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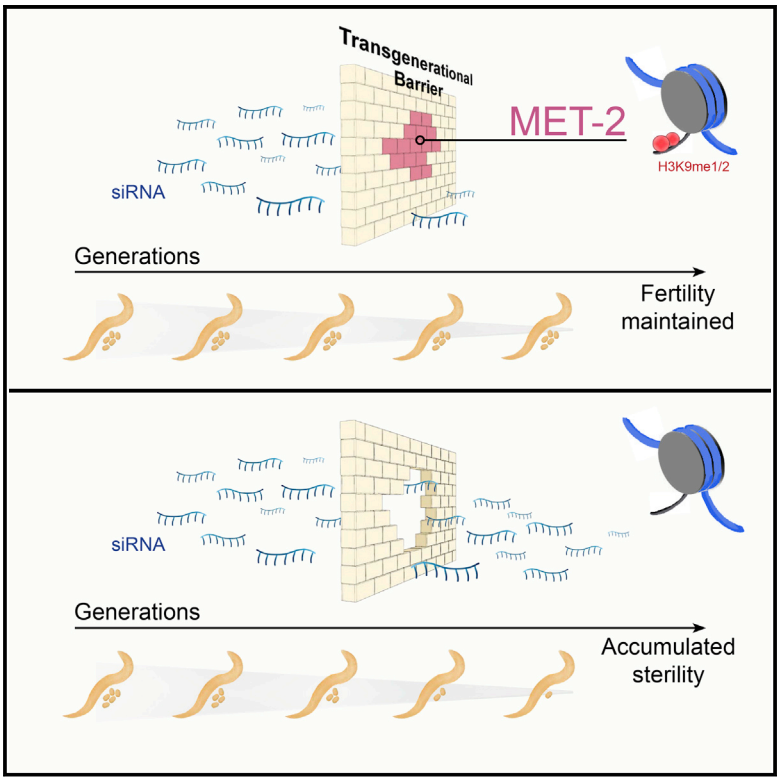
MET-2-Dependent H3K9 Methylation Suppresses Transgenerational Small RNA Inheritance

Graphical Abstract



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In Brief

Lev et al. show that MET-2, an enzyme essential for H3K9 methylation, is required for termination of heritable RNAi. *met-2* mutants display an enhanced RNAi phenotype, and dsRNA induces stable multigenerational silencing of germline genes. Moreover, *met-2* mutants become progressively sterile due to accumulation of HRDE-1-dependent small RNAs.

Highlights

- Strong RNAi inheritance in *met-2;set-25* double mutants defective in H3K9 methylation
- RNAi is stably inherited transgenerationally in *met-2* mutants, defective in H3K9me1/2
- The Mrt phenotype of *met-2* mutants stems from accumulation of aberrant small RNAs
- MET-2 affects RNAi inheritance indirectly by affecting global small RNA levels

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MET-2-Dependent H3K9 Methylation Suppresses Transgenerational Small RNA Inheritance

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SUMMARY

In *C. elegans*, alterations to chromatin produce transgenerational effects, such as inherited increase in lifespan and gradual loss of fertility. Inheritance of histone modifications can be induced by double-stranded RNA-derived heritable small RNAs. Here, we show that the mortal germline phenotype, which is typical of *met-2* mutants, defective in H3K9 methylation, depends on HRDE-1, an argonaute that carries small RNAs across generations, and is accompanied by accumulated transgenerational misexpression of heritable small RNAs. We discovered that MET-2 inhibits small RNA inheritance, and, as a consequence, induction of RNAi in *met-2* mutants leads to permanent RNAi responses that do not terminate even after more than 30 generations. We found that potentiation of heritable RNAi in *met-2* animals results from global hyperactivation of the small RNA inheritance machinery. Thus, changes in histone modifications can give rise to drastic transgenerational epigenetic effects, by controlling the overall potency of small RNA inheritance.

INTRODUCTION

Resetting of the parental gene expression program in every generation prevents environmental responses from becoming heritable [1]. Erasure of acquired epigenetic information is important for the totipotency of the germline and ensures stereotypic, species-appropriate development [2, 3]. Nevertheless, recent evidence shows that specific chromatin modifications persist across generations, also in humans, by escaping from reprogramming, through largely unknown mechanisms [4]. In theory, retention of some ancestral responses could be beneficial, as it could help prepare the progeny for the challenges that the ancestors met [5]. It is not clear how particular histone modifications become heritable [6]. In addition to passive incorporation of naive histones behind the replication fork [7], active mechanisms such as active replacement of histones, upregulation of histone de-methylases and de-acetylases, and active DNA demethylation have evolved to erase parental modifications in the germline and in the early embryo [8, 9]. How then, is the memory of certain histone marks maintained transgenerationally?

In *C. elegans*, several studies have detected heritable histone modification patterns and showed that transiently deactivating certain chromatin modifiers affects the progeny [10, 11]. The nature of the transgenerational epigenetic effects that arise when the epigenome is modified depends on a complex network of interactions between different chromatin modifiers [12]. For instance, *met-2* mutants, which are deficient in H3 lysine 9 methylation (H3K9) [13], and *spr-5* mutants, defective in H3K4 demethylation [11], display a "mortal germline" (Mrt) phenotype and become sterile after many generations [12, 14, 15]. The *spr-5;met-2* double mutant exhibits a synthetic phenotype and becomes sterile immediately, after just one generation [14].

We hypothesized that transgenerational memory of histone modifications in *C. elegans* can be maintained in a heritable small RNA cache. In many different organisms, nuclear-acting small RNAs were shown to direct deposition of histone modifications [16–18]). In *C. elegans*, "repressive" chromatin marks (H3K9me3, H3K27me3) are deposited on loci that are targeted by double-stranded RNA (dsRNA)-derived small RNAs that enforce RNAi. RNAi can produce long-term heritable responses that affect progeny that were not directly exposed to the original trigger [19]. Through an engagement with components of the nuclear RNAi pathway, heritable small RNAs establish a H3K9me3 [20] and H3K27me3 [21] footprint, which can persist on the targeted locus, for a few generations. While positive feedback interactions between small RNAs and histone marks have been described in other organisms [22], it is unknown whether and how chromatin modifications affect small RNA biogenesis and inheritance in *C. elegans*.

Heritable small RNA responses are maintained in the progeny owing to RNA-dependent RNA polymerase (RdRP)-mediated amplification [23], which can in theory perpetuate the RNAi response ad infinitum, by synthesizing additional "secondary" small RNAs in every generation [24]. However, an active regulatory transgenerational feedback process, between heritable endogenous small interfering RNAs (endo-siRNAs) and small RNA inheritance genes, restricts the duration of epigenetic responses [25]. Mutants defective in components of this feedback response, dubbed *motek* genes (modified transgenerational epigenetic kinetics), display RNAi inheritance responses that differ in their length from those seen in wild-type worms [25] and, in certain cases, exhibit unusually long RNAi inheritance durations [25, 26].

Similarly, while the mechanisms are unknown, chromatin modifiers were shown to be required for long-term RNAi inheritance [19]. Among the many chromatin marks that could affect

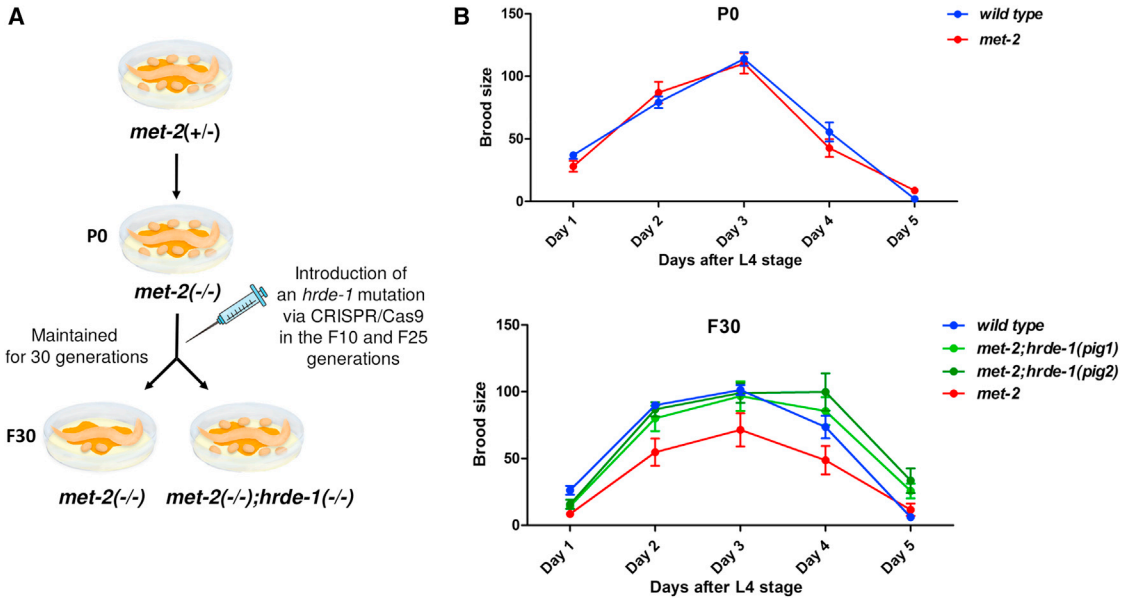

Figure 1. The Mortal Germline Phenotype of *met-2* Mutants Is Dependent on HRDE-1

(A) Experimental scheme. *met-2(+/−)* mutant worms were maintained as heterozygotes for two generations. Homozygous *met-2(−/−)* worms were isolated and assayed for their brood size in the P0 and F30 generations. In the F10 and F25 generations, two different *hrde-1* deletion alleles were introduced using the CRISPR/Cas9 system.

(B) Brood size of homozygous *met-2* mutants in P0 (top) and F30 (bottom) after isolation of *met-2* homozygous animals. The average brood sizes per worm per day are presented (mean ± SEM). The brood size of ten animals was tested per genotype. No significant differences were observed in the P0 generation. In the F30 generation, a significant reduction in the brood size of *met-2* mutants was observed ($p < 0.001$, two-way ANOVA). However, no significant differences between the wild-type and *met-2;hrde-1* mutants were observed.

See also Figure S1 and Tables S1, S2, and S3.

RNAi inheritance, H3K9me was most extensively studied: the H3K9me3 methyltransferases SET-25 and the H3K9me binding protein HPL-2 (Heterochromatin Protein Like-1) were suggested to be required for multigenerational heritable silencing [27, 28].

Here, we found that the Mrt phenotype of *met-2* mutants stems from MET-2's influence on biogenesis of heritable small RNAs. We demonstrate that MET-2 is required for restricting the transgenerational duration of RNAi, as in *met-2* mutants RNAi responses persist indefinitely. Thus, histone modifications are important switches that control the duration of heritable small RNA-mediated epigenetic responses.

RESULTS

The MET-2 methyltransferase was shown to be required for deposition of the first two methyl groups on H3K9 and also indirectly for the deposition of the third methyl, that is successively added by SET-25, to create tri-methylated H3K9 [13]. The Mrt phenotype of histone modifier mutants such as *met-2* was suggested to depend on an interaction between multiple chromatin regulators, that were hypothesized to maintain the heritable memory by balancing euchromatic H3K4 and heterochromatic H3K9 methylation levels [12].

We examined whether the transgenerational memory that leads to sterility in *met-2* mutants is maintained by heritable small RNAs. We isolated homozygote *met-2(−/−)* mutants from heterozygous parents and measured the mutants' fertility in the P0 generation and 30 generations after establishment of homozygosity (Figure 1A). Consistently with previous studies [14], while in the P0 generation *met-2* mutants were as fertile as wild-type animals, they became progressively sterile, and after 30 generations their brood size was reduced to ~50% compared to wild-type worms (Figure 1B). To examine the dependency of this epigenetic memory on inheritance of small RNAs, we knocked out the *hrde-1* gene in *met-2* animals, in the F10 and F25 generations. HRDE-1 (Heritable RNAi Defective 1) is a nuclear and germline-expressed argonaute protein required for carrying small RNAs across generations [29]. *hrde-1* mutants exhibit a Mrt phenotype but only when grown in stressful temperatures (25°C) [29], and our experiments were conducted at 20°C. Since *met-2* and *hrde-1* are tightly linked, we created the double mutants by utilizing the CRISPR/Cas9 system, and not through crossing. Two different null alleles were created: *hrde-1(pig1)* and *hrde-1(pig2)*. Strikingly, the sterility of *met-2* mutants after 30 generations was completely reversed when *hrde-1* was knocked out (Figure 1B). This suggests that the accumulation of aberrant changes, which ultimately lead to sterility in *met-2* mutants, is memorized by heritable small RNAs.

To test how small RNA biogenesis is altered in *met-2* mutants, we sequenced small RNAs from the wild-type and *met-2* mutants, at different generations after establishment of *met-2* homozygosity. We focused on changes in "secondary" endogenous small RNAs (endo-siRNAs), which align perfectly in the antisense orientation to exons of specific genes and cover the entire length of the mRNA target. We refer to units of such endo-siRNAs that target particular individual genes, as "small

RNAs targeting specific genes'' or STGs for short [25, 30]. Over generations, many STGs in *met-2* animals became progressively misexpressed. Differential expression analysis of the STGs revealed drastic changes in the endogenous small RNA pools in the P0 *met-2* mutants (3,846 differentially expressed STGs), which exacerbated over generations (3,919 and 5,269 differentially expressed STGs in the F3 and F15 generations, respectively; Figures S1A and S1B; Table S2). A principle component analysis suggested that the small RNA pools of different biological replicates of *met-2* were more variable in comparison to replicates of wild-type samples (Figure S1A). We found that STGs that align to protein-coding genes, which in wild-type animals are marked by H3K9me2 near their transcription start site (a MET-2-dependent modification [31]), are depleted in *met-2* mutants (1.55-fold enrichment in significantly downregulated STGs, $p < 0.001$). The STGs that became more differentially expressed in *met-2* over generations align preferentially to genes that are expressed in the germline (1.5- and 0.85-fold enrichment for upregulated and downregulated STGs, respectively; $p < 0.001$; Figure S1C) and, in particular, to sperm-expressed genes (1.5- and 0.8-fold enrichment for upregulated and downregulated STGs, respectively; $p < 0.001$; Figure S1C). We detected only very mild enrichments for STGs that target oocyte-expressed genes (Figure S1C). These results support an hypothesis according to which misexpressed small RNAs accumulate in *met-2* mutants over generations, which could lead to sterility [32, 33].

Also in support of this hypothesis, different germline-expressed GFP transgenes, which were otherwise fully expressed in the wild-type background, were silenced in *met-2* mutants. For example, 97.5% of *met-2* worms (196/201 animals) silenced a single-copy GFP transgene expressed under the control of the *pie-1* promoter. Previously, transgenerational silencing of this transgene, in response to anti-*gfp* dsRNA, was used to study heritable RNAi [24, 25, 27].

We directly tested whether the stochastic silencing in *met-2* results from an inability to terminate heritable small RNA responses. We compared the duration of dsRNA-induced transgenerational silencing of different gene targets in the wild-type and in *met-2*, *set-25*, and *met-2;set-25* double mutants, all of which were suggested to be altogether devoid of H3K9me3 [13, 21, 34]. We exposed parents expressing either the *mex-5::GFP* transgene (Figures 2A and 2B) or the *pie-1::GFP* transgene (that also exhibits strong stochastic silencing; Figure S2) to anti-*gfp* dsRNA-producing bacteria and moved the progeny to control bacteria that did not produce dsRNAs. In the wild-type, as previously described [35], the dsRNA-induced silencing persisted for at least three generations and gradually petered out (Figure 2B). The heritable silencing in *met-2* mutants (n4256 allele) was stable (>30 generations), and we could not detect any GFP signal in any of the progeny (Figure 2B). The effects of *met-2* on the RNAi responses was validated using an additional *met-2* allele (RB1789 strain, ok2307 allele; Figure S3). These mutants also stochastically silenced the *mex-5::GFP* transgene, even without exposure to anti-*gfp* dsRNA (85/574 animals,14.8%).

As previously reported [27], *set-25* mutants were defective in RNAi inheritance compared to the wild-type. However, we detected weak heritable silencing effects, as RNAi-treated *set-25* mutants dimly expressed the transgene in the F3 (Figure 2C; on average, 60% of the *mex-5::gfp* expression at the F3 generation compared to the progeny of untreated worms; $p < 0.0001$). These results suggest that SET-25 and H3K9me3 promote but are not completely required for heritable silencing.

Also in *met-2;set-25* double mutants, RNAi inheritance was much stronger and persisted for many more generations in comparison to the wild-type (Figure 2B). While in wild-type worms above 50% of the population re-expressed the GFP transgene already after two or three generations, in *met-2;set-25* double mutants, inheritance lasted for more than ten generations ($p < 0.0001$, two-way ANOVA; Figure 2B). Thus, mutating *met-2* rescues the ability of *set-25* mutants to strongly inherit RNAi. RNAi inheritance in *met-2;set-25* double mutants was weaker in comparison to the inheritance observed in *met-2* animals, supporting the conclusion that MET-2 and SET-25 have opposing effects on RNAi inheritance.

In addition to examining the duration and intensity of the silencing responses that were raised against foreign genes, we targeted with RNAi the redundant and germline expressed *oma-1* gene. We used mutants bearing the *zu405* allele, a temperature-sensitive dominant-lethal allele that was used in the past to study the dynamics of heritable RNAi responses raised against endogenous genes [25, 29]. In this system, only the eggs of worms that inherit anti-*oma-1* RNAi develop in the restrictive temperatures [35]. We observed strong enhancement of dsRNA-induced heritable silencing of *oma-1* in *met-2* mutants and in *met-2;set-25* double mutants ($p < 0.0001$, two-way ANOVA; Figure 2D). Surprisingly, in comparison to the *wild-type*, we detected stronger inheritance of *oma-1* silencing also in the *oma-1;set-25* double mutants, suggesting that SET-25-dependent H3K9me3 affects the intensity of heritable RNAi in a gene-specific manner ($p < 0.0001$, two-way ANOVA; Figure 2D). Altogether, these experiments, in which both foreign and endogenous genes were targeted by RNAi, suggest that MET-2 is required for termination of RNAi inheritance and that SET-25 is not essential for heritable silencing and can either support or inhibit RNAi inheritance, depending on the targeted locus.

Which mechanism potentiates RNAi inheritance in *met-2* mutants? We reasoned that the chromatin state of the locus of the RNAi-targeted gene could be different in *met-2* mutants. As MET-2 was suggested in the past to be required for germline chromatin reprogramming [14], we hypothesized that, in every generation, MET-2-dependent methylation ''reprograms'' the RNAi-targeted locus and thus terminates production of heritable small RNA. We examined H3K9me2 levels, a MET-2-specific mark, by preforming chromatin immunoprecipitation (ChIP). We found that exposing wild-type animals to anti-*gfp* RNAi results in strong accumulation of H3K9me2 on the targeted locus in the P0 and generates a very weak but statistically significant trace of H3K9me2 in the F1, F2, and F3 generations (Figure 3A; $p = 0.0008$, $p = 0.0255$, $p = 0.0190$, and $p = 0.0162$, respectively, two-way ANOVA). If the presence of H3K9me2 on the targeted locus was to reprogram the heritable RNAi response, then the levels of this mark would be expected to rise in every generation following RNAi, until the heritable silencing effect would be ''erased.'' Since the opposite dynamics are observed—strong H3K9me2 in the RNAi-treated P0s and then rapid elimination of the H3K9me2 signal in the next generations—these results

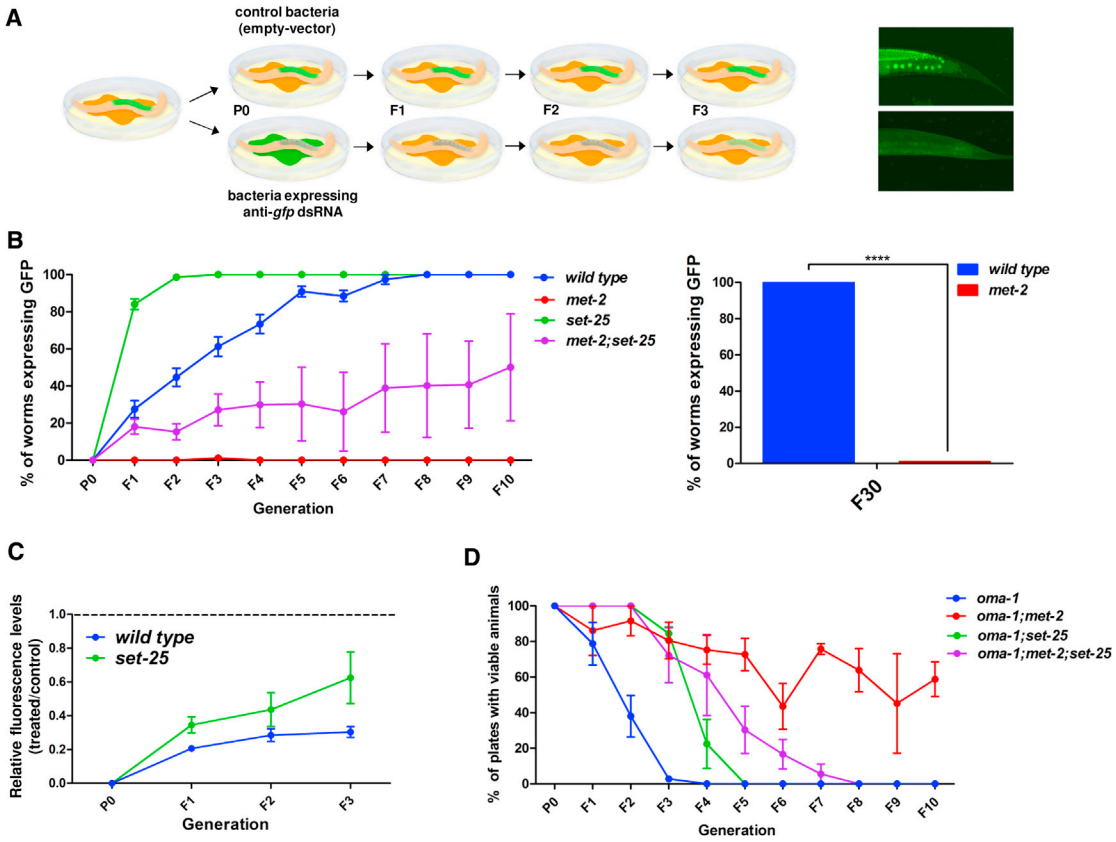

Figure 2. The Effects of Loss of H3K9 Methyltransferases on RNAi Inheritance

(A) A general scheme for the RNAi inheritance experiments. Worms were fed on bacteria expressing *anti-gfp* dsRNA or on control bacteria containing an empty-vector. At each generation, the worms were transferred to plates with bacteria that do not produce dsRNA. Shown are representative pictures of animals expressing GFP in the germline, under the control of the *mex-5* promoter (right, top), and animals that silence the GFP after exposure to anti-*gfp* dsRNA (right, bottom).

(B) Transgenerational inheritance of GFP silencing in the wild-type and in *met-2*, *set-25*, and *met-2;set-25* mutants (left). Percentages of worms positive for GFP expression are indicated for each generation (mean ± SEM). Three biological repeats (n > 60) were performed. *met-2* mutants exhibit stable inheritance also 30 generations after the initial exposure to anti-*gfp* RNAi (right).

(C) Relative GFP fluorescence levels in *anti-gfp* RNAi-treated animals versus untreated controls in the wild-type and *set-25* mutants (mean ± SD). The results of three biological repeats (n > 30) are presented.

(D) Transgenerational inheritance of silencing of a temperature-sensitive allele of *oma-1* in *met-2(+);set-25(+)* animals and in *met-2*, *set-25*, and *met-2;set-25* mutants. Percentages of fertile worms are presented (mean ± SEM). The results of three biological repeats (n = 12) are shown.

See also Figures S2 and S3.

suggest that the very strong RNAi inheritance in *met-2* mutants does not result from absence of H3K9me2 on the targeted locus.

We next examined the possibility that in *met-2* mutants heritable RNAi is potentiated due to the accumulation of H3K27me3. H3K27me3 is another RNAi-induced histone modification [21] that is associated with suppression of transcription [36], which could perhaps compensate for the absence of H3K9me. In accordance with previous work [21], we detected a weak inherited H3K27me3 footprint on the RNAi-silenced GFP locus in wild-type worms (Figure S4; p = 0.0059, two-way ANOVA). Although H3K27me3 levels in *set-25* mutants were higher than in the wild-type, the difference was highly variable and not statistically significant. However, in *met-2* mutants, in which the strongest silencing is observed, the levels of the RNAi-induced H3K27me3 were comparable to the levels observed in the wild-type (Figure S4; p = 0.092 and p = 0.1487, respectively, two-way ANOVA). We found that depleting the H3K27 methyltransferases does not affect heritable silencing in *met-2* mutants (Figure S5), and, since H3K27me3 levels are not significantly increased in H3K9me mutants, it is unlikely that this mark is responsible for the very strong silencing observed in *met-2* mutants.

While a global analysis previously suggested that embryos of *met-2* and *met-2;set-25* are devoid of H3K9me3 [13], in germline-bearing *met-2* adults, H3K9me3 was nevertheless detected [37, 38]. In these studies, it was not examined whether dsRNA-triggered RNAi can induce H3K9me3 deposition on the targeted loci in the mutants. Consistent with previous studies [20], we observed an RNAi-induced H3K9me3 footprint in wild-type worms that was inherited until the F3 generation (Figures 3B and 3C). Upon RNAi, we could not detect a statistically significant heritable H3K9me3 signal in *set-25 mutants* (two-way ANOVA, p = 0.77 in F1 generation; Figure 3B). In *met-2* and *met-2;set-25* mutants, we identified an H3K9me3 footprint

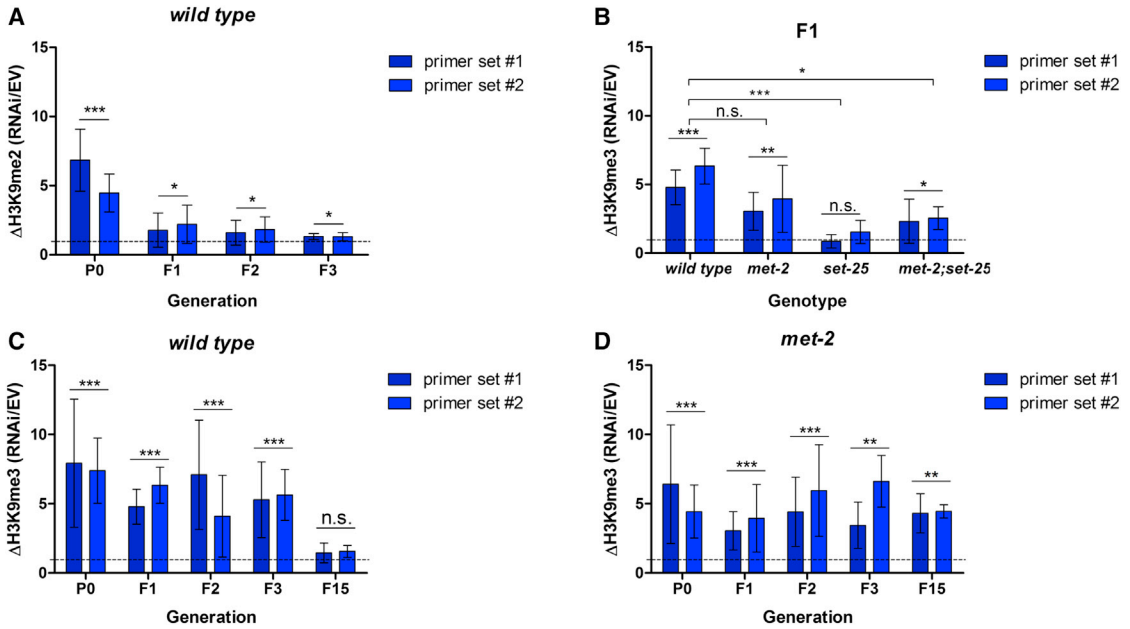

Figure 3. Inheritance of RNAi-Induced H3K9me in Different Methyltransferase Mutants

The inheritance of H3K9me marks on the RNAi-targeted GFP locus was measured by ChIP assays and quantified by real-time qPCR. Two primer sets that span the GFP locus were applied for qPCR detection. The data are expressed as ratios between H3K9me levels in worms treated with RNAi and untreated worms (mean ± SD). *eft-3* and *dpy-28* were used as internal controls for normalization. Asterisks above lines that flank the bars of the two primer sets denote statistically significant changes between RNAi-treated worms and untreated controls. Asterisk above lines that flank bars of different genotypes denote statistically significance differences between the two genotypes. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Sidak's multiple comparisons test was used. Three biological repeats were performed for examination of each genotype.

(A) The H3K9me2 levels of the RNAi targeted GFP across generations.

(B) Comparison of the RNAi-induced H3K9me3 signal in F1 progeny of the wild-type, *met-2*, *set-25*, and *met-2;set-25* double mutants.

(C) The H3K9me3 footprint on the RNAi-targeted GFP in the wild-type across generations.

(D) H3K9me3 footprint on the RNAi-targeted GFP in *met-2* mutants across generations.

See also Figures S4 and S5.

that was comparable or weaker in comparison to the signal found in wild-type worms (Figures 3B–3D). RNAi inheritance is much stronger in both *met-2* and *met-2;set-25* animals in comparison to the wild-type, although the RNAi-induced H3K9me3 signal is either comparable or much weaker in the mutants; thus, the potentiation of heritable silencing in the *met-2* mutant background does not depend on maintenance of higher H3K9me3 levels.

Since the levels of repressive marks on the targeted locus cannot explain the strong inheritance in *met-2* mutants, we hypothesized that these animals inherit more small RNAs. The heritable silencing in *met-2* mutants was found to depend entirely on heritable small RNAs, as knocking out *hrde-1* using CRISPR/Cas9 fully rescued the otherwise complete MET-2-dependent silencing of the GFP transgenes. GFP was re-expressed in *met-2* mutants that stochastically silenced the *pie-1::gfp* transgene (60/60) and in *met-2* lineages that stably silenced the *mex-5::gfp* transgene following RNAi (Figures 4A and 4B). Knocking down of NRDE-2 (Nuclear RNAi Defective 2), a nuclear RNAi protein that cooperates with HRDE-1 [39], also resulted in partial re-expression of the silenced GFP in *met-2* animals (Figures 4C and 4D). Heritable dsRNA-derived small RNAs that are carried by HRDE-1 direct H3K9me3 [29]; however, such small RNAs are detected before H3K9me3 can be identified on the targeted locus in the progeny [40].

Examination of our small RNA sequencing (RNA-seq) data showed that in the P0 generation anti-*gfp* dsRNA-treated *met-2* mutants had higher levels of anti-*gfp* small RNAs compared to the wild-type (~1.34 times the fold change, $p < 0.0001$, Tukey's multiple comparisons test; Figure 5). In the F3 generation, the levels of the anti-*gfp* small RNAs were reduced compared to the levels observed in the P0 generation in all genotypes except for in the *met-2* mutants, which maintained high levels of anti-*gfp* small RNAs, that were comparable to the levels detected in *met-2* P0 animals (Figure 5). Because in the F3 generation the heritable H3K9me3 levels in *met-2* and the wild-type were found to be equal (Figures 3C and 3D), H3K9me3 modification of the targeted locus cannot explain the increase in the intensity of the heritable small RNAs in *met-2*. Thus, another mechanism generates strong RNAi responses in *met-2* animals, and the abundant heritable small RNAs in *met-2* could be the molecules that directly potentiate heritable silencing. Interestingly, while the levels of anti-*gfp* small RNAs in *set-25* mutants were equal to the levels found in the wild-type in the P0 generation, *set-25* mutants did not inherit anti-*gfp* small RNAs at all (Figure 5). This is, to our knowledge, the first time that *set-25* mutants are directly shown to be deficient in maintenance of heritable small RNAs. *met-2;set-25* mutants had lower levels of anti-*gfp* small RNAs compared to *met-2* ($p < 0.0001$; Figure 5) but significantly higher anti-*gfp* small RNAs in comparison to wild-type animals or *set-25* mutants ($p < 0.05$ and

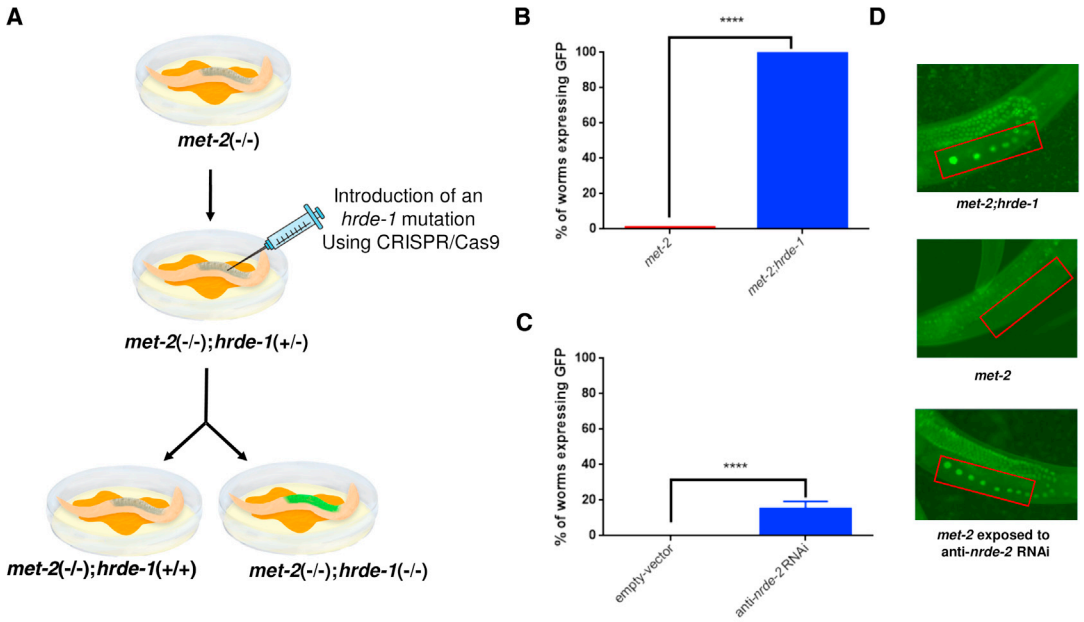

Figure 4. RNAi Inheritance in *met-2* Mutants Is Dependent on Heritable Small RNAs

(A) A scheme describing the rescue of germline GFP expression by CRISPR/Cas9-mediated introduction of an *hrde-1* mutation to *met-2* mutants. *met-2* mutants that stably silence GFP after exposure to anti-*gfp* dsRNA are introduced with an *hrde-1* mutation using the CRISPR/Cas9 system. Isolated homozygote *met-2;hrde-1* double mutants and *met-2* single mutants are assayed for germline GFP expression.

(B) The percentages of worms expressing GFP in *met-2* and *met-2;hrde-1* mutants are shown (mean ± SD). Three biological repeats (n = 60) were performed. ****$p < 0.0001$, Student's t test.

(C) The percentages of GFP-expressing *met-2* worms among animals treated or untreated with anti-*nrde-2* dsRNA are shown (mean ± SD). ****$p < 0.0001$, Student's t test. Three biological repeats (n = 60) were performed. $p < 0.0001$, Student's t test.

(D) Typical pictures of *met-2;hrde-1* mutants that fully express GFP (top), *met-2* mutants that silence GFP (middle), and *met-2* mutants treated with anti-*nrde-2* RNAi that re-express the otherwise silenced GFP (bottom).

$p < 0.0001$, respectively; Figure 5). To conclude, SET-25 supports the maintenance of anti-*gfp* small RNAs, while MET-2 suppresses both the initial synthesis of small RNAs in the parents and the maintenance of heritable small RNAs in the progeny.

Even in the 15th generation after exposure to anti-*gfp* dsRNA, we detected very high levels of anti-*gfp* small RNAs in *met-2* mutants, when the silencing response is long gone in the wild-type (Figure 5). The levels of anti-*gfp* small RNAs that were detected in the F15 generation were not significantly different from the levels detected in the P0 generations (Figure 5; $p > 0.05$), suggesting that the *met-2* mutants can maintain anti-*gfp* small RNAs indefinitely. While the RNAi-induced heritable H3K9me3 trace peters out in the wild-type in the F15, in *met-2* the H3K9me3 levels on the GFP transgene were comparable to the levels detected in the P0 (Figure 3D). These results, together with the fact that *set-25* mutants were found to be incapable of maintaining heritable small RNAs, suggest that also in *C. elegans*, as is the case in fission yeast and plants [22], a feedback with H3K9me3 could support the production of heritable small RNAs.

In addition, we sequenced small RNAs from *met-2;set-25* double mutants that expressed the *pie-1::gfp* transgene, which exhibited complete spontaneous silencing in *met-2* mutants, and partial, accumulated spontaneous silencing in *met-2;set-25* double mutants (Figure S6A). We detected a significant rise along generations in the levels of anti-*gfp* small RNAs in *met-2;set-25* double mutants, which was not observed in wild-type worms, when the worms were fed on the empty-vector producing bacteria (Figure S6B). This result suggests that the stochastic silencing exhibited in the *pie-1::gfp* transgene in the *met-2;set-25* background stems from an upregulation in spontaneous generation and accumulation of heritable anti-*gfp* small RNAs that silence the transgene.

To understand why exogenous small RNAs are upregulated in *met-2* mutants, we examined the proportions that particular small RNA species comprise out of the total small RNA pool in *met-2* and wild-type animals. In *met-2* animals, in comparison to the wild-type, we detected a downregulation in the proportion of small RNAs that target transposable elements, repeats, and pseudogenes ($p < 0.0001$, chi-square test; Figure 6A). It was previously shown that repetitive elements are enriched for H3K9me2 [42], and recently it was shown that repetitive elements are expressed in H3K9me mutants [34]. We have observed an enrichment among the *met-2* downregulated STGs for small RNAs that target H3K9 di-methylated genes (1.55-fold enrichment, $p < 0.001$). It would be intriguing in future studies to test the hypothesis, raised also in [32, 33, 43], that changes in heritable small RNAs that target transposons, repeats, or essential genes could lead to sterility. Moreover, we found significant changes in the levels of endo-siRNAs associated with particular small RNA pathways. Specifically, among the small RNAs downregulated in *met-2* mutants we detected enrichment for HRDE-1, WAGO-1, and Mutator-class-associated small RNAs (Figure 6B).

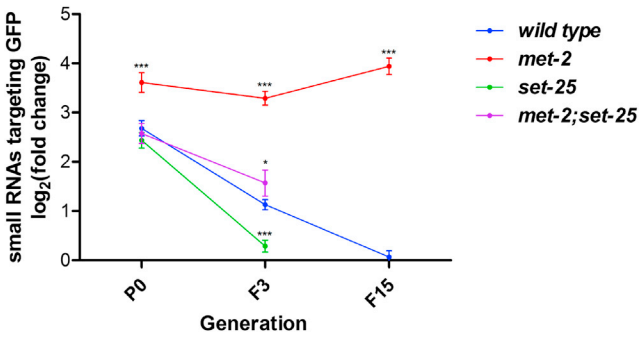

Figure 5. Sequencing-Based Analysis of dsRNA-Induced Small RNA Inheritance in H3K9me Mutants

Shown are log2 fold changes (anti-*gfp* RNAi-treated worms/control empty-vector worms) in normalized antisense small RNA reads that align to the GFP locus in *met-2*, *set-25*, and *met-2;set-25* mutants. Three biological repeats were performed. Data are presented as mean ± SD.
See also Figure S6.

Among the upregulated small RNAs in *met-2*, we detected enrichment for ALG-3/4 class small RNAs, known to affect spermatogenesis [44]. CSR-1-associated small RNAs were under-represented among both up- and downregulated STGs. Competition between different small RNA pathways over shared protein components was shown in many studies to affect the potency of the RNAi system [45–47]. Specifically, depletion of different endogenous small RNA species results in enhanced exogenous RNAi responses and extended small RNA inheritance [25]. We therefore tested whether in *met-2* animals the overall activity of the exo-RNAi machinery is potentiated. First, to test whether *met-2* mutants exhibit an "enhanced RNA interference" (Eri) phenotype, we measured the intensity of silencing responses raised against the *pos-1*, *dpy-11*, and *glp-1* genes, by exposing worms to different dilutions of dsRNA-producing bacteria. Silencing of *pos-1* and *glp-1*, using different dilutions of dsRNA, was used in the past as an assay for assessing germline Eri phenotypes [48]. These assays revealed that *met-2* animals are hypersensitive to RNAi (Figures 6C and 6D) and moreover that the Eri phenotype of *met-2* animals was at least as strong as the Eri phenotype of another known Eri gene, *rrf-3* (Figures 6C and 6E). The *met-2;set-25* double mutants also exhibited an Eri phenotype, however, which was weaker than the Eri phenotype exhibited by *met-2* mutants (Figure 6F). Silencing of *pos-1* occurs in the cytoplasm [49], and we found that *nrde-2* mutants (lacking nuclear RNAi) silence *pos-1* more efficiently than wild-type worms (Figure 6E). Thus, the enhanced silencing of *pos-1* in *met-2* animals (Figure 6E) indicates that in these mutants cytoplasmatic, non-NRDE-dependent silencing, is also potentiated. Enhancement of cytoplasmic RNAi should not dependent on changes to the chromatin of the targeted gene. Together, our results suggest that *met-2* mutants enhance heritable silencing responses indirectly, due to systemic hyperactivation of the exogenous small RNA machinery.

DISCUSSION

We showed that H3K9 methyltransferases produce heritable epigenetic effects by controlling small RNA production and specifically that MET-2 is required for termination of heritable RNAi responses. MET-2 appears to affect heritable RNAi indirectly, not through its influence on histone modifications on the targeted locus, but via its overall potentiation of the RNAi system. We detected global and drastic changes in endo-siRNAs in *met-2* mutants, and it is possible that these worms prolong RNAi inheritance responses because of the disturbance in the global landscape of euchromatic and heterochromatic marks, affecting the balance between different small RNA pathways, which makes more resources (HRDE-1, for instance) available for inheritance of exo-siRNAs. These global changes in the levels of heritable endogenous small RNAs could also explain the Mrt phenotype of these animals. Indeed, we found that both the sterility and the stable RNAi inheritance responses that were observed in *met-2* mutants are completely reversed upon *hrde-1* knockout.

The induction of H3K9me3 by nuclear-acting small RNAs has been observed in a number of different organisms, such as *Arabidopsis thaliana* and *Schizosaccharomyces pombe* [22]. In these organisms, a self-reinforcing epigenetic feedforward loop was described, where nuclear small RNAs direct H3K9me3 deposition at peri-centromeric regions. In turn, the nuclear small RNAs are synthesized in response to the presence of H3K9me3 at these loci [22]. Importantly, in *A. thaliana* and *S. pombe*, mutants lacking H3K9me3 methyltransferases or H3K9me3 binding proteins are depleted of peri-centromeric small RNAs, and mutants that lack small RNAs are depleted of peri-centromeric H3K9me3 [22]. In contrast, while an interaction between Piwi-interacting RNAs (piRNAs) and H3K9me3 exists also in *Drosophila melanogaster*, it was recently shown that transposons that are regulated by piRNAs can escape silencing, even when the H3K9me3 signal on the transposons is intact [50].

Is H3K9me3 required for small RNA inheritance in worms? We observed that while dsRNA induces anti-*gfp* small RNA biogenesis in P0 *set-25* mutant worms, small RNAs are not inherited to the F3 progeny. Although RNAi inheritance is weakened significantly in *set-25* mutants, weak silencing effects are nevertheless detected (Figure 2C). Since in *set-25* mutants we cannot detect heritable small RNAs or H3K9me3, it would be fascinating in the future to study how this weak inherited silencing is enforced. We discovered that *set-25* animals produce very strong heritable silencing responses (stronger than wild-type) when the endogenous *oma-1* gene was targeted by dsRNA. Thus, SET-25-dependent H3K9me3 might differentially affect the inheritance of RNAi responses against different targets. While 3,846 STGs that target endogenous genes are differentially expressed in *met-2* mutants, only 279 STGs that target endogenous genes are differentially expressed in *set-25* mutants. It is possible that SET-25 preferentially affects foreign genes, such as transgenes. Even when transgenes are concerned, the *set-25* gene is not essential for RNAi inheritance, since *set-25;met-2* double mutants exhibit enhanced RNAi inheritance responses.

It was previously suggested that *met-2*, similarly to *spr-5*, is required for chromatin germline reprogramming [14]. Our results suggest that *met-2* is also required for reprogramming of heritable small RNAs. It is thus possible that MET-2 is part of a dedicated protein machinery that ensures that heritable small RNAs

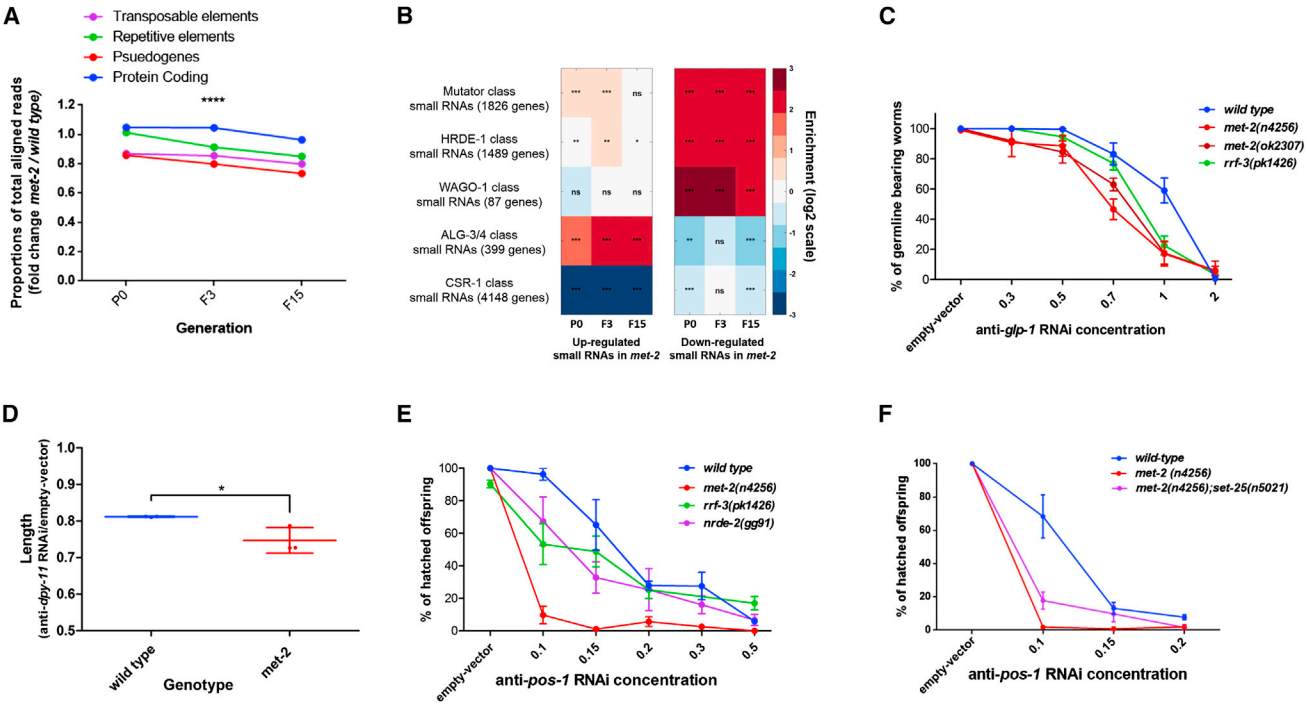

Figure 6. *met-2* Mutants Have Lower Levels of Different Types of Endogenous Small RNAs and Exhibit an Enhanced RNAi Phenotype

(A) Analysis of the proportions of different endogenous small RNA species in *met-2* mutants. Fold changes in the proportions of particular small RNA sub-classes in *met-2* mutants compared to the wild-type are presented. Proportions were calculated out of the number of all aligned reads. A chi-square test was conducted on each biological repeat for three biological repeats in every generation. ****$p < 0.0001$.

(B) An enrichment analysis of STGs associated with particular small RNA pathways. Small RNAs associated with particular pathways (Mutator class, HRDE-1, WAGO-1, ALG-3/4, and CSR-1) were examined for their enrichment among differentially expressed STGs in *met-2* mutants compared to the wild-type, across generations (*$p < 0.5$, **$p < 0.01$, ***$p < 0.001$; see the Experimental Procedures).

(C) *glp-1* RNAi sensitivity assay. Wild-type animals and *met-2* and *rrf-3* mutants were tested for their response to increasing concentrations of bacteria producing anti-*glp-1* dsRNA, which inhibits germline proliferation. The percentage of germline-bearing animals is indicated (mean ± SEM). Three biological repeats ($n > 60$) were performed. The Eri phenotype of two different null alleles of *met-2* was examined. Significantly enhanced responses to anti-*glp-1* dsRNA (compared to the wild-type) are exhibited in both *met*-2 alleles, in dsRNA concentrations of 0.7 ($p < 0.0001$ for n4256, $p < 0.05$ for ok2307) and 1 ($p < 0.0001$ for both alleles), Dunnett's multiple comparisons test.

(D) *dpy-11* RNAi assay. Wild-type animals and *met-2* mutants were grown on bacteria producing anti-*dpy-11* dsRNA, which affects the worm's body size and empty vector control plates. The worms' length was measured using the Worm-Sizer software [41]. The normalized averages worm length (RNAi-treated worms versus controls) is presented per biological repeat (mean ± SD). Three biological repeats ($n > 100$) were performed.

(E) *pos-1* RNAi sensitivity assay examining the potency of the cytoplasmic RNAi pathway. Wild-type animals and *met-2*, *nrde-2*, and *rrf-3* mutants were tested for their response to increasing concentrations of bacteria producing anti-*pos-1* dsRNA, which causes embryonic lethality. The percentages of offspring hatching per worm are indicated (mean ± SEM, $n = 6$ per concentration). Significantly enhanced responses to anti-*glp-1* dsRNA (compared to the wild-type) are exhibited in dsRNA concentrations of 0.1 and 0.15 in *met-2* ($p < 0.0001$) and *nrde-2* ($p < 0.005$) and of 0.1 in *rrf-3* ($p < 0.0001$), Dunnett's multiple comparisons test.

(F) *pos-1* RNAi sensitivity assay examining the potency of the cytoplasmic RNAi pathway in *met-2* and *met-2;set-25* mutants. The percentages of offspring hatching per worm are indicated (mean ± SEM, $n = 6$ per concentration). Significantly enhanced responses to anti-*glp-1* dsRNA (compared to the wild-type) are exhibited in dsRNA concentration of 0.1 in *met-2* ($p < 0.001$) and *met-2;set-25* ($p < 0.0001$). *met-2* exhibit enhanced response compared to *met-2;set-25* ($p = 0.029$), Dunnett's multiple comparisons test.

would be erased after a number of generations, to limit inheritance of acquired traits.

EXPERIMENTAL PROCEDURES

Cultivation of the Worms

All of the experiments were performed at 20°C, except for maintenance of the *oma-1* strains, which was done at 15°C. Before RNAi, standard culture techniques were used to maintain the nematodes on nematode growth medium (NGM) plates seeded with OP50 bacteria, and HT115 bacteria that express dsRNAs were used for RNAi induction, as previously described [51]. Extreme care was taken to avoid contamination or starvation, and contaminated plates were discarded from the analysis.

C. elegans Strains

C. elegans strains employed in this work are as follows: wild-type Bristol N2 strain; EG6089, *unc-119(ed3) III*, *oxTi38* (*Cbr-unc-119(+) Ppie-1::gfp*); TX20, *oma-1 (zu405)*; NL2099: *rrf-3 (pk1426) II*; YY186, *nrde-2 (gg91) II*; GW638, *set-25(n5021) III;met-2(n4256) III*; RB1789, *met-2(ok2307) III*; MT13293, *met-2 (n4256) III*; MT17463, *set-25 III*; SX1263, *mjIs134 II (Pmex-5::gfp::h2b::tbb-2)*; BFF9, *met-2(n4256);hrde-1(pig1);SX1263*; BFF10, *met-2(n4256);hrde-1(pig2); SX1263*; and BFF11, *met-2(n4256);hrde-1(pig3);EG6089*.

RNAi Inheritance Assay

RNAi HT115 bacteria were inoculated into Lysogeny broth (LB) containing Carbenicillin (25 μg/mL) at 37°C overnight with shaking. Bacterial cultures were seeded onto NGM plates containing isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM) and Carbenicillin (25 μg/mL) and grown overnight in the dark

at room temperature. Five L4 animals were placed on RNAi bacteria plates and control empty-vector bearing HT115 bacteria plates and maintained at 20°C for 2 days before being removed. The offspring hatching on these plates was termed the P0 generation. For creation of the F1 generation, five P0 L4 animals were transferred to NGM plates seeded with OP50 bacteria. The next generations were transferred in the same fashion.

ACCESSION NUMBERS

The accession number for the raw sequencing files and the processed data reported in this paper is NCBI GEO: GSE94798.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.03.008.

AUTHOR CONTRIBUTIONS

I.L., U.S., and O.R. conceived and designed the experiments; I.L., U.S., S.A., and R.B. performed the experiments; H.G., U.S., and I.L. performed data analysis; and I.L., U.S., and O.R. wrote the paper.

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