HISAT2 (version 2.2.1).

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All data will be made available on request. RNA sequencing datasets have been deposited onto the NCBI Sequence Read Archive (SRA) under the BioProject accession number of PRJNA819556.
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Sample size: Sample sizes were chosen based off of an estimated number of samples that appeared to reflect the variability of the population. No statistical method was used to predetermine sample size.

Data exclusions: Data were only excluded from the analysis if the control feature used for normalizing between samples and conditions appeared aberrant. Such instances were infrequent.

Replication: Experiments were conducted multiple times using RNAI triggers against three different genes. Similar observations were made for these three triggers.

Randomization: Samples were allocated into experimental groups by genotype and RNAI condition. Covariates were controlled for by maintaining all samples in the same growth and media conditions.

Blinding: Investigators were not blinded for these experiments, as the identity of each condition could always be identified through examination of the RNA level alone.

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Antibodies

Antibodies used: anti-FLAG M2 primary antibody (Millipore Sigma; Cat #: MF1804) and Goat Anti-Mouse IgG1 HRP-conjugated secondary antibody (JacksonImmuno; Cat #: 115-035-205)

Validation: Antibodies were self validated via western blot on a strain lacking the epitope, and no bands were detected (see Fig. S8A).

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Laboratory animals: Experiments were conducted on adult C. elegans hermaphrodites, embryos, and L1s. All strains were of the N2 background.

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