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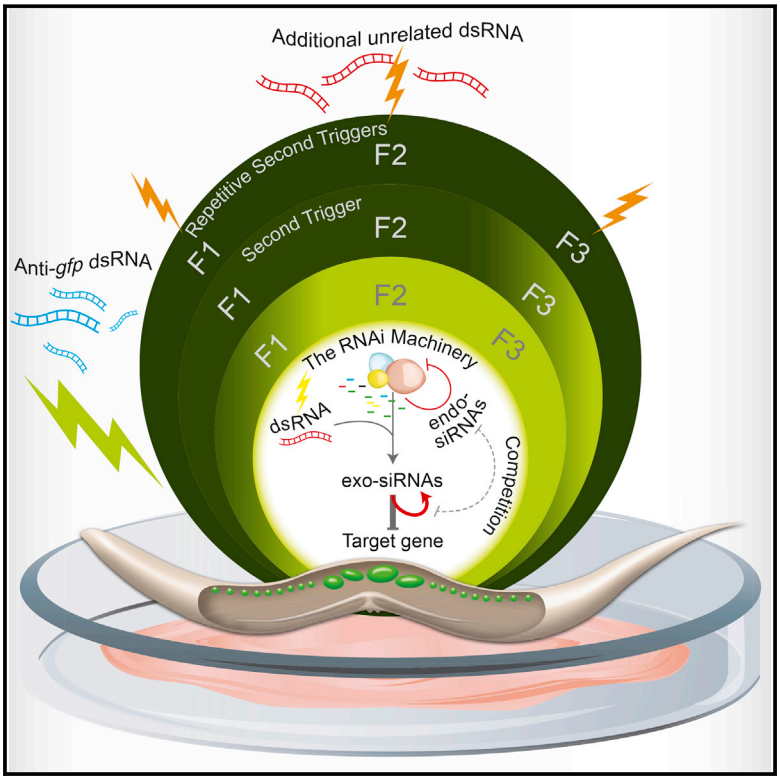
A Tunable Mechanism Determines the Duration of the Transgenerational Small RNA Inheritance in *C. elegans*

Graphical Abstract



Highlights

- New RNAi episodes extend the duration of heritable epigenetic effects
- Amplification of heritable exo-siRNAs occurs at the expense of endo-siRNAs
- A feedback between siRNAs and RNAi genes determines heritable silencing duration
- Modified transgenerational epigenetic kinetics (MOTEK) mutants are identified

Authors

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In Brief

The duration of epigenetic responses underpinning transgenerational inheritance is determined by an active mechanism relying on the production of small RNAs and modulation of RNAi factors, dictating whether ancestral RNAi responses would be memorized or forgotten.

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A Tunable Mechanism Determines the Duration of the Transgenerational Small RNA Inheritance in *C. elegans*

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SUMMARY

In *C. elegans*, small RNAs enable transmission of epigenetic responses across multiple generations. While RNAi inheritance mechanisms that enable "memorization" of ancestral responses are being elucidated, the mechanisms that determine the duration of inherited silencing and the ability to forget the inherited epigenetic effects are not known. We now show that exposure to dsRNA activates a feedback loop whereby gene-specific RNAi responses dictate the transgenerational duration of RNAi responses mounted against unrelated genes, elicited separately in previous generations. RNA-sequencing analysis reveals that, aside from silencing of genes with complementary sequences, dsRNA-induced RNAi affects the production of heritable endogenous small RNAs, which regulate the expression of RNAi factors. Manipulating genes in this feedback pathway changes the duration of heritable silencing. Such active control of transgenerational effects could be adaptive, since ancestral responses would be detrimental if the environments of the progeny and the ancestors were different.

INTRODUCTION

Epigenetic responses are dynamic and, in most cases, short lived (Anava et al., 2015). In recent years, it became clear that in different organisms dedicated mechanisms enable some epigenetic effects to transfer across multiple generations (Weigel and Colot, 2012). Specific transgenerational responses are maintained despite the reprograming of the germline, which is a prerequisite for development (Heard and Martienssen, 2014). Still, the conditions that dictate which particular inherited epigenetic "memories" would be retained remain unknown (Crews et al., 2014; Jablonka and Lamb, 2008).

In parallel to the discovery of double-strand RNA (dsRNA)-induced RNAi in *Caenorhabditis elegans* nematodes, it was found that silencing spreads across the worm's tissues, and even from the soma to the germline (Fire et al., 1998). Moreover, it was later shown that in certain cases RNAi responses could last for multiple generations (Vastenhouw et al., 2006).

Both exogenously derived small interfering RNAs (exo-siRNAs) and endogenous small RNAs such as endo-siRNAs and PIWI-interacting small RNAs (piRNAs) can trigger heritable RNAi (Anava et al., 2015). Heritable RNAi responses establish immunity against genomic parasites (Ashe et al., 2012; Luteijn et al., 2012; Rechavi et al., 2011; Shirayama et al., 2012) and are affected by starvation (Koonin, 2014; Rechavi et al., 2014) and cultivation in high temperatures (Schott et al., 2014). The exogenous and endogenous siRNA pathways compete over common resources such as over the activity of the sole Dicer protein, DCR-1, which is essential for the production of exo-siRNAs and microRNAs, and certain endo-siRNAs (Duchaine et al., 2006; Sarkies et al., 2013; Wu et al., 2011; Zhuang and Hunter, 2012). As a consequence, mutants that are defective in the production of endo-siRNAs are hypersensitive for exogenous RNAi, and exo-siRNA mutants produce more endo-siRNAs (Zhuang and Hunter, 2012).

In *C. elegans*, amplification of the original dsRNA-induced reaction by RNA-dependent RNA polymerases (RdRPs) is required for potent, full-blown RNAi responses (both exogenous and endogenous) (Aoki et al., 2007; Gent et al., 2010; Smardon et al., 2000; Vasale et al., 2010), and for inheritance of silencing (Gu et al., 2012; Rechavi et al., 2011; Sapetschnig et al., 2015). "Primary" small RNAs of different sources—such as 21U piRNAs, small RNAs that are produced from exogenously supplied dsRNA, or 26G endogenous small RNAs (endo-siRNAs) (Billi et al., 2014)—trigger the production of much more abundant "secondary" small RNAs, which are mostly 22G endo-siRNAs (Billi et al., 2014). Primary small RNAs can guide the synthesis of secondary small RNAs by recruiting RdRPs to their target mRNAs, which serve as templates for the production of the secondary endo-siRNAs (Maniar and Fire, 2011).

Secondary small RNAs associate with multiple argonautes (the *C. elegans* genome encodes for 27 argonautes) (Yigit et al., 2006) are shuttled from the cytoplasm to the nucleus and regulate target genes mostly through cooperation with nuclear acting RNAi factors (e.g., Nuclear RNAi Deficient genes, NRDE genes). Nuclear small RNAs regulate transcription by recruiting chromatin-modifying factors to cognate nascent RNA transcripts (Buckley et al., 2012; Guang et al., 2008). Changing chromatin modifications (either by nuclear RNAi or

by manipulation of chromatin modifiers) also produces heritable effects (Gaydos et al., 2014; Greer et al., 2011, 2015; Gu et al., 2012; Kelly, 2014). Two nuclear argonaute proteins, HRDE-1 (heritable RNAi deficient-1) and CSR-1 (chromosome segregation and RNAi deficient-1), carry heritable small RNAs in the germline and are required specifically for RNAi inheritance (Ashe et al., 2012; Buckley et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). Small RNA binding to CSR-1 promotes expression of cognate genes and counteracts the heritable silencing effects of HRDE-1. CSR-1 and HRDE-1 associate with different populations of endo-siRNAs; however, the two proteins also compete over binding to similar small RNA molecules through an unknown mechanism (Cecere et al., 2014; Seth et al., 2013; Tu et al., 2015; Wedeles et al., 2013).

Injecting, feeding, or soaking worms in dsRNA that corresponds to certain genes (mostly germline-expressed genes) triggers RNAi responses that are heritable, but in most cases the effect is terminated after one to four generations (Alcazar et al., 2008). The degree of heritable silencing effects varies in populations of isogenic worms (Vastenhouw et al., 2006). While in every generation some worms lose heritable silencing, continuous selection of worms that exhibit silencing enables the propagation of long-lasting responses that can persist for more than 80 generations (Vastenhouw et al., 2006). Low concentrations of dsRNA trigger can limit the transgenerational duration of RNAi inheritance effects, but even under high dsRNA concentrations a sharp reduction in silencing (a "bottleneck") is observed in the transition between the F3 and F4 generations (Alcazar et al., 2008). It has been suggested that the reduction in silencing over the course of generations occurs because some RNA agent, which is required for RNAi inheritance, is diluted in every generation, until it reaches levels that are too low to allow efficient gene silencing (Alcazar et al., 2008). However, passive dilution of a limited RNA agent cannot explain the dynamics of RNAi inheritance decay. Every *C. elegans* nematode produces ~250 eggs, and therefore the overwhelming dilution factor (~3.906 billion after four generations) could not permit transgenerational responses. While it is unclear why epigenetic responses peter out at a certain rate, it is also unknown why RNAi inheritance ever decays, instead of being perpetuated indefinitely. A feedforward reaction was shown to allow amplified "secondary" small RNAs to guide additional rounds of amplification, which lead to stable silencing of certain silencing responses that are established in the germline by piRNAs (Sapetschnig et al., 2015).

We describe here a tunable feedback system that times the duration of heritable RNAi effects, effectively dictating whether ancestral RNAi responses would be memorized or forgotten.

RESULTS

Upon analysis of published RNA sequencing (RNA-seq) data, we noticed that abundant endo-siRNAs (fold enrichment = 4.9, $p < 7.800e-23$ [Maniar and Fire, 2011]), and specifically heritable endo-siRNAs (fold enrichment = 4.7, $p < 8.851e-85$ [Claycomb et al., 2009]), align in the antisense orientation to multiple endo-siRNA biogenesis genes. We therefore hypothesized that a feedback exists between heritable small RNAs and regulated RNAi inheritance and biogenesis genes. If exogenous RNAi responses could activate this hypothetical transgenerational feedback, then RNAi could affect the duration of the heritable silencing by switching OFF the RNAi inheritance machinery in the progeny. In theory, initiation of new exogenous RNAi responses could again turn the same system ON.

To test this hypothesis, we examined whether there is an interaction between distinct inherited RNAi responses, aimed against different and unrelated genes, when the separate dsRNA triggers are administered at different time points along a worm's ancestry (see the scheme in Figure 1A). We first used as a target for RNAi a green fluorescent protein (GFP), which is expressed in the germline (under the control of the *pie-1* promoter) off an integrated, single-copy transgene. As expected, feeding these worms on bacteria that produce anti-*gfp* dsRNA induced silencing of *gfp* in the treated worms as well as in the progeny (Figures 1B and 1C). Consistent with the previously reported "bottleneck" to transgenerational RNAi, the inherited RNAi effect dissolved after approximately four generations. In the second stage, after the parents were treated with anti-*gfp* dsRNA, the progeny was transferred to plates with bacteria that expressed a control empty vector, or vectors that encode for different dsRNA triggers (hereon referred to as "second dsRNA triggers"). Surprisingly, progeny that was exposed to the different "second dsRNA triggers" exhibited much stronger inherited GFP silencing than progeny that was exposed to the empty vector, even though no additional anti-*gfp* dsRNA triggers were added (Figures 1C and S1A). Exposure to the "second dsRNA triggers" on its own did not affect GFP levels (Figure S1B). Thus, an RNAi response that targets a particular gene can extend the duration of an ancestral heritable RNAi response, aimed against a different and unrelated gene.

Extension of the transgenerational duration of GFP silencing was achieved both by "second dsRNA triggers," which targeted somatically expressed genes (e.g., *dpy-2*), and "second dsRNA triggers," which targeted germline-expressed genes (e.g., *Pdpy-30::mcherry*). To examine the generality of the effect, we compared 11 different dsRNA "second triggers," which target genes that function in different cellular processes, and that on their own do not affect GFP levels. We found that all these "second triggers" extended the transgenerational duration of ancestral heritable silencing responses aimed against *gfp* (see Figure S2).

To examine whether continuous activation of the RNAi machinery by dsRNA administration would perpetuate the ancestral anti-*gfp* response, we performed experiments in which the progeny was challenged with the "second dsRNA trigger" in every consecutive generation. We observed that consecutive anti-*dpy-2* dsRNA "second triggers" strongly prolonged and enhanced the transgenerational silencing of GFP for additional generations (Figure 1C). Therefore, applying consecutive dsRNA triggers of RNAi can continually counteract the termination of separate ancestral RNAi responses. The results of these experiments reject the possibility that termination of heritable silencing occurs solely due to the dilution of the original inherited agent and support an alternative hypothesis: that a systemic property of the organism (perhaps the activation state of the RNAi system) determines whether an RNAi response would persist or terminate.

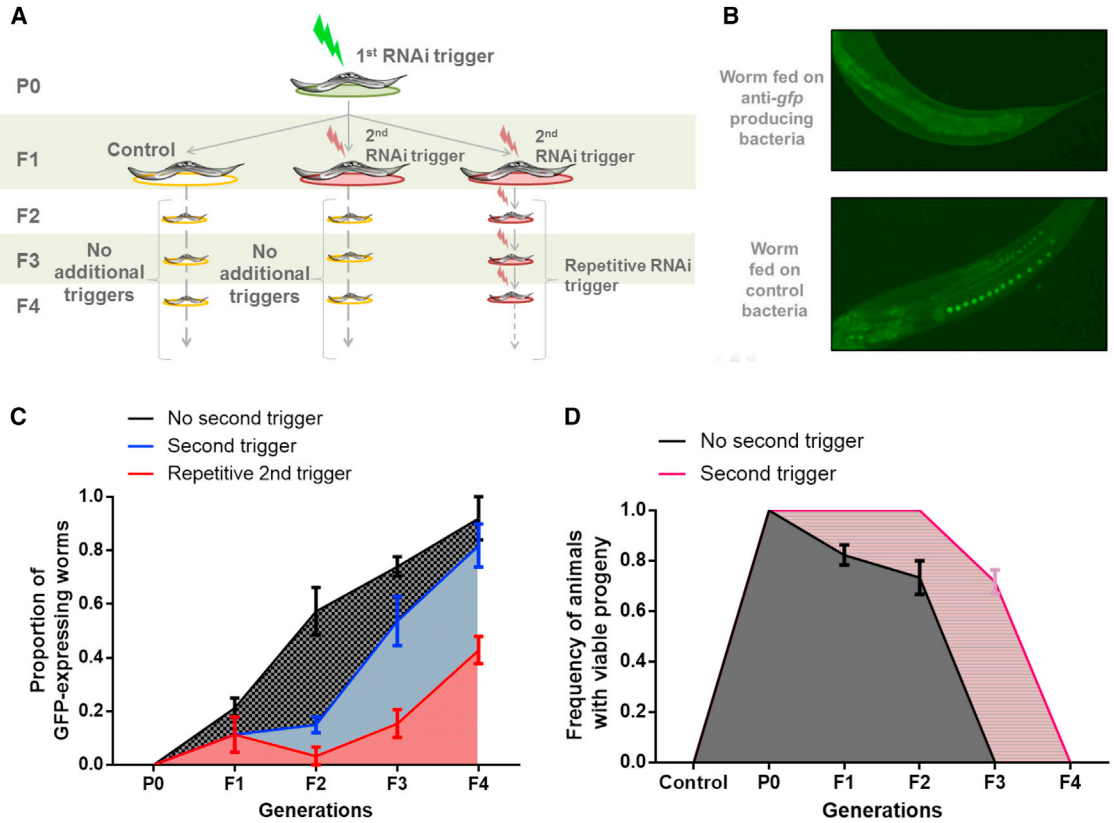

Figure 1. Extension of Heritable RNAi Responses by Recurrent Exposure to dsRNA Triggers

(A) A general scheme for the RNAi inheritance experiments: after the exposure to the first dsRNA trigger (anti-*gfp* or anti-*oma-1*), the progeny is transferred either to control plates (with bacteria that contain an empty expression vector) or to plates with bacteria that transcribe an unrelated dsRNA trigger ("second trigger"). At the F2 generation, the "second trigger"-treated worms are either transferred to control plates or moved again to plates that contain bacteria that produce dsRNA ("repetitive second trigger"). Thus, we also examined worms that were consistently exposed to "second triggers." The heritable silencing in response to the original dsRNA trigger is scored in each generation ($n > 50$, see Experimental Procedures).

(B) RNAi silences germline-expressed GFP: an example of (1) GFP silencing following exposure to bacteria that transcribe anti-*gfp* dsRNA and (2) fully expressed GFP in worms that were fed with control bacteria ("empty vector"). The worms contain a single-copy integrated *Ppie-1::gfp::H2B* transgene, which drives GFP expression in germ cells' nuclei.

(C) Extension of inherited GFP silencing by "second triggers": extension of the heritable silencing effects following the introduction of a "second dsRNA" (anti-*dpy-2* dsRNA) trigger, introduced at the F1 generation, and the extension of heritable silencing by consecutive exposure to the "second trigger" ("repetitive second triggers"). The proportion of worms that exhibit silencing is scored in each generation (see Experimental Procedures). Data are represented as mean ± SEM.

(D) Extension of inherited OMA-1 silencing by "second triggers": the duration of inherited OMA-1 silencing following RNAi was quantified by scoring the number of worms that lay more than five viable progeny in each generation, as previously described (Alcazar et al., 2008).

*Experiments in which a "second dsRNA trigger" extended the heritable silencing of a previously initiated silencing response were repeated more than 20 times and were conducted by more than five different students.

See also the related Figures S1, S2, and S3.

The timing of the exposure to the "second trigger" is important: when the ancestral anti-*gfp* RNAi response was separated from the "second dsRNA trigger" by more than one generation, the "second dsRNA trigger" lost its ability to extend the duration of the original anti-*gfp* RNAi response (Figure S3A). When the "second trigger" proceeded by one generation, the exposure to anti-*gfp* dsRNA (*mcherry* dsRNA administered to the P-1 generation), inheritance of anti-*gfp* silencing was enhanced. However, "second triggers" that were administered to the F1 generation were more efficient (Figure S3B). Thus, consecutive "second triggers" affect RNAi inheritance, and there is a "*critical period*," one generation after the original trigger is administered, during which "second triggers" are most effective in extending previously initiated, inherited RNAi responses.

As *gfp* is a foreign gene, we next conducted similar experiments in which we examined the inheritance of an RNAi response aimed against an endogenous gene, using as target the temperature-sensitive dominant lethal allele of the redundant germline expressed gene, *oma-1* (as previously described, Alcazar et al., 2008). In this system, only the eggs of worms that inherit anti-*oma-1* RNAi develop in the restrictive temperatures. The transgenerational duration of the RNAi response aimed against *oma-1*, similar to the heritable RNAi responses that were aimed against the *gfp* transgene, was dramatically

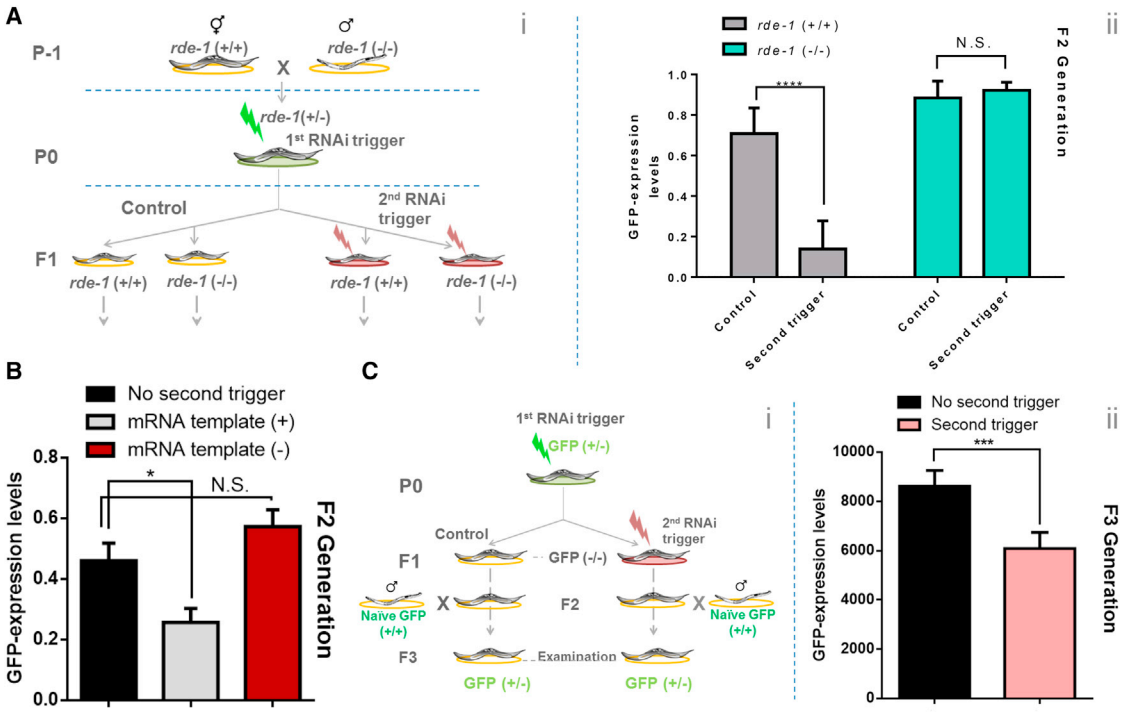

Figure 2. The Requirements for Enhancement of Ancestral RNAi Responses by "Second Triggers"

(A) Examining whether the ability of the "second trigger" to enhance ancestral RNAi responses depends on processing of the dsRNA that serves as a "second trigger". By crossing, we manipulated the statues of *rde*-1 in the generation that was exposed to the "second trigger," to examine whether RDE-1's activity (removal of the passenger strand from the dsRNA) is required for "second triggers" to effectively enhance the duration of ancestral anti *gfp* silencing responses. The genotypes of the worms were verified using PCR; wild-type (WT) and homozygous mutants were scored for GFP expression. (i) Scheme of the cross, which also specifies when exposure to the dsRNA triggers took place. (ii) Experimental results (GFP levels were measured to track heritable silencing, mean ± SEM).

(B) Examining whether the ability of the "second trigger" to enhance ancestral RNAi responses depends on the presence of an mRNA template. RdRPs require an mRNA template in order to transcribe "secondary small RNAs." Animals without the *mcherry* gene in the genome were exposed in the F1 generation either to an anti-*mcherry* "second trigger" ("mRNA template [–]") or to an anti-*dpy-2* "second trigger" ("mRNA template [+]"). Shown are GFP levels as measured in the F2 generation, which are indicative to the intensity of heritable silencing (mean ± SEM).

(C) Examining whether the action of the "second trigger" depends on changes to the GFP genomic locus. The original *gfp* locus that was present when the "first trigger" (dsRNA that targets *gfp*) was administered was crossed out. The "second trigger" was administered when no *gfp* locus was present in the genome. In the next generation, a new *gfp* locus was crossed in (a "naïve" *gfp*), and GFP silencing was scored at the F3 generation (reduction in GFP levels is indicative of heritable silencing). (i) Scheme of the cross and the exposure to the different dsRNA triggers. The genotypes of the worms were verified using PCR. (ii) Experimental results (GFP levels were measured to track heritable silencing, mean ± SEM).

prolonged when the next generation was treated with a "second trigger" consisting of *mcherry* dsRNA (see Figure 1D).

Synthesis of dsRNA is required for replication of RNA viruses and transposons, and therefore dsRNA constitutes a "danger signal" in many organisms, including humans, where it activates the interferon response (Wang et al., 2002). As in worms, RNAi is important for anti-viral defense (Lu et al., 2005); it is possible that the mere "sensing" of dsRNA (for example, by pattern recognition mechanisms [Melo and Ruvkun, 2012]) is sufficient to activate the RNAi system, regardless of whether the dsRNA molecule is further processed to trigger an RNAi response or not. To examine whether a "second trigger" has to trigger a full-blown RNAi response to extend ancestral RNAi responses, we tested whether a "second trigger" could prolong inherited responses in *rde-1* mutants. RDE-1 removes the passenger strand from the dsRNA precursor and is therefore required for the first step in RNAi responses, the production of primary siRNAs (Steiner et al., 2009). RDE-1 is required for initiation of RNAi in the parents but not for the inheritance of the response to the progeny (Grishok et al., 2000). We challenged GFP-expressing *rde-1* heterozygous mutant animals with anti-*gfp* dsRNA ("first trigger") and next administrated their *rde-1* homozygous mutant progeny with anti-*dpy-2* dsRNA (the "second trigger"). We found that the "second trigger" that was presented to F1 *rde-1* homozygous mutants did not extend the transgenerational duration of the ancestral anti-*gfp* RNAi response (Figure 2A). Thus, the production of "primary siRNAs" is required for efficient extension of ancestral RNAi by "second dsRNA triggers."

We next tested whether amplification of "secondary siRNAs" is required for the establishment of a potent "second trigger" (that effectively extends ancestral responses). Since amplification of secondary siRNAs requires an mRNA template, we examined whether dsRNA against *mcherry* could re-initiate the transgenerational RNAi effect of anti-*gfp* RNAi, in animals that do not possess the *mcherry* gene in their genome. We found that administration of dsRNA aimed against *mcherry* in *mcherry*(–)

animals did not extend heritable silencing of ancestral anti-*gfp* RNAi (Figure 2B). These results indicate that a full-blown RNAi response is required for ''second triggers'' to strongly extend transgenerational inheritance of past RNAi responses.

We noticed that in all our experiments anti-*dpy-2* ''second dsRNA triggers'' were more potent than anti-*mcherry* ''second dsRNA triggers'' in enhancing the duration of ancestral RNAi responses. We extended this observation by comparing the potency of multiple ''second dsRNA triggers'' and detected a very replicable difference in the degree to which exposure to each ''second trigger'' enhanced ancestral silencing (while all the ''second dsRNA triggers'' were effective, targeting certain genes produced an especially strong effect) (see Figure S2). Thus, the identity of the mRNA that particular ''second triggers'' silence changes the intensity of the induced effect.

RNAi responses in *C. elegans* can be inherited transgenerationally even in the absence of the DNA locus that encodes for the targeted mRNA (Grishok et al., 2000; Rechavi et al., 2011; Sapetschnig et al., 2015). To understand whether the ''second dsRNA trigger'' enhances ancestral heritable RNAi responses by affecting the genomic locus of the gene that was originally targeted, we tested whether the ability of an anti-*dpy-2* dsRNA ''second trigger'' to extend the inheritance of an ancestral anti-*gfp* RNAi response depends on the presence of the DNA locus that encodes for the GFP protein. One generation after we subjected the worms to anti-*gfp* RNAi, we crossed out the targeted *gfp* allele and challenged the worms with a second dsRNA, which corresponded to the *dpy-2* gene (see scheme in Figure 2C). We next crossed in an identical ''naïve'' *gfp* allele and examined whether the original RNAi response against *gfp* was extended. Silencing of the newly introduced ''naïve'' *gfp* allele was significantly stronger in lineages that were exposed in the past to the second dsRNA trigger. Therefore, the extension of ancestral silencing responses through administration of ''second dsRNA triggers'' does not depend on changes in the chromatin of the gene that was originally targeted (Figure 2C).

To examine whether the ''second trigger'' leads directly to amplification of heritable anti-*gfp* small RNAs, we sequenced small RNAs from lineages of worms that were exposed to anti-*gfp* RNAi, and from lineages of worms that were exposed in addition to an anti-*mcherry* ''second trigger'' (all the sequencing experiments were done in triplicates). Typical of exogenous RNAi responses, anti-*gfp* dsRNA triggered the production of both sense and antisense ''primary'' small RNAs, which are mostly 23 nt long, and also the production of much more abundant ''secondary'' small RNAs, which are mostly 22Gs, and align exclusively in the antisense orientation to exons of the *gfp* gene (Figure 3A). The number of primary anti-*gfp* small RNAs, as estimated by the number of small RNAs that align to *gfp* in the sense orientation, decreased sharply in the progeny of the anti-*gfp* dsRNA-treated worms. Practically no primary small RNAs that align to *gfp* in the sense orientation can be found in F1 worms (Figure 3A). Similarly to the reduction that was observed in the number of primary small RNAs, the number of secondary 22G anti-*gfp* small RNAs also decreased as generations passed. However, the decrease in secondary small RNAs was gradual, and significant levels of heritable 22Gs were found after the F1 generations (Figure 3A). In agreement with the phenotypic results (extension of heritable silencing of GFP), challenging the F1 worms with the anti-*mcherry* dsRNA ''second trigger'' led to a highly significant ''boost'' ($\sim$1.5-fold $p < 0.0001$, Figure 3A) in the number of heritable secondary anti-*gfp* small RNAs in the F2 and F3 generation (Figure 3A). The ability of anti-*mcherry* dsRNAs to induce amplification of heritable anti-*gfp* small RNAs indicates that dsRNA that targets specific genes can affect the overall functionality of the RNAi system. Explicitly, these findings show that a specific dsRNA trigger can lead to the amplification of other small RNAs.

According to the current model, exogenously triggered RNAi responses produce siRNAs, such as anti-*gfp* siRNAs, that are carried over in the germline by HRDE-1, and not by CSR-1 (the other argonaute that carries small RNAs across generations). Since this hypothesis was never formally tested, we examined whether anti-*gfp* siRNAs display the molecular signatures that characterize HRDE-1 or CSR-1-bound siRNAs. Untemplated poly-uridine ''tails'' are added to siRNAs that bind CSR-1, by CSR-1's binding partner, the nucleotidyltransferase CDE-1 (van Wolfswinkel et al., 2009). We thus tested whether the 3′ ends of the anti-*gfp* siRNAs undergo untemplated poly-uridylation. The analysis shows that throughout the heritable response, and also in response to ''second trigger'' exposure, anti-*gfp* siRNAs are completely devoid of Poly-Us (Figure S4), characteristically to HRDE-1-bound siRNAs (de Albuquerque et al., 2015).

Production of exo-siRNAs affects, and is effected by, the production of endogenous small RNA molecules (Zhuang and Hunter, 2012). A model that supports a competition between the exo and endo RNAi pathways is supported by three main findings: (1) endo-siRNA mutants are hypersensitive to exogenous RNAi; (2) in endo-siRNA mutants and in animals that are exposed to dsRNA, genes that are normally silenced by microRNAs are overexpressed; (3) overexpression of DCR-1, a limiting RNAi factor needed for both microRNAs, endo-siRNAs and exo-siRNAs biogenesis, sensitizes the worm for dsRNA-induced RNAi (Zhuang and Hunter, 2012).

As described above, analysis of published databases revealed that endo-siRNA biogenesis genes are regulated by endo-siRNAs and specifically by heritable endo-siRNAs (Claycomb et al., 2009; Maniar and Fire, 2011). We examined whether the levels of endo-siRNAs that regulate endo-siRNA biogenesis genes are affected by exogenous RNAi responses. To find RNAi genes that are dynamically regulated in response to dsRNA-induced RNAi, we targeted by RNAi foreign genes (*gfp* and *mcherry*) that have no function in worms, to avoid compromising of physiological processes. We reasoned that if RNAi triggers a feedback response between endo-siRNAs and regulated RNAi inheritance genes, then identification and manipulation of the genes at the heart of the feedback pathway could affect the duration of heritable silencing.

We parsed the small RNA pools that were sequenced from the samples of the different experimental conditions into specific small RNA families (see Supplemental Experimental Procedures) and found support for the ''competition model.'' Induction of RNAi by dsRNA administration changes the balance between different RNAi pathways: upon exposure to exogenous dsRNA, we observed a highly statistically significant downregulation in the proportion of several endogenous small RNA pathways

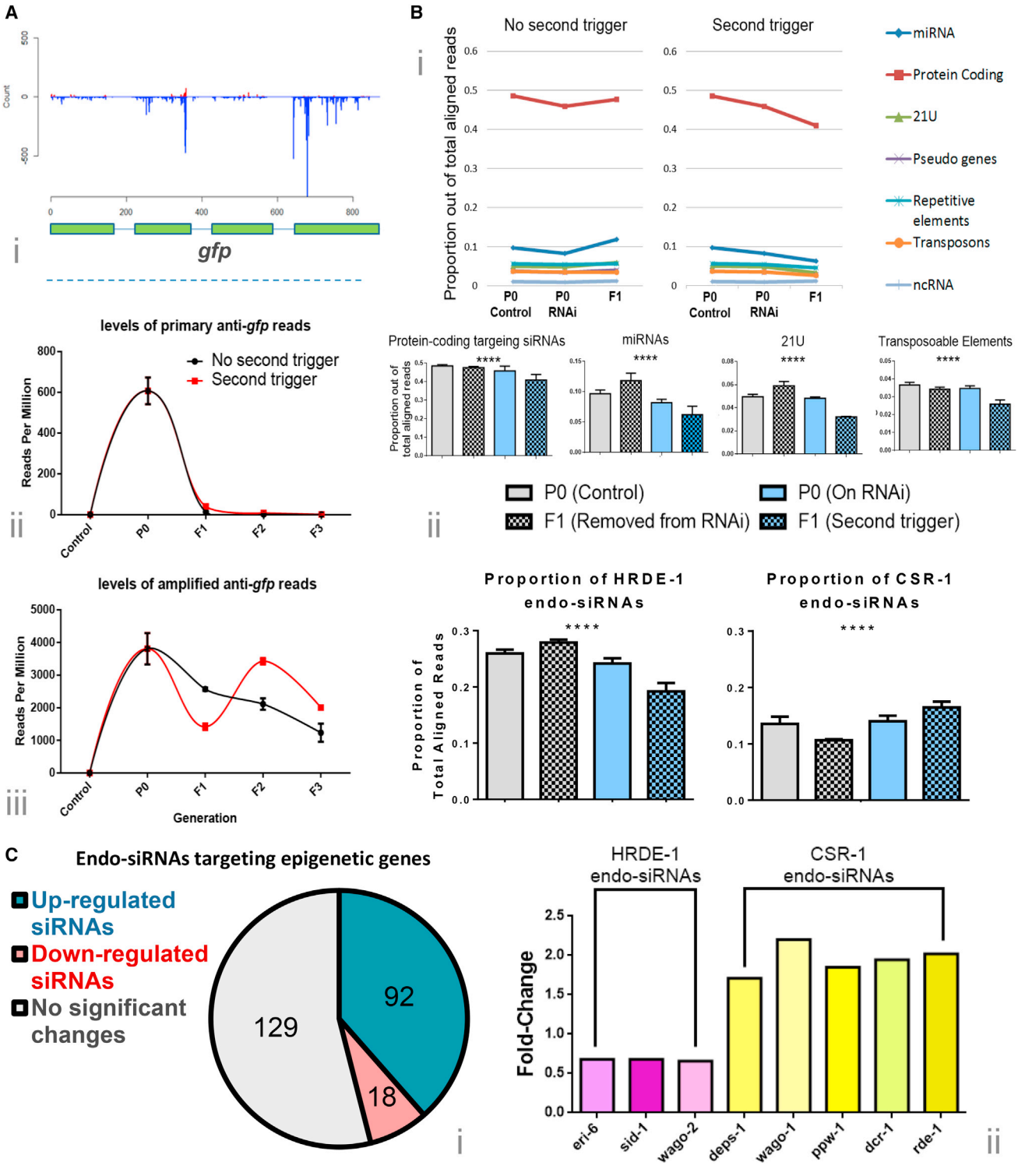

Figure 3. Changes in Anti-*gfp* Small RNAs Levels and in Endogenous Small RNA Levels over the Course of a Heritable RNAi Response

(A) Dynamic changes in anti-*gfp* small RNAs following RNAi and as a response to administration of a "second trigger": (i) The distribution of small RNA reads over the *gfp* gene following anti-*gfp* RNAi (P0 generation). Sense reads are shown in red; antisense reads are shown in blue. (ii) The dynamics of the primary response ("primary small RNAs) against *gfp* across generations, and the changes following administration of the "second trigger." Primary small RNAs were measured based on the levels of sense aligning small RNA reads. (iii) The dynamics of the "secondary" (RdRP-amplified) response against *gfp* across generations, and as a response to the "second trigger." Secondary small RNAs levels were measured based on the levels of anti-sense aligning small RNA reads (mean ± SEM).

(legend continued on next page)

(Figure 3B). Downregulation in microRNAs and endo-siRNAs levels was observed in the P0 worms that were exposed to anti-*gfp* RNAi (15% average decrease in microRNAs, $p < 0.0001$; 6% average decrease in endo-siRNAs that align to protein coding genes, $p < 0.0001$), and an even stronger downregulation was detected in the F1 worms that were repetitively targeted by the two different RNAi triggers (36% decrease in microRNAs, $p < 0.0001$; 16% decrease in endo-siRNAs that align to protein coding genes, $p < 0.0001$) (Figure 3B). A decrease in the levels of 21U was also observed in worms that were triggered with two different RNAi triggers (35%, $p < 0.0001$, Figure 3B).

Upon closer examination of the changes in endo-siRNAs following RNAi exposure (single or repetitive), we found that the downregulation in heritable endo-siRNAs levels stems from downregulation of endo-siRNAs, which were shown to bind HRDE-1 (90% of the differentially expressed HRDE-1-bound endo-siRNAs were downregulated, $p < 0.000e+00$, Figure 3B). In striking contrast, ~92% of the differentially expressed CSR-1 endo-siRNAs were upregulated upon RNAi exposure ($p < 0.000e+00$, Figure 3B).

The F1 progeny of the anti-*gfp*-treated parents (that were restored to plates with bacteria that do not produce dsRNA) displayed the exact reverse image to the changes seen upon RNAi exposure. Upon removal from RNAi, we observed an upregulation in the proportion of microRNAs and HRDE-1 endo-siRNAs and downregulation of CSR-1 endo-siRNAs (Figure 3B). We examined whether known HRDE-1 targets are 3′ poly-uridylated throughout the heritable response (and in response to the "second trigger"), which could indicate that these endo-siRNAs "shift" from HRDE-1 to CSR-1 binding. Such differential 3′ poly-uridylation was not observed in any experimental condition (Figure S4).

These changes in the endogenous small RNA pool are in line with the competition model and suggest that upon dsRNA-induced RNAi the RNAi system adopts a "state" that supports production of particular heritable small RNA species. Specifically, these experiments expose a dynamic "switch" that controls the balance between HRDE-1 and CSR-1 endo-siRNAs (see more in the Discussion).

Genes involved in epigenetic regulation (see Figure 3C and Table S1) were highly enriched among the putative targets of the siRNAs that were differentially expressed upon RNAi exposure (fold change >2.7, p value <3.317e-15, when strict cutoffs of false discovery rate [FDR] <0.01 and log2 fold change >0.5 were used). 79% of these endo-siRNAs, which target epigenetic genes and were differentially expressed following RNAi, are CSR-1 siRNAs (Figure 3C; Claycomb et al., 2009).

Some of the RNAi genes that we find to be targeted by differentially expressed endo-siRNAs (see Table S2) are known to be defective in specific stages of RNAi inheritance (mutants show HRDE phenotypes). For example, we found that endo-siRNA, which change following dsRNA-induced RNAi responses, target *rde*-1. RDE-1 is essential for initiation of heritable RNAi responses in the parents but is dispensable for maintenance of silencing in the inheriting progeny (Grishok et al., 2000). We found that RDE-1 displays a "modified transgenerational epigenetic kinetics" phenotype (here termed "MOTEK" phenotype), since RDE-1 is required also for extension of heritable RNAi responses by "second triggers" (see Figure 2A). *rde-2* and *mut-7* (which encode for a novel protein and an exoribonuclease that work as a complex) and *nrde-4* (which encodes for a nuclear RNAi factor), that unlike RDE-1 are required in the progeny for RNAi inheritance (Burton et al., 2011; Grishok et al., 2000), were also found in our experiments to be targeted by RNAi-induced endo-siRNAs (see Table S2). In addition, we found that endo-siRNAs that change following RNAi target the *rrf-1* gene. RRF-1 is an RdRP that is known to be required for RNAi inheritance (Gu et al., 2012; Rechavi et al., 2011; Sapetschnig et al., 2015), and our experiments demonstrate that *rrf-1* mutants are less sensitive to extension of heritable responses by "second triggers" (Figure S5A).

We examined whether manipulating additional RNAi genes, not known to display HRDE or MOTEK phenotypes, could change the duration of transgenerational silencing. We focused on RNAi genes that we found to be targeted by differentially expressed endo-siRNAs following RNAi, and which exhibited in addition changes in their mRNA levels (see Table S3). Two mutants, *deps-1* and *ppw-1*, were found to display a MOTEK phenotype:

The *deps-1* gene encodes for an auto-regulating, unfamiliar P-granule-associated protein (Spike et al., 2008). We found that *deps-1* mutants cannot maintain heritable RNAi (Figure 4A). DEPS-1 regulates a number of RNAi factors, including *rde-4* (positive regulation) (Spike et al., 2008), which encodes for a dsRNA-binding protein that is required only for initiation, but not for maintenance of heritable RNAi responses (Grishok et al., 2000).

ppw-1 mutants displayed the most interesting MOTEK phenotype. This is the first gene to our knowledge that upon manipulation extends the duration of RNAi inheritance (Figure 4B). *ppw-1* mutants were shown in the past to be germline RNAi defective (Tijsterman et al., 2002). Our analysis shows that PPW-1 is required in the parents but not in the progeny for propagation of RNAi responses (Figure 4B). After we crossed a wild-type worm to *ppw-1* mutants, and treated the F1 heterozygous with anti-*gfp* dsRNA, all the derived lineage [irrespectively of whether the progeny was *ppw-1(+)* or *ppw-1(–)*] exhibited strongly enhanced transmission of heritable RNAi, and silenced GFP for more than six generations. Surprisingly, this extension in the transgenerational duration of RNAi is dependent on the P-1 mother being *ppw-1(–/–)* (Figure S5B). Interestingly, in contrast

(B) Changes in the levels of different small RNA sub-classes following RNAi administration. (i) Changes in the levels of different small RNA species following RNAi and in response to the "second trigger." (ii) Changes in the levels of HRDE-1 and CSR-1 endo-siRNAs following RNAi and in response to the "second trigger." (C) Changes in small RNAs that align to genes that affect epigenetic processes following RNAi. The small RNAs pools of F1 worms that were exposed to the "second trigger" were compared to the small RNA pools of F1s that were removed from RNAi. (i) Changes in the levels of different small RNAs that affect epigenetic processes following RNAi. (ii) Changes in CSR-1 or HRDE-1 endo-siRNAs, which target epigenetic genes.
See also the related Figure S4.

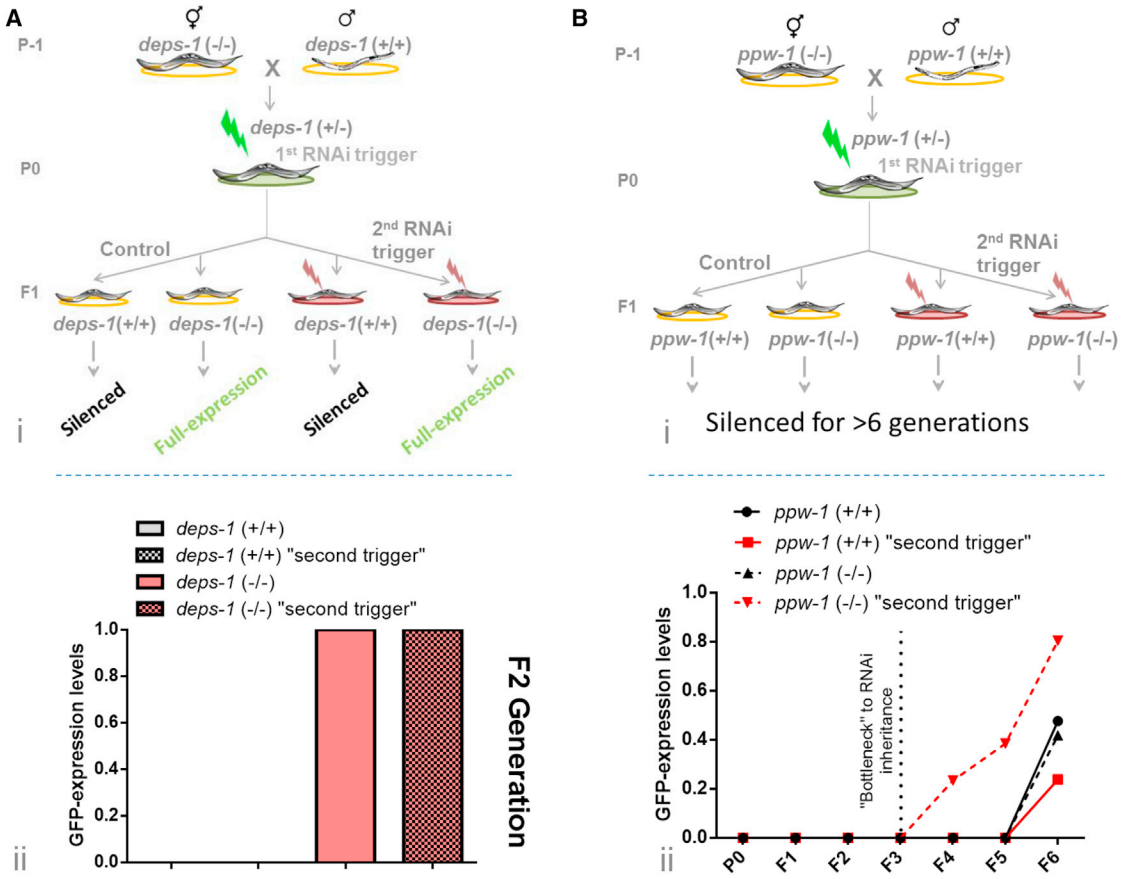

Figure 4. *ppw-1* and *deps-1* Mutants Exhibit a Modified Transgenerational Epigenetic Kinetics Phenotype

(A and B) *deps-1* (A) or *ppw-1* (B) heterozygous mutants were exposed to anti-*gfp* dsRNA. At the F1 generation, progeny of the different genotypes (including homozygous mutants) was treated either with an anti-*dpy-2* "second trigger" or with a control "empty vector." (i) Scheme of the crosses that indicates when each dsRNA trigger was administered. The genotypes of the worms were verified using PCR; WT and homozygous mutants were scored for GFP expression. (ii) Experimental results (GFP levels were measured to track heritable silencing, mean ± SEM). See also the related Figures S5 and S6.

to the effects seen in *wild-type* animals, exposure of *ppw-1*(−/−) worms to a "second trigger," for yet unknown reasons, reduced the transgenerational duration of GFP silencing (although these worms still silence GFP for longer durations, in comparison to *wild-type* animals) (Figure 4B).

In summary, intervention in the feedback response, through manipulation of genes that affect RNAi processes, which were targeted by heritable endo-siRNAs following RNAi, alters the normal duration of heritable RNAi responses.

DISCUSSION

Our results suggest that the RNAi inheritance machinery can acquire different "states" that either support or restrict exogenous small RNA inheritance. Initiation of an RNAi response turns the exo-siRNA inheritance system ON, by enhancing the production of exo-siRNAs at the expense of endogenous small RNA populations (and by altering the balance between CSR-1 and HRDE-1 endo-siRNAs). A feedback response returns the siRNA inheritance mechanism back to the OFF state by altering the regulation of endo-siRNAs on genes required for the inheritance of endo-siRNAs. This "transgenerational timer" is being reset by initiation of new RNAi responses, and therefore "second triggers" extend the inheritance of ancestral silencing (see scheme in Figure 5).

To qualitatively assess the constraints that such a mechanism would have, we built a minimal mathematical model, which abstracts the system's basic features (Figure 5; Supplemental Information). Simulations conducted using this model faithfully recapitulated the dynamics of heritable silencing and the effects of "second triggers" that were observed experimentally (Figure 5). The model allows estimating the dependency between the different components that determine the duration of heritable RNAi responses: (1) the starting conditions (intensity of the original RNAi response), (2) the degree of passive decay, (3) the competition between the different RNAi pathways, and (4) the negative feedback response (see Supplemental Information). The model also explains how selection of individuals with strong heritable responses could stabilize heritable responses for multiple generations (Figure 5) (Vastenhouw et al., 2006). The mathematical model is available in the Supplemental Information (see Data S1 and S2) and can also be expanded or used in the future

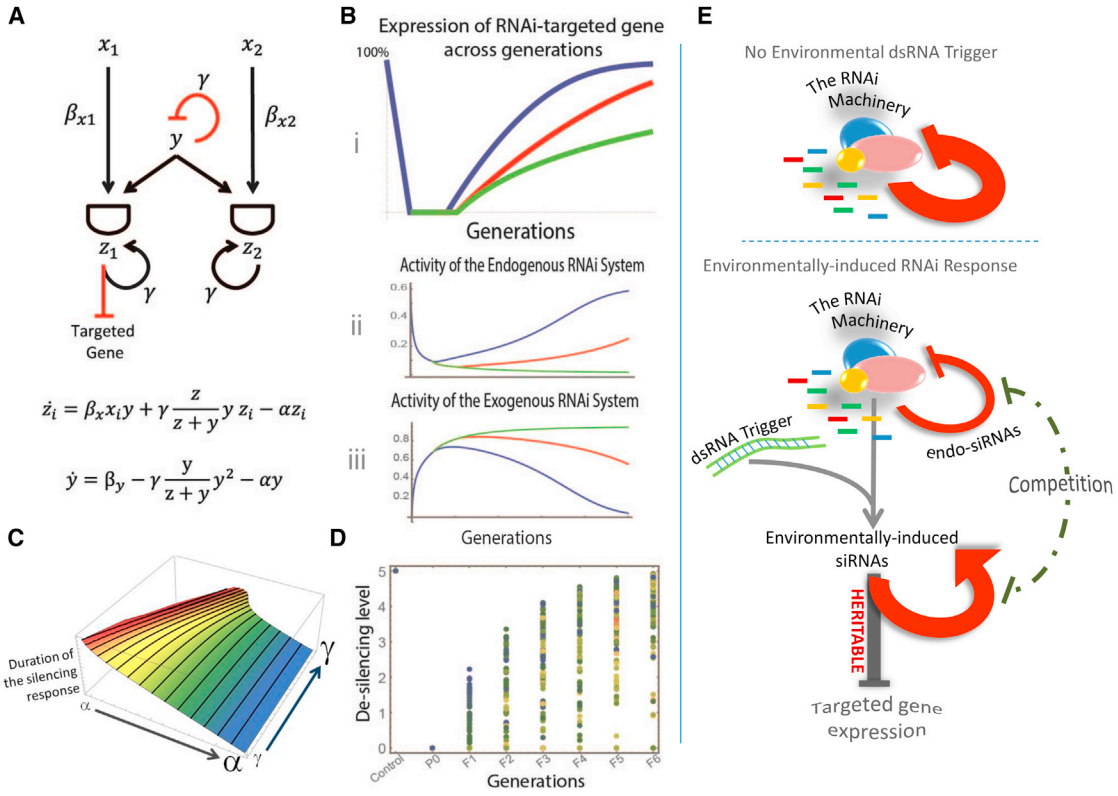

Figure 5. A Simplified Mathematical Model for Simulating RNAi Inheritance Dynamics

(A) A schematic description of the model (see a detailed description in the Supplemental Information). x_1 and x_2 represent two external dsRNA triggers, which induce RNAi responses against different genes at a β_x rate. y represents the activity of the RNAi machinery; z_1 and z_2 represent the levels of the amplified small RNAs (products of x_1 and x_2). The strength of the feedback between the small RNAs and the RNAi machinery is represented by the parameter γ. The α parameter represents the intensity of the passive decay.

(B) The predictions of the model for the following: (i) The expression levels of the gene that was originally targeted. (ii) Transgenerational changes in the activity of the endogenous RNAi-machinery (the pathway that supports production of heritable endogenous small RNAs). (iii) Transgenerational changes in the activity of the exogenous RNAi-machinery (the pathway that supports production of heritable exogenous small RNAs). The levels of the above i–iii were modeled in response to three different treatments: when only a "first trigger" is administered (blue), when an additional "second trigger" was administered at the F1 generation (red), and when repetitive "second triggers" were administrated repetitively, across generations (green). The displayed results were obtained when the following parameters were used: $\beta_x = 10$, $\beta_y = 1$, $\gamma = 1$, $x_1 = x_2 = 1$ $\alpha = 1$.

(C) The duration of the inherited silencing response. Shown are the dependencies between the rate of the passive decay (α) and the intensity of the active feedback and the amplification of the response (γ), when only 1 dsRNA trigger is administered. Color and height represent the inheritance duration.

(D) Changes in GFP levels across generations at the population level. Individual worms show variance in heritable responses dynamics. To examine whether the source of this variability could stem form different feedback intensities, at each generation the parameter γ that represents the strength of the feedback between the RNAi and the RNA-machinery was randomly picked from a normal distribution. Each point represents a different worm, and the color represents the γ chosen (blue - low γ levels; red - high γ levels). The simulation shows that it is possible to maintain long term silencing by selecting worms with high γ.

(E) A diagram summarizing the interactions between different heritable RNAi responses, and the feedback loop that times the duration of transgenerational silencing.

as a platform for testing hypothesis regarding heritable RNAi dynamics.

In summary, despite the acknowledged limitations of the "dilution" model, no alternative models that could recapitulate the dynamics of epigenetic responses were previously provided (Alcazar et al., 2008). While the "dilution" model is incompatible with long-term RNAi inheritance also for theoretical considerations, dilution of heritable effects over time is often qualified as the main criterion based on which transgenerational effects are marked as "epigenetic" instead of "genetic" (as changes in the DNA sequence are permanent). In contrast, we described an active process that based on a set of conditions dictates whether particular epigenetic effects would persist or terminate. The worm's capacity to time transgenerational epigenetic inheritance in response to dsRNA triggers suggests that RNAi inheritance is an evolved mechanism and not an epiphenomenon of RNAi.

The view of epigenetic inheritance as "passive," which is contrasted by our results, appears to resonant with 19th century ideas regarding genetics; until the re-discovery and acknowledgment of Mendel's principles, inheritance was explained using a "blending inheritance" hypothesis, according to which the traits of the parents passively "dilute" and "blend" in the progeny (in the "blood line") (Weldon, 1902).

Different RNAi responses can segregate together if linked in time, and repetitive activation of the RNAi system can perpetuate specific silencing episodes. The identified "critical period" during which two discreet epigenetic responses can be "entangled" could restrict non-adaptive pairing of unrelated epigenetic effects. If unrelated ancient epigenetic responses would nevertheless influence the dynamics of newly elicited responses, irrelevant heritable silencing would be carried over to the progeny, which would likely be detrimental.

Our results show that, while many "second triggers" are effective in extending heritable RNAi effects, targeting particular genes by RNAi produces an especially strong response. These results suggest that sensing the levels of genes that are targeted by specific "second triggers" could contribute to the second trigger's ability to shift the state of the RNAi inheritance system to a state that supports exo-siRNA inheritance (an immunological mechanism that enables sensing of dsRNA-induced mRNA silencing was recently described [Melo and Ruvkun, 2012]).

It would be important to examine whether feedback interactions between small RNAs and other epigenetic mechanisms (chromatin modifications, DNA marks) can perpetuate RNAi in higher organisms as well. In theory, it could be deleterious to maintain epigenetic responses if environmental conditions change rapidly in proportion to the organism's generation time. While heritable effects have been demonstrated in many organisms (Jablonka, 2013), the mechanisms that enable long-lasting multigenerational epigenetic effects are better understood in nematodes, which have short generation times (3–4 days), and in plants, which are sessile organisms (Heard and Martienssen, 2014). Perhaps, similarly to worms, organisms with longer generation times can regulate the duration of heritable effects, using homologs "transgenerational timer" mechanisms. If this is the case, long-term transmission of epigenetic responses could be adaptive also in "higher" organisms, for which the parental environment is often very different from that of the progeny. Adaptive control over the duration of environmental responses could affect the process of evolution.

EXPERIMENTAL PROCEDURES

Cultivation of the Worms

All 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 NGM plates seeded with OP50 bacteria, and HT115 bacteria that express dsRNAs were used for RNAi induction, as previously described (Kamath et al., 2001), see more in Supplemental Experimental Procedures. These strains were employed in this work: wild-type Bristol N2 strain; EG6089 *unc-119(ed3)* III, *oxTi38 cb-unc-119(+) Ppie-1::GFP* I, EG4885 *oxIs320 (CB-unc-119(+) Pdpy-30::mCherry::histone*) II, *unc-119(ed3)* III, *oma-1* (TX20), *rde-1* (WM27), *rrf-1* (RB798), *ppw-1* (NL3511), *deps-1* (DG3226), *rrf-3* (NL2099). The nematodes were kept well fed for at least five generations before the beginning of each experiment. Extreme care was taken to avoid contamination or starvation, and contaminated plates were discarded from the analysis.

RNAi Treatment

The standard assay for RNAi by feeding was carried out as previously described (Kamath et al., 2001). In each stage of the different experiments, worms were cultivated either on HT115 bacteria that transcribe specific dsRNA (e.g., targeting *gfp*, *oma-1*, *mcherry*, *dpy-2*) or on control HT115 bacteria that only contain an empty vector that does not lead to dsRNA transcription and gene silencing. Transferring onto dsRNA-producing bacteria or off RNAi (onto plates that contain bacteria that express an empty vector) was performed at the L4 stage.

Small RNA-Seq Analysis

For details, see the Supplemental Experimental Procedures. In brief, the adaptor sequences were removed using CutAdapt (Martin, 2011). Clipped reads were mapped to version ce10 of the *C. elegans* genome using Butter (v.0.3.3) (Axtell, 2014). Reads that aligned in the antisense orientation to genes were counted using htseq-count (Anders et al., 2015) and Ensembl-based gff file. We used DESeq2 (Love et al., 2014), an R package, to determine differential expression of small RNAs that target specific genes, and considered a small RNAs cluster to be differentially expressed if its assigned FDR value was less than 0.01.

ACCESSION NUMBERS

The raw sequencing files and the processed data are available under GEO: GSE77654.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, three tables, and two data files and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.02.057.

AUTHOR CONTRIBUTIONS

O.R. and L.H.-Z conceptualized the ideas and designed the experiments; L.H.-Z, L.F., I.A.T., Y.D., L.A., and L.D. performed the experiments; L.H.-Z conducted the bioinformatic analyses. O.R. and L.H.-Z. wrote the manuscript; U.A., Y.K., and H.S. constructed the mathematical model.

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