poly(UG)-tailed RNAs in genome protection and epigenetic inheritance

Transposable elements are mobile parasitic genetic elements that are present in all genomes. Transposons threaten genome integrity and can cause disease by disrupting genes or inducing non-allelic recombination. RNA interference (RNAi) is a conserved gene-silencing mechanism initiated by double-stranded RNA (dsRNA). Forward genetic screens to identify factors required for either transposon silencing or RNAi in the model metazoan C. elegans identified an overlapping set of genes, indicating that an RNAi-related process silences transposons. One gene required for both efficient transposon silencing and RNAi in C. elegans is rde-3 (also known as mut-2), which encodes a protein with homology to ribonucleotidyltransferases (rNTs). rNTs add non-templated ribonucleotides to RNAs and other substrates. Recently, C. elegans RDE-3 was shown to add perfectly alternating U and G nucleotides to the 3′ termini of RNAs during transposon silencing and/or RNAi in C. elegans.

RDE-3 pUGylates mRNAs targeted by RNAi

We first tested whether pUG tails are added to RNAs targeted by RNAi in C. elegans. We used an (AC)₉ oligo to reverse transcribe total RNA extracted from worms exposed to dsRNA targeting the germline-expressed gene oma-1, and then performed nested PCR to detect oma-1 RNAs modified with 3′ pUG repeats (Fig. 1a). This approach (termed pUG PCR) detected PCR products that were dependent on oma-1 dsRNA (Fig. 1b), as well as an independent result of priming from genomically encoded UG-rich sequences. RDE-3 adds long stretches of alternating 3′ U and G nucleotides to the 3′ termini of RNAs during transposon silencing and/or RNAi in C. elegans.

Mobile genetic elements threaten genome integrity in all organisms. RDE-3 (also known as MUT-2) is a ribonucleotidyltransferase that is required for transposon silencing and RNA interference in Caenorhabditis elegans. When tethered to RNAs in heterologous expression systems, RDE-3 can add long stretches of alternating non-templated uridine (U) and guanosine (G) ribonucleotides to the 3′ termini of these RNAs (designated poly(UG) or pUG tails). Here we show that, in its natural context in C. elegans, RDE-3 adds pUG tails to targets of RNA interference, as well as to transposon RNAs. RNA fragments attached to pUG tails with more than 16 perfectly alternating 3′ U and G nucleotides become gene-silencing agents. pUG tails promote gene silencing by recruiting RNA-dependent RNA polymerases, which use pUG-tailed RNAs (pUG RNAs) as templates to synthesize small interfering RNAs (siRNAs). Our results show that cycles of pUG RNA-templated siRNA synthesis and siRNA-directed pUG RNA biogenesis underlie double-stranded-RNA-directed transgenerational epigenetic inheritance in the C. elegans germline. We speculate that this pUG RNA–siRNA silencing loop enables parents to inoculate progeny against the expression of unwanted or parasitic genetic elements.

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pUG RNAs drive gene silencing

pUG tails could either mark mRNA fragments for degradation or convert mRNA fragments into active mediators of gene silencing. To differentiate between these possibilities, we tested whether in vitro-transcribed pUG RNAs possess gene-silencing activity. Indeed, injection of a gfp pUG RNA (that is, 18′-terminal pUG repeats appended to the first 369 nt of gfp mRNA) into worms expressing a germline-expressed gfp::h2b transgene was sufficient to silence gfp::h2b expression (Fig. 2a). The same gfp mRNA fragment without a 3′ tail or with 18′-terminal pAU, pGC or pAC repeats lacked gene-silencing activity (Fig. 2a). To control for potential dsRNA contamination in our in vitro transcription reactions, all RNAs were injected into rde-1(ne219) worms, which cannot respond to dsRNA3 (Fig. 2a, b). The ability of a pUG tail to confer gene-silencing activity on an mRNA fragment was both general and sequence-specific, oma-1(zu405ts) worms lay arrested embryos at 20 °C unless oma-1(zu405ts) is silenced4. An in vitro-transcribed S41-nt-long oma-1 mRNA fragment with 18′ pUG repeats (hereafter, oma-1 pUG RNA)—but not 18′ pAU, pGC or pAC repeats—silenced oma-1(zu405ts) (Fig. 2b). In addition, an oma-1 pUG RNA injection did not silence gfp::h2b and, similarly, a gfp pUG RNA injection did not silence oma-1 (Extended Data Fig. 2c, d). Finally, although RDE-3 was required for efficient oma-1 RNAi (Extended Data Fig. 3a), this requirement could be bypassed by injection of an oma-1 pUG RNA (Extended Data Fig. 3b), establishing that RDE-3-mediated pUGylation is necessary for RNAi. We conclude that pUG tails convert otherwise inert mRNA fragments into agents of gene silencing.
an *oma-1* mRNA fragment with a 3′ pUG tail triggered *oma-1(2u40S)* silencing. *oma-1* mRNA fragments with 5′ or internal UG repeats did not (Fig. 2c). Finally, the *oma-1* segment of an *oma-1* pUG RNA had to possess the sense coding sequence (Extended Data Fig. 4a) and be more than 50 nt in length for pUG RNA functionality (Extended Data Fig. 4b). Together, these data show that a pUGRNA must contain more than 8 3′ UG repeats appended to more than 50 nt of sense RNA to trigger gene silencing.

### RDE-3 pUGylates germline-expressed RNAs

We next investigated whether endogenous mRNAs are pUGylated in *C. elegans*. Tc1 is the most abundant DNA transposon in the *C. elegans* genome. In the absence of RDE-3, Tc1 transposase RNA is upregulated and Tc1 is mobilized, suggesting that Tc1 RNA might be pUGylated in wild-type worms. Indeed, using a Tc1-specific pUG PCR assay (Fig. 1a), we observed RDE-3-dependent pUG tails appended to Tc1 RNA fragments (Fig. 3a, Supplementary Table 1). In addition, Tc1 mobilization caused by rde-3 mutation was suppressed by injection of a Tc1 pUG RNA (Fig. 3b). We conclude that RDE-3-dependent pUGylation silences the Tc1 transposon in *C. elegans*.

To identify additional targets of pUGylation, we analysed mRNA by RNA sequencing (RNA-seq) in wild-type and rde-3(-) worms and identified 346 RNAs that were upregulated in rde-3(-) worms (Supplementary Table 2, adjusted P < 0.05 and log₂(fold change) > 1.5), including Tc1, as well as six other DNA transposons (TcA4, Tc5, MIRAGE1, CEMUDR1 and Chapaev-2), several long terminal repeat retrotransposons (Cer9, Cer9 and Cer13) and 294 predicted protein-coding RNAs (Extended Data Fig. 5a, Supplementary Table 2). Directed pUG PCR analyses confirmed that Tc4v, Tc5, Cer3 and four of five predicted protein-coding RNAs tested from our list of RNAs most upregulated in rde-3(-) mutants were pUGylated in an RDE-3-dependent manner (Extended Data Fig. 5b, c). pUG tails were not detected on RNAs whose expression is unchanged in rde-3 mutants, including *oma-1*, *gfp* and *dpy-11* (Extended Data Fig. 2a, b) as well as two additional RNAs selected at random (Extended Data Fig. 5d). We conclude that RDE-3 adds pUG tails to endogenous RNAs in *C. elegans*, which include, but are not limited to, transposon RNAs.

### pUG RNAs localize to germ granules

Germ granules are liquid-like condensates that form near the outer nuclear membrane in most animal germ cells and probably promote germ-cell totipotency by concentrating germline determinants—including maternal RNAs and proteins—into developing germ-line blastomers. *C. elegans* RDE-3 localizes to perinuclear germ granules called *Mutator foci*. RNA fluorescence in situ hybridization (FISH) using a fluorescein-labelled (AC)₉ probe to detect pUG RNAs showed that pUG RNAs localized to perinuclear puncta in germ cells of wild-type, but not rde-3(-) worms (Fig. 3c). pUG FISH coupled with immunofluorescence to detect a GFP- and deg-3 tag showed that pUG RNA FISH colocalized with RDE-3 and, therefore, *Mutator foci* (Fig. 3d). This suggests that pUG RNAs are produced, function, and/or are stored in *Mutator foci* in the *C. elegans* germline. Indeed, *gfp-(1q224)* worms, which when grown at 25 °C (hereafter, *gfp-(1ts)*) lose around 99% of their germ cells, failed to produce detectable Tc1 pUG RNAs (Fig. 3e) or *oma-1* dsRNA-induced *oma-1* pUG RNAs (Extended Data Fig. 6a) at 25 °C, confirming that pUG RNAs are produced or stored in germ cells. Incidentally, when *gfp-(1ts)* worms were treated with dsRNA targeting the hypodermis-expressed *dpy-11* gene, *dpy-11* pUG RNAs were detected in somatic cells (Extended Data Fig. 6a), consistent with previous reports showing that RDE-3 promotes RNAi within and between cells in the *C. elegans* soma. We subsequently focused on the biogenesis and function of pUG RNAs in the germline.

To further investigate the relationship between germline pUG RNAs and *Mutator foci*, we tested whether the glutamine–asparagine motif-rich protein MUT-16, which is required for *Mutator* focus assembly in germ cells, was needed for pUG RNA biogenesis or function. *mut-16(pk710)* worms, which harbour a nonsense mutation in *mut-16*, produced increased levels of *oma-1* pUG RNAs in response to *oma-1* dsRNA (Extended Data Fig. 6b, c) and decreased levels of Tc1 pUGRNAs
pUG RNAs are templates for RdRPs

To understand how pUG tails might convert RNAs into agents of gene silencing, we sought to identify proteins that bind to UG repeats. We conjugated 5′ biotinylated RNA oligonucleotides consisting of 18 UG repeats, which conferred gene-silencing activity to oma-1 and gfp mRNA fragments (Fig. 2), to streptavidin beads. These beads were incubated with extracts from wild-type C. elegans om-1(SNP) RNA fragments (Fig. 2), to streptavidin beads. These beads were incubated with extracts from worms expressing HA::TagRFP::RRF-1. Immunoblotting with HA antibody. Data are representative of two biologically independent experiments. (Extended Data Fig. 6d), suggesting that Mutator foci help to coordinate pUG RNA biogenesis. mut-16(pk710) worms were completely defective for silencing of oma-1 after an oma-1 pUG RNA injection (Fig. 3f), indicating that Mutator foci are required for pUG RNA-dependent gene silencing, downstream of pUG RNA biogenesis.

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pUG RNAs are vectors for TEI

RNAi-triggered gene silencing can be inherited for multiple generations in C. elegans, making RNAi inheritance a robust example of transgenerational epigenetic inheritance (TEI)28–33. Notably, a one-time exposure of worms to oma-1 dsRNA both initiated the production of oma-1 pUG RNAs (Fig. 1a, b) and caused oma-1 pUG RNA to be expressed for four additional generations (Fig. 5a), concomitant with oma-1 gene silencing (Extended Data Fig. 5a), suggesting that pUG RNAs may contribute to TEI. To test this idea, we injected worms with gfp or oma-1 pUG RNAs and monitored gfp or oma-1 silencing over generations. gfp or oma-1 pUG RNAs were sufficient to silence gfp (Fig. 5b) or oma-1 (Extended Data Fig. 5b), respectively, for multiple generations. We conclude that pUG RNAs are sufficient to induce TEI.

How might pUG RNAs drive TEI? We speculated that if pUG RNA-templated siRNAs (Fig. 4d, Extended Data Fig. 7a) could direct de novo mRNA pUGylation, then generationally repeated cycles of
generations are required for TEI. We crossed which showed that these de novo pUGylation events in inheriting Fig. 10). C. elegans triggers and thus propagate gene silencing across generations. biogenesis could be maintained in the absence of initiating dsRNA synthesis, supporting the idea that repeated pUG–siRNA cycling occur during the inheriting generations of RNAi-directed TEI and that that pUG RNA biogenesis, and therefore pUG–siRNA cycling in progeny, is necessary for TEI maintenance. We conclude that pUG tails convert otherwise inert RNA fragments into drivers of an RNA-based memory system, which is probably propagated across generations via iterative cycles of sense pUG RNA and antisense siRNA biogenesis.

pUG RNA–templated siRNA synthesis and siRNA-directed pUG RNA biogenesis could be maintained in the absence of initiating dsRNA triggers and thus propagate gene silencing across generations. Three lines of evidence support this ‘pUG–siRNA cycling’ model for RNAi-directed TEI. First, RdRP-derived secondary siRNAs in C. elegans can engage twelve Argonaute proteins (termed WAGOS) to mediate gene silencing. MAGO12 worms, which harbour deletions in all twelve WAGOS35, produced oma-1 pUG RNA after oma-1 RNAi (Fig. 5c). Progeny of RNAi-treated MAGO12 worms, however, failed to produce oma-1 pUG RNAs (Fig. 5c). Thus, the secondary siRNA system is needed to maintain pUG RNAs specifically during the inheriting generations of TEI, consistent with a pUG–siRNA cycling model for TEI. Of note, pUG RNAs derived from the endogenous pUGylation targets c38d9.2 and Tc1 were also dependent on the WAGOS (Extended Data Fig. 5e), suggesting that the endogenous targets of RDE-3 also undergo heritable silencing via pUG–siRNA cycling.

Second, when we injected worms with an oma-1(SNP) pUG RNA, oma-1 pUG RNAs were detectable in subsequent generations (Extended Data Fig. 8d), but did not contain the engineered SNP (Fig. 5d). Similarly, less than 1% of siRNAs sequenced from the progeny of worms injected with oma-1(SNP) pUG RNA harboured the SNP complement (Extended Data Fig. 8e). This suggests that de novo pUGylation events occur during the inheriting generations of RNAi-directed TEI and that these newly derived pUG RNAs become templates for further siRNA synthesis, supporting the idea that repeated pUG–siRNA cycling mediates TEI.

Third, we conducted a genetic analysis (Extended Data Fig. 8f), which showed that these de novo pUGylation events in inheriting generations are required for TEI. We crossed oma-1 RNAi-treated wild-type hermaphrodites with rde-3(ne298) males, isolated rde-3(+) and rde-3(ne298) F1 progeny and then assayed the F2 generation of this cross for oma-1 pUG RNA expression (Fig. 5e) and oma-1 gene silencing (Extended Data Fig. 8f). rde-3(ne298) progeny lacked oma-1 pUG RNAs (Fig. 5e) and failed to silence the oma-1 locus (Extended Data Fig. 8f) during the inheriting generations of TEI, supporting the idea that pUG RNA biogenesis, and therefore pUG–siRNA cycling in progeny, is necessary for TEI maintenance. We conclude that pUG tails convert otherwise inert RNA fragments into drivers of an RNA-based memory system, which is probably propagated across generations via iterative cycles of sense pUG RNA and antisense siRNA biogenesis.

Discussion

Here we show that RDE-3 adds pUG tails to germline- and soma-expressed RNAs in C. elegans and demonstrate a role for this modification in transposon silencing and TEI. We find that RdRPs are recruited, directly or indirectly, to pUG tails and use pUG RNAs as templates for siRNA synthesis. Assembly of RDE-3 and other proteins, such as the endonuclease RDE-836 and the RdRP RRF-112, into germline condensates termed Mutator foci probably coordinates RNA target recognition, cleavage, pUGylation and siRNA amplification (Extended Data Fig. 9). Functional pUG tails consist of more than eight pairs of perfect or near-perfect 3′ UG repeats. These precise length and sequence requirements for pUG tail function suggest that long pUG tails may impart stability to siRNA fragments and/or form a structure that helps to recruit, and possibly prime, RdRPs—similar to the proposed role of poly(U)-tailing in small RNA-based gene silencing in Tetrahymena13. In addition, our data show that proteins other than RdRPs, such as TDP-1, the C. elegans orthologue of the mammalian U12-AS binding protein TDP-4337,38, also interact with pUG repeats. We speculate that these other proteins may regulate the localization, stability or function of pUG RNAs. Extended Data Figure 9 relates our findings to a previous report suggesting that RDE-3 may uridylate targets of RNAi12.

Furthermore, our data show that pUG RNAs act as informational vectors for TEI when they engage in feed-forward amplification cycles with RdRP-generated secondary siRNAs (Extended Data Fig. 9). These pUG–siRNA cycles may enable C. elegans to ‘remember’ past gene-silencing events and to inoculate progeny against expressing unwanted and/or dangerous genetic elements. Experimental RNAi-initiated pUG–siRNA cycles endure for several generations (Fig. 5a) but are not permanent—suggesting that C. elegans possesses systems to prevent pUG–siRNA cycles from propagating in perpetuity. Notably, we find that RNAi-initiated pUG RNAs shorten progressively during TEI, suggesting that pUG RNA shortening—which may be an inevitable consequence of RdRP-dependent secondary siRNA synthesis (Extended Data Fig. 10)—could function as one such brake on TEI. By contrast, the natural targets of pUGylation, such as transposons, are constitutively silenced by the pUG–siRNA system, suggesting that genetic systems such as genomically encoded Piwi-interacting RNAs (piRNAs) or endogenous dsRNAs probably reinforce and refocus epigenetic pUG–siRNA silencing at these loci at each generation (Extended Data Fig. 9).

The logic of sense pUG RNA–antisense siRNA cycling resembles that of fly and mammalian piRNA ‘ping-pong’ systems in which iterative base pairing between genomically encoded sense–antisense transposon RNAs and piRNAs derived from these RNAs mediates stable transposon silencing39. We speculate that related sense–antisense RNA systems could contribute to other biological processes for which long-term memories of past expression states are needed, such as antiviral immunity, development or inheritance of environmentally acquired traits. Finally, our data show that long non-templated and non-homopolymeric tracts ofribonucleotides can be appended to, and confer novel functions to, RNAs in C. elegans. It will be interesting to determine whether pUG RNAs or RNAs bearing other tail sequences
are restricted to *C. elegans* or are, instead, emissaries of a new class of eukaryotic RNA.

**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/s41586-020-2323-8.

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Methods

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Genetics

*C. elegans* culture and genetics were performed as described previously. Unless otherwise noted, all *C. elegans* strains (Supplementary Table 5) were maintained at 20 °C on nematode growth medium (NGM) plates and fed OP50 *Escherichia coli* bacteria.

RNAi

To perform RNAi experiments, embryos were obtained by hypochlorite treatment (egg prep) of gravid adult hermaphrodites and dropped onto RNAi plates (standard NGM plates with 1 mM isopropyl β-D-1-thiogalactopyranoside and 25 μg ml−1 carbencillin) seeded with HT115 *E. coli* bacteria expressing either L4440 (Addgene, #1654) empty vector control or L4440 carrying inserts to trigger the production of dsRNA against a gene of interest. To perform pUG RNA analysis after RNAi treatment, gravid adults were washed off plates after 3–4 days using M9 + Triton X-100 buffer, collected in TRIzol, flash frozen in liquid nitrogen and stored at −80°C until total RNA extraction (see below). To investigate *oma-1* pUG RNAs across generations after RNAi, embryos were dropped onto plates seeded with HT115 bacteria expressing either empty vector control or dsRNA of interest. Some gravid adults were collected for the F₀ generation sample and the remaining were egg prep’d onto plates without dsRNA every generation, for the indicated number of generations. Each generation, some adult animals were collected while some were egg prep’d to obtain the next generation. pUG RNAs were then detected as described below. To measure percentage of embryos hatch after *oma-1* RNAi, embryos obtained from animals harbouring the *oma-1*(zu4051) allele were dropped onto plates seeded with HT115 bacteria expressing either empty vector control or *oma-1* RNAi and grown at 20 °C. Six adults were then single per treatment for each strain or genotype and allowed to lay embryos overnight. The total number of embryos laid was counted, and then embryos were allowed to hatch for 24 h, after which the total number of embryos that hatched was counted. For transgenerational RNAi experiments, empty vector control-treated and *oma-1* RNAi–treated adults were egg prep’d onto plates without dsRNA every generation and percentage of embryos hatched was counted as just described until embryos no longer hatched. The dpy-11 RNAi clone and the *oma-1* RNAi clone used, unless noted below, came from the *C. elegans* RNAi collection (J. Ahringer laboratory). The second *oma-1* RNAi clone (referred to as pAS74 and used for Fig. 5a and Extended Data Figs. 1b, 8a, 10a, 10b) was a custom clone made to target exon 6 of *oma-1*. The *gfp* RNAi clone was obtained from the A. Fire laboratory.

pUG PCRs and RT–qPCRs

Total RNA was extracted using TRIzol Reagent (Life Technologies, 15596018). Five micrograms of total RNA and 1 pmol of reverse transcription oligonucleotide was used to generate first-strand cDNA using the Superscript III First-Strand Synthesis System (Invitrogen, 18080051). Note: total RNA was heated with dNTPs and reverse transcription oligonucleotide to 65 °C for 5 min and immediately chilled on ice before proceeding with remaining cDNA synthesis steps. One microlitre of cDNA was used for the first PCR (20 μl volume) performed with Taq DNA polymerase (New England Biolabs, M0273) and primers listed in Supplementary Table 5. First PCR reactions were diluted 1:100, and then 1 μl was used for a second PCR (50 μl volume) using primers listed in Supplementary Table 5. *gpa-1*, which has an 18-nt-long genomically encoded pUG repeat in its 3′-untranslated region (UTR), served as a control for all pUG PCR analyses. PCR reactions were then run on agarose gels. For Sanger sequencing, lanes of interest were cut out from agarose gels and gel-extracted using a QIAquick Gel Extraction Kit (Qiagen, 28706). Three microlitres of gel-extracted PCR product was used for TA cloning with the pGEM-T Easy Vector System (Promega, A1360) according to the manufacturer’s instructions. Ligation reactions were incubated overnight at 4 °C. Transformations were performed with 5-α Competent *E. coli* cells (NEB, C2987H) and plated on LB, ampicillin, IPTG, X-gal plates (according to pGEM-T Easy Vector System manufacturer’s instructions). On the next day, white colonies were selected and inoculated, and then liquid cultures were mini-prepped using QIAprep Spin Miniprep Kit (Qiagen, 27106). Plasmids were Sanger sequenced using a universal SP6 primer (5′-CATACGATTAGTGCACATAG-3′) (Dana-Farber–Harvard Cancer Center DNA Resource Core, Harvard Medical School). Reverse transcription with quantitative PCR (RT–qPCR) was performed using 2 μl of 1:100 diluted first PCRs as a template with qPCR primers (Supplementary Table 5) and iQtaq Universal SYBR Green Supermix (Bio-Rad, 1725121) according to the manufacturer’s instructions.

MiSeq

*oma-1* pUG PCRs were sequenced on an Illumina MiSeq from animals fed HT115 bacteria expressing L4440 empty vector control plasmid (two biological replicates), *oma-1* RNAi clone from the J. Ahringer RNAi library or our custom *oma-1* RNAi clone (pAS74). F₀ to F₃ descendants from pAS74-fed animals were obtained as described above and also sequenced (1 replicate each generation). A first round of PCR was performed with the same primers as described above (Supplementary Table 5). Primers were modified for the second PCR to contain Illumina p5 and p7 sequences, read 1 and 2 sequencing primers, a unique index (reverse primer only) for multiplexing and unique molecular identifiers (NNN) (Supplementary Table 5). PCR reactions were then pooled, run on an agarose gel and gel purified as described above. Gel-purified DNA was sequenced on an Illumina MiSeq (Biopolymers Facility, Harvard Medical School) to obtain paired-end reads (67 bp for read 1 and 248 bp for read 2).

MiSeq analysis

First, unique molecular identifiers (UMIs) were removed from each read pair and appended to the end of the read name using UMI-tools v.1.0.0. Then, Cutadapt v.2.5 was used for the following: (1) low-quality bases (quality score <20) were trimmed from the 3′ ends of reads; (2) read pairs containing the inline portion of the 5′ adaptor (AACAGCAGA AGATCGATGA) in read 1 were selected for and then trimmed; (3) read pairs containing the inline portion of the 3′ adaptor (GGCGTTCGA TCTTACACACACACACACACAC) in read 2 were selected for and trimmed; and (4) if the 5′ adaptor was present in any read 2 sequences, the adaptor was trimmed from those sequences. After adaptor trimming, read 2 sequences were screened for additional pACs at the 5′ end: reads that did not contain additional pACs (and therefore did not have a pUG tail longer than the adaptor) were discarded; reads that did contain additional pACs were retained, and the pACs were trimmed using Cutadapt v.2.5 (pAC and pCA sequences were provided as non-internal 5′ adaptors). After pAC trimming, read 2 sequences shorter than 5 nt were discarded. The remaining read 2 sequences were aligned to the *C. elegans* genome (WormBase release WS260) using STAR v.2.7.0f. SAM and BED files of unique alignments were generated using SAMtools v.1.9 and BEDtools v.2.27.1 and then imported into R for subsequent analyses (https://rstudio.com). Alignments were deduplicated based on the combination of the UMI and end coordinate. Alignments that mapped to the + strand and/or to coordinates outside of the *oma-1* gene were discarded.

To systematically define the *oma-1* and pUG portions of each read, the pre-pAC-trimmed version of the read was reverse-complemented and then split as follows. By default, the aligned portion of the read
was designated as 'oma-1', and any sequence downstream of the aligned portion was designated as the pUG. Then, the 'oma-1' portion was matched to an oma-1 reference sequence (spliced + UTRs) using Biostrings v.2.50.2 (https://doi.org/10.18129/B9.bioc.Biostrings). If the first 1–6 nt that occurred 3' of the match were the same in the oma-1 reference as they were in the read before pAC trimming (and therefore had the potential to be templated), then those nucleotides were reassigned to the oma-1 portion of the read. End coordinates of the alignments were adjusted accordingly. A small portion of reads (<1%) were misannotated with the above approach, largely due to soft-clipping at the 3' end during alignment. To systematically filter out such reads, reads for which the annotated pUG started with a base other than U or G and/or contained two or more bases other than U or G within the pUG sequence were discarded. A list of oma-1 pUG RNA reads can be found in Supplementary Table 1. The abundance of each pUGylation site (Extended Data Fig. 1a) was plotted in R using Sushi v.1.20.0. For pUG RNAs sequenced from wild-type animals fed the oma-1 RNAi clone from the RNAi collection. To generate the pUG site logos shown in Extended Data Fig. 1b, a list of unique pUG sites was combined for pUG RNAs sequenced from wild-type animals fed the oma-1 RNAi clone from the RNAi collection and pAS74 (our custom oma-1 RNAi clone). This combined list was sorted by the last nucleotide of the oma-1 portion and then plotted in R using ggseqlogo v.0.14.

pUG RNA injections

For gfp and oma-1 pUG RNA injections, pUG RNAs were synthesized in vitro using MEGAScript T7 Transcription Kit (Invitrogen, AM1334). DNA templates for in vitro transcription reactions were gel-purified PCR products (150 ng per in vitro transcription reaction) amplified using primers listed in Supplementary Table 5. Reactions were incubated overnight at 37 °C. In vitro-transcribed RNA was purified using TRIzol Reagent (Life Technologies, 15596018) and stored at −80 °C. In vitro transcription reactions were performed under the same conditions using 50 ng of in vitro-transcribed RNA and 2.5 ng of co-injection marker (mCherry::h2b). mCherry expression using the Plan-Apochromat 20× 0.8 M27 objective on an Observer.Z1 fluorescent microscope (Zeiss) using the Plan-Apochromat 20× 0.8 M27 objective on an Axios Observer.Z1 fluorescent microscope (Zeiss). Images were taken with the Plan-Apochromat 63×1.4 Oil DIC M27 objective. For translational inheritance experiments, five F1 progeny expressing mCherry per injected animal were picked under an Axio Zoom.V16 fluorescence dissecting microscope using a PlanNeofluar Z 1× 0.25 FWD 56 mm objective to new plate to lay an F2 brood. Five random F1 animals were picked to a new plate to lay F3 progeny, while the remaining F1 adults (~40 per injected animal) were scored for gfp expression as described above, but without regard for mCherry expression. This process was continued for several generations until 100% of animals expressed gfp. For all experiments, no-injection control animals or animals injected with other RNA species were scored as described for pUG RNA-injected animals. For oma-1 pUG RNA injections, oma-1(zu405ts); rde-1(ne219). UTR was matched to an oma-1 aligned portion was designated as the ‘pUG’. Then, the ‘oma-1’ portion and any sequence downstream of the ‘oma-1’ was designated as ‘oma-1’ RNAi clone. This combined list was sorted by the last nucleotide of the ‘oma-1’ portion and then plotted in R using ggseqlogo v.0.14.

RNA FISH and immunofluorescence

Approximately 30 animals were dissected in 15 μl of 1× egg buffer (25 mM HEPES (pH 7.3), 118 mM NaCl, 48 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂) on SuperFrost Plus Adhesion slides (VWR, 631-0448) to isolate gonads. A coverslip was placed on top of dissected tissue, excess buffer was soaked up using a Kimwipe and slides were placed onto a metal block pre-chilling on dry ice for 10 min. Coverslips were removed and slides were submerged in methanol at −20 °C for 10 min. Slides were then washed twice, 5 min per wash in 1× PBS + 0.1% Tween-20 (PBSTW). Samples were then fixed with 4% paraformaldehyde solution in 1× PBS for 20 min, followed by two 5 min washes in PBSTW. Samples were then incubated at 37 °C for 6 h in a humid chamber with a 1:50 dilution of fluorescent RNA FISH probe in hybridization buffer (10% formamide, 2× SSC, 10% dextran sulfate (w/v)). The RNA FISH probe to detect pUG RNAs (S/Alexa647/CACACACACACACACACA) was ordered from Integrated DNA Technologies and stored at a stock concentration of 100 μM at −20 °C. The RNA FISH probe to detect oma-1 mRNA was ordered from Stellaris (MF-6011-1). After 6 h, slides were washed twice, 10 min per wash, in FISH wash buffer (2× SSC, 10% formamide, 0.1% Tween-20). Samples were then washed for 5 min in 2× SSC. Slides were sealed using 15 μl of Vectashield Antifade Mounting Medium (H-1000) with DAPI. For experiments in which RNA FISH and immunofluorescence were combined, RNA FISH was first performed as described. After the final 2× SSC wash, slides were washed once with PBST for 5 min, samples were incubated overnight at room temperature in a humid chamber with a 1:1000 dilution of GFP antibody (Abcam, ab290) in PBSTW. Slides were then washed three times, 10 min per wash, in PBSTW and incubated in a 1:100 dilution (in PBSTW) of goat anti-rabbit secondary antibody, Alexa Fluor 555 (Invitrogen, A-21429) for 2 h at room temperature in a humid chamber. Slides were then washed three times, 10 min per wash, in PBSTW and then sealed with 15 μl of Vectashield Antifade Mounting Medium (H-1000) with DAPI. All imaging was performed on an Axios Observer.Z1 fluorescent microscope (Zeiss) using the Plan-Apochromat 63×1.4 Oil DIC M27 objective. All image processing was done using Fiji 16.

RNA-seq

Total RNA was extracted using TRIzol Reagent (Life Technologies, 15596018). RNA quality (RIN) and quantity were assessed on the TapeStation 2200 (Agilent). Two rounds of mRNA purification were performed on 1μg total RNA using the Dynabeads mRNA DIRECT Kit (Invitrogen, 61011). First-strand cDNA was generated using the SuperScript III First-Strand Synthesis System (Invitrogen, 18080051), followed by second-strand synthesis using DNA polymerase I (Invitrogen, 19170101).
cdNA libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, FC-131-1024). Libraries were sequenced on the Illumina NextSeq500 platform (Biopolymers Facility, Harvard Medical School) and 75-bp paired-end reads were obtained.

RNA-seq analysis

Reads were trimmed to remove sequencing adapters and low-quality bases using Trim Galore v.0.4.4.dev (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Trimmed reads were then aligned to the C. elegans genome (UCSC cell/WBcel235) using STAR v.2.7.0a40. Differential expression analysis of genes and repeat elements was performed using the TETranscripts package in TEtoolkit v.2.0.342. Gene annotations were obtained from Ensembl (WormBase release WS260)40. Repeat annotations were obtained from UCSC by downloading the RepeatMasker (rmsk) table in the Table Browser program. The table was reformatted to a GTF file using the Perl script makeT-Egtf.pl (http://labshare.cshl.edu/shares/mhammellab/www-data/TETranscripts/TE_GTF/). Features with an adjusted P-value < 0.05 and a log2(fold change) > 1.5 were reported. Overlap (Extended Data Fig. 5a) was determined between mRNAs upregulated in rde-3(−) animals and published lists of: (1) RNAs targeted by CSR-1 bound endo-siRNAs49, (2) piRNA-targeted mRNAs (http://cosb16.ee.ncu.edu.tw/piRTarBase/, stringent and clash list)49, and (3) WAGO-class mRNAs49.

CRISPR

The CRISPR strategy described previously was used to revert the missense mutation in rde-3(−) animals to wild type and to tag the N terminus of rrf1 with ha::TapRFP. SapTrap cloning2,53 and the selection-based CRISPR strategy described previously was used to tag rde-3 at the N terminus with gfp::degron and to introduce 3′-flag::rde-3 (with 2 kb upstream of the AGT and 2 kb downstream of the stop codon) in the LGM MosSCI site ttt5650 into rde-3(ne3370) animals. All guide RNAs were designed using the guide RNA selection tool CRISPOR44.

Small-RNA sequencing

rde-1(ne219); oma-1(zu405ts) animals were injected with an oma-1 pUG or pGC RNA (oma-1 mRNA fragment with (UG)18 or (GC)18 tail) in which the oma-1 mRNA was modified to contain a SNP in exon 4 (ATTCAATCCGA > T CATCTGACCCA). Injection mix was prepared as described above. For P0 analysis (Fig. 4d and Extended Data Fig. 7a), -100 rde-1(ne219) animals were injected per experiment. After recovering for 1–4 h at room temperature, injected animals were collected for total RNA extraction. For F1 analysis (Extended Data Fig. 8e), -20 rde-1(ne219) animals were injected per experiment. Injected animals recovered at 15 °C for 2 days and were returned to room temperature. Approximately 500 adult co-injection marker-expressing progeny of injected animals were collected at 15 °C for 2 days and cDNA was prepared for each of these animals.

Small-RNA sequencing analysis

A custom Python script was used to select reads starting with the last 4 nucleotides of the 5′ adaptor (either AGCG or CGTC). Cutadapt 1.14 was then used to trim the 3′ adaptor (CTGAGCCACATCATAATGCGAAGAC) and the in-line portion of the 5′ adaptor (AGCG and CGTC) (both with a minimum phred score of 20), allowing only sequences ≥ 16 nt after trimming to pass (cutadapt -q 20 -m 16 -u 4 -a CTGAGCGCACAATCATAATGCGAAGAC -d -c). The quality of the trimming was assessed with FastQC 0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). For downstream analysis, custom Python scripts were used to select reads that were 22 nt in length and began with a G (22G siRNA reads). TopHat 2.1.1 was then used to map 22G siRNA reads to the C. elegans genome (WBcel235). Gene annotations were obtained from Ensembl (WormBase release WS269) and custom shell scripts were used to select protein-coding genes only. One mismatch was allowed to identify 22G siRNAs with SNPs. Using Sambtools v.0.1.19, only uniquely mapping sequences were retained. See Supplementary Table 4 for a list of all small RNA reads mapping to oma-1 and antisense 22G siRNAs mapping to oma-1. 22G siRNA pileup figures were generated as follows: first, bam files generated from TopHat v.2.1.1 were normalized by DeepTools v.3.0.249 based on counts per million and only antisense reads were kept for further analysis (bamCoverage -bs 2–normalizeUss–CPM –samFlagExclude 16). Then, the normalized antisense 22G small RNA sequences (bedGraph files) were visualized using Sushi 1.20.0 in R. The number of reads mapping antisense to each gene was calculated by featureCounts v.1.6.049 (featureCounts -s 2-a -g txon-gene_name). All custom scripts used in this section are available at: https://github.com/Yuhan-Fei/pUG-analysis.

pUG RNA chromatography

Wild-type adult animals (~1–2 full 10-cm plates per experiment) were frozen in liquid nitrogen as small droplets and ground into powder with a mortar and pestle. Powder was dissolved in lysis buffer (5 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, 3 mM MgCl2, 0.5 mM EDTA (pH 8.0), 5% glycerol, 0.25% Triton X-100, 0.5 mM DTT, 1 mM PMSF, 1 tablet of Complete protease inhibitor (Roche, 11679498001)) and rotated for 30 min at 4 °C. The resulting lysate was centrifuged at top speed for 10 min at 4 °C. Supernatant was distributed evenly among experiments, and RNaseOUT recombinant RNase inhibitor (Invitrogen, 10777019) was added to lysate (1 μl per 100 μl lysate). For each experiment, 160 pmol of biotinylated RNA was conjugated to 400 μg Dynabeads MyOne Streptavidin beads (Invitrogen, 65001) according to the manufacturer’s instructions. Beads were added to lysates and rotated at room temperature for 1 h. Beads were separated from supernatant on a magnetic rack, and the supernatant (‘sup’ fraction) was collected and saved. Beads were washed 3 times with lysis buffer and rotated for 5 min at 4 °C in lysis buffer. To perform liquid chromatography with tandem mass spectrometry (LC–MS/MS), beads were incubated in 500 mM NH4OH and shaken at 37 °C for 20 min after the last wash with lysis buffer. Beads were then pelleted using a magnetic rack, and the supernatant was removed and vacuum dried until NH4OH had completely evaporated. 100 μl of 100% TCA and 400 μl of pre-chilled water was added to each sample, followed by a 15 min incubation on ice. Samples were spun at top speed at 4 °C for 20 min. The supernatant was removed, and the previous step was repeated using 1 ml of 10% TCA solution. Samples were then washed two times with acetone and centrifuged for 10 min at 4 °C after each wash. Acetone was removed, and samples were dried in a speed vacuum to remove all residual acetone. Samples were analysed using LC–MS/MS (Taplin Mass Spectrometry Facility, Harvard Medical School). Note: a beads-only pull-down served as a control for this experiment. To analyse the LC–MS/MS data, 1 peptide count was assigned to all proteins with 0 peptide counts, and the peptide counts for each pull-down sample were then normalized by the total number of peptides identified for that sample (Supplementary Table 3). Only proteins with normalized peptide counts that were at least twofold more enriched in the (UG)18 pull-down versus the beads-only control pull-down were kept for further analysis (Fig. 4a and Supplementary Table 3). For gel electrophoresis and western blotting experiments,
lysates were made from animals harbouring rrf-1 tagged with ha and TagRFP at the endogenous locus. pUG RNA chromatography was performed as above. After the last wash with lysis buffer, beads (pull-down fraction) and sup were dissolved in 2× Laemmli sample buffer (Biorad, 1610373, final concentration = 1×) with 5% 2-mercaptoethanol and heated for 5 min at 95 °C and chilled on ice. Pull-down and sup fractions were loaded into 4–15% Mini-Protein TGE Precast protein gels (Biorad, 4561086) and run in Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Proteins were then transferred to nitrocellulose membrane (BioRad) at 100 V for 1 h in electrophoresis buffer (50 mM Tris, 40 mM glycine, 0.2% methanol, 0.5% SDS). Blotted membranes were blocked with 5% milk in PBST (phosphate-buffered saline, 1.0% Tween-20) for 1 h at room temperature and probed with primary antibody (1:1,000 HA-Tag Rabbit mAb, Cell Signalling 3724, in 5% milk) overnight at 4 °C. After washing with PBST 3 times, membrane was probed with secondary antibody (1:10,000 IRDye 800CW Goat anti-Rabbit IgG, LI-COR 926-32211, in 5% milk) for 1 h at room temperature. Membrane was washed with PBST 3 times before imaging using Odyssey FC Dual-Mode Imaging System (LI-COR).

Heterozygous experiment

To perform the experiment in Fig. 5e and Extended Data Fig. 8f, embryos were obtained via hypochlorite treatment of oma-1(24O4Sts) gravid adult hermaphrodites and dropped onto RNAi plates seeded with HT115 bacteria expressing dsRNA targeting oma-1. L1 hermaphrodites were then transferred, along with rde-3(ne298)/oma-1(24O4Sts) males, onto RNAi plates seeded with 25 µl small area of food to encourage mating of oma-1 dsRNA-expressing bacteria. Once hermaphrodites were adults, they were singled onto NGM plates seeded with OP50 and allowed to lay F1 progeny. Twelve to fifteen F1s were single from three independently mated hermaphrodites and genotyped to ensure that they were heterozygous for rde-3(ne298). To obtain F2 animals, 12–15 F1 worms per F1 (as verified to be heterozygous for rde-3(ne298)) were single to 15 °C for (as to avoid embryonic arrest due to temperature) and allowed to lay a brood. F2s were then single-worm genotyped to identify rde-3(+) and rde-3(ne298) homozygous animals. Then, per cent embryonic arrest was calculated by pooling 5 L4 stage F3 animals per F2 at 20 °C until they had laid a brood of 50–200 progeny and counting the number of embryos that were laid versus hatched on the following day. rde-3(+) and rde-3(ne298) homozygous F2 broods were pooled for all plates that were derived from the same P0 and oma-1 pUG PCR was performed as described above.

Reporting summary

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

Data availability

All DNA and RNA sequencing data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus61 (GEO) under accession number GSE148134. Processed MiSeq and small-RNA sequencing reads are also available in Supplementary Tables 1 and 4, respectively.

Code availability

Descriptions of custom scripts used to analyse MiSeq and RNA-seq data are provided in the Methods and the scripts are available upon request from the corresponding author. Custom Python scripts used to analyse small-RNA sequencing data have been deposited at https://github.com/Yuhan-Fei/pUG-analysis.

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Tables 3, 4. D.J.P., J.G. and J.G.S. contributed to Supplementary Table 2. A.E.D. contributed to Fig. 4a, Extended Data Figs. 1a, b, 5a, 10b, Supplementary Tables 1, 3. Y.F. contributed to Fig. 4d, Extended Data Figs. 7a–c, 8e, Supplementary Table 4. A.S., M.W. and S.K. conceived the project. S.K. supervised the project. A.S. and S.K. wrote the manuscript.

Competing interests M.W. has a patent (US20160145666A1) through Wisconsin Alumni Research Foundation (Madison, WI) for methods, kits and compositions of matter relating to poly(UG) polymerases.

Additional information Supplementary information is available for this paper at https://doi.org/10.1038/s41586-020-2323-8.

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Peer review information Nature thanks Rene Ketting, Taiowa Montgomery and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Extended Data Fig. 1 | Analysis of oma-1 pUGylation sites. a, Illumina MiSeq was performed (n = 1 biological experiment) on oma-1 pUG PCR products derived from WT and rde-3(-) worms, with or without oma-1 dsRNA. The number of sequenced pUG RNAs (y-axis) mapping to each pUGylation site (x-axis) is shown. Inset, total number of sequenced oma-1 pUG RNAs from indicated samples and total number of these sequenced pUG RNAs in which the oma-1 sequence was spliced. b, MiSeq-sequenced oma-1 pUG RNAs were sorted into four groups on the basis of the nucleotide at the last templated position (−1) of the oma-1 mRNA. The percentage of oma-1 pUG RNAs (MiSeq reads) with each nucleotide in the −1 position is shown. Logo analysis was then performed on each of the four groups to determine the probability of finding each nucleotide at the first position of the pUG tail (+1), as well as at the second-to-last templated nucleotide of oma-1 (−2). This analysis showed that if the last templated nucleotide of the oma-1 mRNA fragment was an A or a C, then RDE-3 was equally likely to add a U or a G as the first nucleotide of an elongating pUG tail. If, however, the last templated nucleotide was a U or G, then RDE-3 preferentially added a G or U, respectively, as the first nucleotide in an elongating pUG tail. Note, to perform the analyses in this figure, we assumed that if a U or G could have been genomically encoded, then it was. If, instead, RDE-3 added the U or G shown in the −1 position as the first nucleotide of the pUG tail, then these data show that the second nucleotide that RDE-3 prefers to add is a G after a U or a U after a G. CCA-adding rNT enzymes modify the 3′ termini of transfer RNAs (tRNAs) with non-templated CCA nucleotides. The mechanism by which these enzymes add non-templated nonhomopolymeric stretches of nucleotides is thought to involve allosteric regulation of the nucleotide-binding pocket by the 3′ nucleotide of a substrate tRNA62. A similar mechanism may explain how RDE-3 can add pUG tails to its mRNA substrates. For instance, when the 3′ nucleotide of an RDE-3 substrate is a U, the rNTP binding pocket of RDE-3 might adopt a structure that preferentially binds G and vice versa when the 3′ nucleotide of an RDE-3 substrate is a G. Such a model could explain how a single rNT enzyme adds perfectly alternating U and G nucleotides to RNA substrates. There are also alternative models for how RDE-3 might add pUG tails to an RNA. These include: (1) the existence of a poly(AC) nucleic acid template used by RDE-3 during pUG tail synthesis, (2) the existence of one or more rNTs that cooperate with RDE-3 to produce pUG tails, or (3) the possibility that RDE-3 binds and incorporates UG or GU dinucleotides. We disfavour the first two possibilities, as these models are difficult to reconcile with the observation that RDE-3 adds UG repeats to tethered RNAs in yeast or in Xenopus oocytes5. The third proposed model may be true, but because our sequencing shows that pUG tails can initiate with either a U or a G (this figure, Supplementary Table 1), then RDE-3 would need to be able to bind both UG and GU dinucleotides. Determining the mechanism by which RDE-3 adds pUG tails will probably involve structural studies and/or in vitro pUGylation assays using recombinant RDE-3 protein.
Extended Data Fig. 2 | RNAi-triggered pUGylation and pUG RNA-directed gene silencing are general and sequence-specific. a, gfp::h2b, rde-3(-); gfp::h2b and WT (no gfp::h2b) worms were fed E. coli expressing either empty vector control or gfp dsRNA. b, WT and rde-3(-) worms were fed E. coli expressing empty vector control and either oma-1 or dpy-11 dsRNA. In a, b, gfp, dpy-11 and oma-1 pUG RNAs were detected using the assay outlined in Fig. 1a. Data are representative of three biologically independent experiments. c, rde-1(ne219); oma-1(zu405ts) worms were injected with either an oma-1 (n = 6) or a gfp (n = 10) pUG RNA. n = 3 for no injection. The percentage of embryos hatched was scored for the progeny of injected worms. Panels below the x-axis show RNAs run on 2% agarose gel to assess RNA integrity. Data are mean ± s.d. d, rde-1(ne219); gfp::h2b worms were injected with either an oma-1 or a gfp pUG RNA (n = 10 for both, 3 for no injection). Data are mean ± s.d. of percentage of progeny with gfp::h2b silenced. In c, d, all pUG tails were 36 nt in length.
Extended Data Fig. 3 | RDE-3-mediated pUGylation is necessary for RNAi.

a, Worms of the indicated genotypes (all harbouring the *oma-1(zu405ts)* mutation) were treated with or without *oma-1* dsRNA. For each experiment, the percentage of embryos hatched was scored at 20 °C and averaged for six individual worms per treatment for each genotype. *rde-1(ne219)* mutants, which cannot respond to dsRNA, serve as a control for this experiment. Data are mean ± s.d of three biologically independent experiments. 

b, Control or *rde-3(ne298)* worms (all *rde-1(ne219); oma-1(zu405ts)* background) were injected with *oma-1* pUG RNAs and the percentage of embryos hatched was scored at 20 °C. *n* = 10 noninjected and 16 injected worms for control. *n* = 8 noninjected and 14 injected worms for *rde-3(ne298)*. Data are mean ± s.d.
Extended Data Fig. 4 | pUG tails must be appended to sense RNAs of >50 nt for functionality. a, b, rde-1(ne219); oma-1(zu405ts) worms were injected with: an oma-1 pUG RNA consisting of the sense or antisense strand of the same 541 nt-long oma-1 mRNA fragment (beginning at the aug) with a 36-nt 3′ pUG tail (a, n = 9 for both; n = 3 for no injection); oma-1 pUG RNAs consisting of oma-1 mRNA fragments of varying lengths (with position 1 starting at the aug of the oma-1 mRNA sequence) all appended to a 36-nt pUG tail (b, n = 6 (no injection), 10 (1–50), 17 (1–100), 8 (1–270), 9 (271–540) and 15 (1–540)). In a, b, percentage of embryonic arrest was scored at 20 °C. Data are mean ± s.d.
Extended Data Fig. 5 | Endogenous targets of pUGylation in *C. elegans.*

**a**, mRNAs upregulated in *rde-3(-)* mutants (Supplementary Table 2) were compared to published lists of: (1) RNAs targeted by CSR-1-bound endo-siRNAs⁴⁹, (2) piRNA-targeted mRNAs (based on predictive and experimental approaches)⁵⁰, and (3) WAGO-class mRNAs²⁶. P values were generated using a one-sided Fisher’s exact test. This analysis showed statistically significant overlap between the mRNAs upregulated in *rde-3(-)* mutants and both piRNA targets and WAGO-class mRNAs. **b–d**, Total RNA was extracted from WT or *rde-3(-)* worms. The assay outlined in Fig. 1a was used to detect pUG RNAs for two DNA transposons (Tc4v and Tc5) and a retrotransposon (Cer3) that were significantly upregulated in *rde-3(-)* worms (**b**); predicted protein-coding mRNAs that were significantly upregulated in *rde-3(-)* worms (**c**); and two randomly selected mRNAs whose expression does not change in *rde-3(-)* mutants (**d**). Results are representative of three biologically independent experiments. The same reverse-transcribed samples were used for **c** and **d** and, therefore, the gsa-1 loading control is the same for both panels.
Extended Data Fig. 6 | Mutator foci probably coordinate pUG RNA biogenesis in germ cells. **a**, dpy-11 and oma-1 pUG PCR (Fig. 1a) were performed on total RNA from glp-1(q224/ts) worms grown at 15 °C (germ cells present) or 25 °C (approximately 99% of germ cells absent), with or without oma-1 and dpy-11 dsRNA. Data are representative of two biologically independent experiments. The samples in a are the same as those used in Fig. 3e and, therefore, the gsa-1 loading control is the same. **b**, oma-1 pUG PCR was performed on total RNA extracted from wild-type, rde-3(-) and mut-16(pk710) worms, with or without oma-1 dsRNA. Data are representative of four biologically independent experiments. **c**, RT-qPCR was used to quantify levels of oma-1 pUG RNAs in wild-type, rde-3(-) and mut-16(pk710) worms, with or without oma-1 dsRNA. Data are represented as fold change in the levels of oma-1 pUG RNAs with or without oma-1 dsRNA (y-axis) for each strain (x-axis). n = 3 biologically independent samples per treatment for each strain. Data are mean ± s.d. **d**, RT-qPCR was used to quantify levels of Tc1 pUG RNAs in wild-type, rde-3(-) and mut-16(pk710) worms. The RNA samples used for d are the same as those used in c, except that the data for the samples with and without oma-1 dsRNA were pooled for each strain. n = 6 biologically independent samples for each strain. Data are mean ± s.d. The analyses in c and d showed that mut-16 mutants produced more oma-1, but fewer Tc1, pUG RNAs than wild-type worms. The increased levels of oma-1 pUG RNAs in mut-16(pk710) worms was also suggested by the results in b. Together, these data suggest that Mutator foci probably have an important role in coordinating pUG RNA biogenesis in germ cells, as pUG RNA levels become misregulated in mut-16(pk710) mutants.
Extended Data Fig. 7 | pUG RNAs are templates for RdRPs. a, A biological replicate of the experiment shown in Fig. 4d was performed. *oma-1*(SNP) pUG or pGC RNAs were injected into *rde-1(ne219); oma-1(zu405ts)* germlines. SNP location is indicated with the dotted line. Injected worms were collected 1–4 h after injection, total RNA was isolated and small RNAs (20–30 nt) were sequenced. The distribution of 22G siRNAs mapping antisense to *oma-1* is shown, with 22G siRNA reads normalized to reads per million total reads. *oma-1* pUG (but not pGC) RNA injection triggered 22G siRNA production near the site of the pUG tail (pUG-specific 22G siRNAs). For unknown reasons, both pUG and pGC RNA injections triggered production of small RNAs around 400 bp 5’ of either tail. b, The length distribution of small-RNA reads mapping antisense to *oma-1* is shown for small RNAs sequenced after *oma-1*(SNP) pUG RNA injections (Fig. 4d and a). c, The proportion of 22-nt small RNAs mapping antisense to *oma-1* containing 5’ A, U, G or C is shown.
Extended Data Fig. 8 | De novo pUGylation events in progeny are required for TEI. a, oma-1(zu405ts) worms were fed bacteria expressing empty vector control or oma-1 dsRNA and the percentage of embryos hatched at 20 °C was scored for six independent experiments. Data are mean ± s.d. of three biologically independent experiments. For each experiment, the percentage of embryos hatched at 20 °C was averaged for six individual worms per treatment for each genotype. b, rde-1(ne219); oma-1(zu405ts) worms were injected with co-injection marker alone (n = 12) or co-injection marker + oma-1 pUG RNA (n = 19) and the percentage of embryos hatched at 20 °C was scored for four generations in lineages of worms established from injected parents (see Methods for details of experimental set-up). Data are mean ± s.d. P values were generated using two-tailed unpaired Student’s t-test. c, c38d9.2 and Tc1 pUG RNA expression quantified by RT–qPCR in embryos collected from wild-type, rde-3(-) or MAGO12 worms. Fold change is normalized to rde-3(-). Each point (n) represents a biologically independent replicate, n = 3 independent replicates per strain. Data are mean ± s.d. d, Same experiment as Fig. 5d. rde-1(ne219); oma-1(zu405ts) worms were injected with an oma-1(SNP) pUG RNA or with co-injection marker only. Co-injection marker-expressing F 1 progeny were picked and allowed to lay their F2 broods. oma-1 pUG PCR was performed on total RNA from F2 progeny. Shown are data from three biological replicates. e, Two biological replicates of small RNAs sequenced from the progeny of rde-1(ne219); oma-1(zu405ts) worms injected with oma-1(SNP) pUG or pGC RNAs are shown. Dotted line indicates the location of the SNP incorporated into oma-1. The distribution of 22G siRNAs mapping antisense to oma-1 is shown, with 22G siRNA reads normalized to reads per million total reads. In Fig. 4d and Extended Data Fig. 7a, small RNAs were sequenced 1–4 h after injection and 100% of 22G siRNAs antisense to the region of the engineered SNP in oma-1 were found to encode the complement of the SNP. Shown here, less than 1% of 22G siRNAs from the progeny of injected worms encoded the SNP complement. siRNAs mapping near the pUG tail were observed only after oma-1(SNP) pUG RNA injection (pUG-specific siRNAs). For unknown reasons, both oma-1(SNP) pUG and pGC RNAs triggered production of small RNAs 5′ of the pUG-specific siRNAs. It is possible that these siRNAs were triggered by systems that respond to foreign RNAs, such as the piRNA system. Further work will be needed to determine the aetiology of these siRNAs. f, Same experiment as Fig. 5e. oma-1(zu405ts) hermaphrodites were fed oma-1 dsRNA and crossed to rde-3(ne298); oma-1(zu405ts) males. F2 progeny from this cross were genotyped for rde-3(ne298). WT and rde-3(ne298) homozygous F2 progeny were phenotyped for the percentage of embryos hatched at 20 °C. Three biologically independent crosses (P0 1–3) were performed. Data are mean ± s.d. P values were generated using two-tailed unpaired Student’s t-tests.
Extended Data Fig. 9 | Working model for pUG RNA–siRNA cycling during RNAi. Initiation: exogenous and constitutive (that is, genomically encoded such as dsRNA or piRNAs) triggers direct RDE-3 to pUGylate RNAs previously fragmented by factors in the RNAi pathway. Maintenance: pUG RNAs are templates for secondary siRNA synthesis by RdRPs. Argonaute proteins (termed WAGOs) bind secondary siRNAs and: (1) target homologous RNAs for transcriptional and translational silencing29,34,63,64, as well as (2) direct the cleavage and de novo pUGylation of additional mRNAs. In this way, cycles of pUG RNA-based siRNA production and siRNA-directed mRNA pUGylation maintain silencing over time and across generations. This model shows germ line perinuclear condensates termed Mutator foci as the likely sites of pUG RNA biogenesis in germ cells for several reasons. RDE-3 localizes to Mutator foci17 and we show, in Fig. 3d, that endogenous pUG RNAs localize to Mutator foci. The fact that enzyme and enzyme product both localize to Mutator foci suggests that Mutator foci may be sites of RNA pUGylation. In addition, although pUG RNAs are still made in mut-16 mutants (Extended Data Fig. 6b–d), which lack Mutator foci, the levels of both dsRNA-triggered and endogenous pUG RNAs are misregulated. Thus, while RDE-3 still has enzymatic activity in the absence of Mutator foci, these perinuclear condensates are probably coordinating target recognition and pUGylation in wild-type worms.

Indeed, both the endonuclease RDE-8, which cleaves mRNAs targeted by dsRNA12, and the RdRP RRF-117 also localize to Mutator foci, further suggesting that pUG RNA–siRNA cycling occurs in Mutator foci. Previous studies have shown that animals lacking RDE-3 still produce some 22G endo-siRNAs, including 22G siRNAs that associate with the Argonaute CSR-1 and whose biogenesis depends upon the RdRP EGO-126,65. Thus, EGO-1 may also produce some 22G siRNAs via a pUG RNA-independent mechanism. A previous study showed that, in rrf-1 mutants that lack germlines, sel-1 RNAi causes a small fraction of sel-1 mRNA fragments to be uridylated in a largely RDE-3-dependent manner in the soma12. These results suggest that, in somatic tissues, RDE-3 may add non-templated Us to the 3′ termini of mRNA fragments generated during RNAi. It was proposed that this uridylation may be important for turnover or decay of RNAi targets12. Our work, combined with this earlier data about RDE-3-dependent uridylation12, suggests two models. First, RDE-3 may possess two distinct catalytic activities: uridylation and pUGylation. According to this model, RDE-3 might add Us or UGs depending on context (for example, cell or tissue type, or developmental timing). Alternatively, the mRNA uridylation observed in the soma could depend on RDE-3 and the pUGylation system, but may be mediated by another, currently unknown, poly(U) polymerase.
Extended Data Fig. 10 | pUG RNA shortening may act as a brake on TEI.

a, The gel shown is the same as in Fig. 5a, except that oma-1 pUG RNAs from the F₁ generation are included for WT and rde-3(-) worms. Data are representative of three biologically independent experiments. b, oma-1 pUG RNA reads from MiSeq (n = 1 biological experiment) were mapped to oma-1 and the length of the oma-1 mRNA portion of each pUG RNA read was determined (y-axis). In the box plot, the box represents the interquartile range (IQR) and the centre line shows the median of lengths at the indicated generations after dsRNA treatment. The y-axis starts at the aug of the oma-1 mRNA. The whiskers extend to values below and above 1.5 × IQR from the first and third quartiles, respectively. Data beyond the end of the whiskers are outliers and plotted as points. These data support the results in a, showing that the oma-1 mRNA fragments appended to get pUG tails, and thus oma-1 pUG RNAs, get shorter in each generation during RNAi-triggered TEI. c, A ratchet model to explain pUGRNA shortening. pUG RNA shortening may be due to the 3′→5′ directionality of RdRPs, which, during the maintenance phase of pUG-siRNA cycling (see model in Extended Data Fig. 9), causes each turn of the pUG–siRNA cycle to trigger cleavage and pUGylation of target mRNAs at sites more 5′ than in the previous cycle. Eventually, pUG RNAs are too short to act as RdRP templates, cycling cannot be maintained and silencing ends. Additional support for the ratchet model comes from Fig. 5c, which shows that RNAi-triggered pUGRNAs are longer in MAGO12 mutant worms than in wild-type worms. Note: the P₀ worms in Fig. 5c were exposed to dsRNA continuously from embryos to adulthood, when they were collected. These longer pUG RNAs are probably due to continued initiation of pUGylation triggered by the exogenously provided dsRNA without downstream pUG-siRNA cycling. In the absence of this cycling, pUG RNA shortening does not occur. Finally, a number of recent studies in C. elegans have reported transgenerational inheritance of acquired traits, which lasts three to four generations⁶⁶–⁷¹. As shown in a, the expression of oma-1 RNAi-directed pUG RNAs also endures for three to four generations. These shared generational timescales of inheritance hint that the inheritance of acquired traits in C. elegans may be mediated by pUG RNAs whose generational ‘half-life’ is limited to three to four generations owing to the built-in brake on TEI provided by pUG RNA shortening.
Reporting Summary

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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

All fluorescence images were acquired using the Carl Zeiss Zen 2 (blue edition) software. qPCR data was acquired using Bio-Rad CFX Manager software (Version 3.1).

Data analysis

A description of custom scripts used to analyze MiSeq and RNA-seq data is provided in the Methods section and scripts are available upon request. Custom Python scripts used to analyze small RNA sequencing data are deposited at: https://github.com/Yuhan-Fei/pUG-analysis. Open source software used for RNA-seq analysis: Trim Galore version 0.4.4_dev, STAR version 2.7.0a, TEToolkit version 2.0.3. Open source software used for MiSeq analysis: UMI-tools 1.0.0, Cutadapt v2.5, STAR v2.7.0f, SAMtools v1.9, BEDtools v2.27.1, Biostrings v2.50.2, Sushi v1.20.0, ggseqlogo v0.1. Open source software used for small RNA sequencing analysis: Cutadapt 1.14, FastQC 0.11.5, Tophat 2.1.1, Samtools v0.1.19, Tophat v2.1.1, DeepTools v3.0.2, Sushi 1.20.0, featureCounts 1.6.0.

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All data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE148134. Processed MiSeq and small RNA sequencing reads (see Methods) are also provided in Table S1 and S4, respectively.
Field-specific reporting
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Life sciences study design
All studies must disclose on these points even when the disclosure is negative.

Sample size
Samples sizes are noted in Figure legends. No statistical methods were used to predetermine sample size. For PCR and qPCR data, 10cM plates of worms were collected in order to obtain sufficient quantities of RNA for reverse transcription. For fluorescence imaging experiments, a minimum of 10 animals were analyzed per experiment and each experiment was repeated 3 times on different days. For injection data, multiple animals were injected for every experiment to control for variability between animals. For all data, positive and negative controls were included whenever possible. Our samples sizes for each experiment are typical of those used in the field.

Data exclusions
No data were excluded from analyses.

Replication
For PCR and qPCR data, every experiment was replicated using independent samples collected and processed on different days to ensure reproducibility of the data (i.e. biologically independent experiments). For injection data, key results were either repeated on multiple days and/or confirmed using a second target gene. LC-MS/MS was performed only once, but hits were verified using pull-down followed by Western blotting. MiSeq was only performed once, but pUG RNAs from the same samples were also Sanger sequenced from three different biological replicates. All attempts at replicating the remaining data were successful and the number of biological replicates performed for each experiment are stated in Figure legends and/or Methods.

Randomization
Our experiments were not randomized and controlling covariates was not necessary. For all experiments, control vs. experimental samples were treated in parallel. Samples were allocated into different groups based on genotype for all genetic/mutant analyses performed. Animals were randomly chosen from stock plates for different injection treatments and control vs. experimental injections were performed in parallel.

Blinding
We were not blinded to group allocation during experiments and outcome assessment. However, control vs. experimental samples were treated equally and in parallel.

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Methods
n/a
☐ Involved in the study
☐ ChiP-seq
☐ Flow cytometry
☐ MRI-based neuroimaging

Antibodies

Antibodies used
Rabbit Anti-GFP antibody - ChIP Grade (ab290), Abcam, 1:1000 dilution. Goat Anti-Rabbit Secondary Antibody, Alexa Fluor 555 (A-21429, Invitrogen), 1:100 dilution. Rabbit Anti-HA antibody (#3724), Cell Signaling Technology, 1:1000 dilution. IRDye 800CW Goat anti-Rabbit IgG Secondary Antibody (926-32211), LI-COR, 1:10000.

Validation
The Anti-GFP antibody (ab290) we used for immunofluorescence is a highly cited anti-GFP antibody (https://www.abcam.com/gfp-antibody-chip-grade-ab290-references.html#active-tab) and was used for immunofluorescence in C. elegans in Zacharias et al., 2015, Plos Genetics. The Anti-HA antibody (#3724) we used for Western blots was recently used for Western blots in Huynh et al., 2019, Nature Communications.
## Animals and other organisms

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| Category               | Details                                                                 |
|------------------------|-------------------------------------------------------------------------|
| Laboratory animals     | Experiments were performed using Caenorhabditis elegans. All strains were derived from the N2 (Bristol) wild-type strain. |
| Wild animals           | This study did not involve the use of any wild animals.                 |
| Field-collected samples| No field samples were collected for this study.                         |
| Ethics oversight       | No ethical approval is required to work with Caenorhabditis elegans.    |

Note that full information on the approval of the study protocol must also be provided in the manuscript.