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POINT OF VIEW

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Plastic germline reprogramming of heritable small RNAs enables maintenance or erasure of epigenetic memories

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ABSTRACT

In *Caenorhabditis elegans* small RNAs can regulate genes across generations. The mysterious tendency of heritable RNA interference (RNAi) responses to terminate after 3–5 generations has been referred to as "the bottleneck to RNAi inheritance." We have recently shown that the re-setting of epigenetic inheritance after 3–5 generations is not due to passive dilution of the original RNA trigger, but instead results from an active, multigenerational, and small RNA-mediated regulatory pathway. In this "Point of View" manuscript we suggest that the process that leads to the erasure of the ancestral small RNA-encoded memory is a specialized type of germline reprogramming mechanism, analogous to the processes that robustly remove parental DNA methylation and histone modifications early in development in different organisms. Traditionally, germline reprogramming mechanisms that re-set chromatin are thought to stand in the way of inheritance of memories of parental experiences. We found that reprogramming of heritable small RNAs takes multiple generations to complete, enabling long-term inheritance of small RNA responses. Moreover, the duration of this reprogramming process can be prolonged significantly if new heritable RNAi responses are provoked. A dedicated signaling pathway that is responsive to environmental cues can tune the epigenetic state of the RNAi inheritance system, so that inheritance of particular small RNA species can be extended.

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Introduction

A barrier to transgenerational epigenetic inheritance

The ancestors' responses to environmental challenges are generally thought to be prevented from affecting the progeny because parentally-acquired epigenetic marks are erased, through a process that is known as "germline reprogramming." In this "Point of View" manuscript we discuss the implications of a new study from our lab that shows that in *C. elegans* parental small RNAs are also "reprogrammed," and that dedicated mechanisms can delay the process for several generations. Indeed, in worms, dsRNA-induced RNA interference (RNAi) can transmit epigenetic memory for multiple generations.[1] We recently described an active mechanism that regulates the duration of heritable silencing.[2] In response to any dsRNA-induced RNAi, small RNA reprogramming is minimized, so that long-term maintenance of heritable non-DNA sequence information is achieved. Before discussing the mechanisms that enable and prevent small RNA inheritance specifically, we will briefly introduce the concept of germline reprogramming.

Germline reprogramming

In animals, ancestrally deposited chromatin modifications are reset in the germline in every generation by both passive and active mechanisms. This "re-setting" is thought to be required for the totipotency of the germline.[3] To prevent deviations from the genomic blueprint and species-inappropriate development, most epigenetic marks that were acquired during the development of the parents are removed or "reprogrammed."[4] This process entails the erasure of epigenetic marks in a genome-wide manner, leaving a limited number of modified loci, such as transposable elements that need to be repressed constitutively to preserve the integrity of the genome.[5] There are additional escapers from reprogramming. For example, while the majority of histones are replaced by protamines in the sperm of humans and mice, there are particular genes on male-provided chromosomes that retain histones and the modifications with which they are decorated.[6,7] Deposition of epigenetic marks on many genetic loci allows organisms to adjust gene activity to changing environmental conditions, and failure to reprogram such modifications could in theory prepare the progeny for the challenges that the parents faced. Empirical evidence suggests, however, that in mammals germline reprogramming by both passive processes (e.g. removal of histones during DNA replication) and active mechanisms (e.g., DNA demethylation by enzymes) ensures that maintenance of acquired DNA and chromatin modifications is infrequent.[5] These mechanisms have evolved possibly since inheriting the parents' responses to changing environments is likely to be inappropriate for the children. If the children are not exposed to similar challenges, preparing in advance (retaining epigenetic memories), by "betting" on the parents' reactions, could be detrimental. This could be especially true when organisms

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with long generation times are concerned, since the progeny's environment is more likely to differ from the parent's.

Nevertheless, some heritable molecules appear to evade germline reprogramming, as different environmental challenges, for example manipulations to the organism's diet, have been shown to produce physiological changes that are carried over for multiple generations through unknown mechanisms in many organisms.[8] Epigenetic information can be transmitted in theory via DNA methylation, histone modifications, and regulatory RNA molecules.[4] As discussed above, most DNA methylation and chromatin modifications are erased in every generation. It is possible that the few loci that escape reprogramming retain critical information that affects specific traits. Alternatively, other epigenetic agents, such as small RNAs, may not be efficiently reprogrammed and may pass the memory of parental experiences to progeny, as we discuss below.

In mice, extensive active reprogramming of cytosine methylation and different histone modifications is tightly regulated and occurs in 2 defined periods, during development of germ cells and during early embryogenesis.[5,9] Indeed, failure to remove some epigenetic marks has been linked to improper development.[10,11] While the consequences of global failure to reprogram different chromatin modifications are probably dire, it is possible that maintaining certain heritable modifications on specific genes for multiple generations could be tolerated, and might even be beneficial.

In nematodes, the extent and importance of germline reprogramming are not well understood, in comparison to mammals, where this subject has been thoroughly investigated. What is clear, however, is that worms are an attractive model system for studying the genetic basis, biochemical mechanisms, and physiological functions of germline reprogramming. This is because removal of enzymes that normally reprogram specific chromatin marks is not lethal in *C. elegans*.

Removal of parental 5-methylcytosine is the germline reprogramming mechanism that has been most extensively studied. *C. elegans* genome does not contain methylated cytosines. However, low levels of N(6)-adenine methylations (6mA) were recently discovered in the worm's DNA.[12] Not much is known yet about the function or reprogramming of these marks. Notably, disruption the 6mA DNA landscape is not lethal in *C. elegans* and results in heritable changes that are maintained for multiple generations in relatively healthy animals.[12] Similarly, worms that lose enzymes that generate certain histone modifications (for example methylation of H3K9 and H3K4) are viable and fertile, and mutant phenotypes are manifested only after multiple generations. The inability to remove certain histone marks results in a "mortal germline" (Mrt) phenotype, sterility that accumulates over time.[13] For example, failure to remove dimethylation of lysine 4 on histone H3 (H3K4me2), in *spr-5* mutants (ortholog of the demethylase LSD1), results in a Mrt phenotype. Disruption of the H3K4me3 complex for one generation, by manipulating the *ash-2, wdr-5* and *set-2* genes produces global heritable changes which are maintained for 3 generations, and counterintuitively extend the worm's lifespan.[14] By manipulating the activity of the parents' Polycomb repressive complex 2 (PRC2), it was shown that H3K27 methylation is transmitted to F1 embryos via both the egg and the sperm (histones are not replaced by protamines in *C. elegans*).[15] In the germline of the F1 worms, the chromosomes displayed a gradual redistribution of H3K27me, which likely reflects "reprogramming" of this chromatin marks. In summary, since reprogramming of different epigenetic marks is not immediately required for viability or fertility, *C. elegans* offers an optimal system for dissecting the underlying mechanisms that lead to removal of parental epigenetic memories and the consequences of these processes.

In this paper we discuss a reprogramming process that is largely ignored: removal of ancestral small RNAs. Since small RNAs transmit heritable responses for multiple generations in *C. elegans*, their elimination is obviously not absolute. In nematodes, heritable small RNAs function also in the nucleus, where they guide deposition of histone modifications.[16-18] Thus, in theory, even if chromatin modifications are removed over the course of development, heritable small RNAs that escape reprogramming could reconstitute parts of the parental chromatin landscape *de novo* in every generation. We will discuss this theory in light of recent findings regarding *C. elegans* transgenerational small RNA inheritance, and will suggest that reprogramming of heritable small RNAs is an active process that can be tuned by environmental information to support long-term maintenance of specific non-DNA sequence-based memory.

Heritable RNAi in C. elegans

Injecting, soaking or feeding worms with dsRNA can initiate systemic and heritable RNA interference (RNAi).[19-21] Worms fed with bacteria that produce dsRNA can silence the targeted gene in the fed parents and in their F1 progeny, even when the progeny are not exposed to the dsRNA-expressing bacteria themselves.[22] RNAi responses against genes expressed in the animal's germline produce long-term silencing responses, which can be transmitted through both the male and female germline, and at the population level typically last 3-5 generations.[22] While RNAi has been extensively studied, the mechanisms that initiate and maintain long-term RNAi inheritance are only now beginning to be elucidated. In the last decade several factors required specifically for transgenerational RNAi inheritance have been identified. The *C. elegans* genome encodes 26 argonaute proteins, most of which are still largely unexplored. The argonautes HRDE-1/WAGO-9, WAGO-1, and CSR-1 were found to carry small RNAs in the germline and across generations.[23-25] The heritable small RNAs that are bound by these argonautes are products of RNA-dependent RNA Polymerase (RdRP)-mediated amplification, display a bias for Guanosine at their 5′ end, and are mostly 22 nt long (22G). RdRPs are guided by different types of "primary" small RNAs, including PIWI-interacting small RNAs (piRNAs), exogenously derived small interfering RNAs (exo-siRNAs), and endogenous small RNAs (endo-siRNAs) to mRNA targets. RdRPs use the targeted mRNA as a template for synthesis of "secondary" small RNAs (22G RNAs).[26] Secondary small RNAs are much more abundant than the primary small RNA species, and are the RNA species that directly regulate gene expression[25,27-29] While production of 22G RNAs appears to take place in the cytoplasm,[30] these small RNAs are shuttled back to the nucleus, where (through still only partly understood mechanisms) they affect gene expression by inducing

deposition of chromatin modifications and by inhibiting Pol II elongation.[16,18,24]

Our study investigated the process that "reprograms" the inheritance of exogenous siRNAs. It is thought that the exo-RNAi pathway evolved to confer immunity against viruses.[31,32] However, it could have additional functions as well. For example, exogenous dsRNA might allow communication of gene responses between conspecifics or even between worms and other organisms.[33] Importantly, the exo-RNAi pathway competes for protein components that serve also for synthesis and utilization of endogenous small RNAs. Processing of the original exogenous dsRNA molecule to primary ~23nt exo-siRNAs depends on the sole *C. elegans* DICER protein (encoded by *dcr-1*), which is required for synthesis of other small RNA species as well (microRNAs and certain endo-siRNAs).[34,35] Similarly to piRNAs and some endo-siRNAs, primary exo-siRNAs guide RdRPs that produce abundant secondary 22G small RNAs, which are carried transgenerationally in the germline by the argonaute HRDE-1 (Heritable RNAi DEfective-1).[24]

The mysterious "bottleneck to RNAi inheritance"

Since measured amounts of dsRNA can be administered to worms at defined time points, exo-siRNA-mediated silencing enables precise analysis of RNAi dynamics and can provide valuable insights into the process of small RNA reprogramming. After feeding or injecting dsRNA to worms, it was found that transgenerational RNAi inheritance typically lasts 3-5 generations. This "barrier to RNAi inheritance" cannot be breached by injecting the worm's gonad with higher doses of dsRNA,[22] leading to questions about the nature and regulation of the barrier.

It was intuitive to assume that RNAi ceases to affect the progeny after 3-5 generations because the original RNA molecules that triggered silencing in the parents are being passively diluted in every generation, such that after a precise number of dilution cycles their numbers are too low to be effective.[17] While at the population level silencing responses indeed fade after 3-5 generations, it has been shown that by selecting, in every generation, individuals that still silence the targeted gene, RNAi responses can be maintained for more than 80 generations.[36] Since *C. elegans* hermaphrodites produce ~250 offspring, the dilution factor of the RNA molecules that derive from the original dsRNA trigger would be huge after 4 generations (the RNA from one germ cell has to be distributed to ~4 billion worms), and could not possibly allow a silencing response to persist. On the other hand, it is not clear why RNAi responses ever stop, since it was found that in the germline secondary small RNAs can continue to induce production of "tertiary" small RNA by triggering RdRP-mediated amplification.[37] Thus, in theory dsRNA-induced silencing responses have the potential to be perpetuated indefinitely. Indeed, some transgenes can become permanently silenced.[37,38]

The more complex heritable RNAi dynamics that are observed in experiments might be explained by an imbalance between passive dilution of the original small RNA response, and an opposing amplification by RdRPs. If the RdRP-mediated amplification process is less efficient than the dilution process, then the response would gradually "fade away." Silencing responses might become permanent by robust RdRP-mediated amplification of the initial silencing molecules and/or by chromatin changes that silence transcription. These hypotheses would suggest that the observed dynamics of small RNA inheritance reprogramming result simply from unregulated, passive, and perhaps stochastic processes. According to this model, while heritable silencing responses could in theory give rise to interesting heritable effects, the process could very well be an epiphenomon, a side-effect of the parental RNAi response. In contrast, we propose that a regulated mechanism has evolved to ensure transmission of certain responses to future generations.

Each exogenous RNAi response re-sets the inheritance "timer"

Upon examination of published data of small RNAs that are carried by the argonaute proteins that mediate heritable small RNAs, CSR-1 and HRDE-1, we hypothesized that RNAi responses "control their own inheritance." In these data we detected a surprising enrichment for small RNAs that target genes that act in RNAi and RNAi inheritance processes in particular, including the *csr-1* and *hrde-1* genes themselves.[2,23,39] This observation raised the possibility that feedback regulation between small RNAs and small RNA biogenesis genes controls heritable RNAi dynamics. We hypothesized that instead of passive transmission across generations the induction of each RNAi response could dynamically switch the RNAi inheritance system ON or OFF by affecting such RNAi genes. Such self-regulating feedback circuits are very common in biology. For example, many transcription factors control their own expression,[40] and many RNA-binding proteins bind their own transcripts.[41] Furthermore, in support of our model, a recent study showed that CSR-1-bound small RNAs regulate *csr-1* transcription.[42]

To test our hypothesis, we first examined whether consecutive triggering of distinct, gene-specific exo-RNAi silencing responses affects the dynamics of RNAi inheritance. We exposed worms to different dsRNA molecules that served as "triggers" (a term that we use hereafter) to induce silencing of specific genes (that have no sequence similarity between them). If the property that underlies the dynamics of RNAi inheritance is passive dilution of the original dsRNA molecules/response, then repetitively exposing worms, in consecutive generations, to unrelated dsRNA triggers should not affect the dynamics of each specific inherited RNAi response. In other words, silencing of one gene should not affect the inherited silencing of a second gene. In contrast, if the activity of the exo-RNAi inheritance system itself changes in response to induction of any exo-RNAi response by any exogenously-provided dsRNA trigger, then repetitive initiation of different RNAi responses, regardless of the identity of the genes being targeted, should affect the transgenerational transmission of gene-specific silencing responses that were initiated in previous generations.

These experiments revealed that inheritance of ancestral exo-RNAi responses, which is normally restricted to 3-5 generations (the "bottleneck to inheritance"), can be significantly prolonged when worms are challenged with additional and unrelated dsRNA triggers (hereafter called "second triggers") in the following generations. In other words, environmentally

induced RNAi responses activate the exo-RNAi inheritance system, in a sequence-independent manner.

Notably, the ability of second triggers to extend the duration of ancestral RNAi responses was found to require the induction of a full-blown RNAi response by the second trigger. It was not enough to simply expose the worms to a second dsRNA (which could constitute a "danger signal" [43]). Instead, the dsRNA that served as a second trigger had to: 1) be processed into primary small RNAs 2) lead to amplification of secondary small RNAs, and 3) induce downregulation of the targeted gene's expression, in order to prolong the duration of the ancestral RNAi response.[2]

RNAi responses in *C. elegans* involve heritable deposition of specific histone modifications on the targeted gene locus.[44] We found that extension of the duration of the ancestral RNAi response by second triggers does not depend on the presence of the original histone marks that the ancestral response induced. Instead we found, using small RNAs sequencing, that repetitive activation of the exo-RNAi system "boosted" the overall production of heritable exo-siRNAs; the second trigger leads to an RdRP-dependent amplification of heritable secondary small RNAs that derive from the ancestral RNAi response.

A competition-based mechanism for germline reprogramming of exogenous heritable RNAi responses

As briefly mentioned above, it was observed in multiple studies that the different arms of the RNAi system, which utilize distinct types of small RNAs, compete for common protein factors, such as DCR-1[45]. For example, mutants defective in synthesis of endo-siRNAs are hypersensitive to RNAi by exogenously supplied dsRNA.[45,46] We reasoned that by tilting the balance between exogenously and endogenously derived small RNAs, dsRNA-induced responses turn the exo-RNAi inheritance ON.

Accordingly, we found that the response to environmentally supplied dsRNA shifts the RNAi system to produce and transmit to the germline exo-siRNAs, at the expense of endogenous small RNAs. While most endogenous small RNAs are downregulated following administration of exogenous RNAi (microRNAs, piRNAs, and 90% of HRDE-1-dependent endo-siRNAs), the absolute majority (92%) of genes which are targeted by CSR-1-bound endo-siRNAs were actually found to have more small RNAs antisense to them following induction of exogenous RNAi response. When activation of the exo-RNAi inheritance pathway stops (when external dsRNA is no longer supplied), the balance between production and utilization of endo- and exo-siRNAs returns over several generations back to the ground state.

The default state of the RNAi machinery, when no exogenous dsRNA is supplied, is to transcribe endogenous small RNAs that regulate protein-coding genes, including genes that are involved specifically in the production and inheritance of endogenous small RNAs. This self-regulation generates a feedback that enables proper tuning of endogenous RNAi responses. A shift in the balance between exogenous and endogenous small RNAs following induction of an exo-RNAi response disrupts the regulation of RNAi genes by endogenous small RNAs. We suggest that the time it takes to restore the "natural" balance between endo- and exo-siRNAs, depends, at least in part, on the action of this feedback mechanism (see Fig. 1).

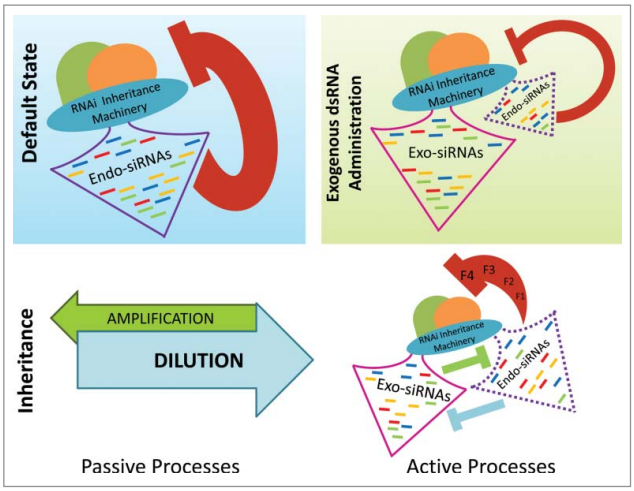

Figure 1. Small RNAs reprogramming in *C. elegans*. In its default state the RNAi inheritance machinery is auto-regulated by endo-siRNAs that target RNAi genes. Upon administration of exogenous dsRNA there is a shift in the balance between production and inheritance of exogenous and endogenous small RNAs. As a result of dsRNA-induced RNAi, the dynamics of the auto-regulatory feedback between endo-siRNAs and the RNAi inheritance machinery change. Reprogramming of heritable small RNAs takes place by both passive and active processes: the interplay between the rate of dilution and the rate of amplification dictates the passive degradation of heritable small RNAs across generations, and the competition between the self-regulating endo-siRNAs and the heritable exogenous small RNAs sets an active "transgenerational timer" that times exogenous RNAi responses.

In addition to changes in heritable endo-siRNAs, we observed changes in the mRNA levels of some of the endo-siRNA-targeted RNAi genes. Therefore, we examined whether corresponding mutants, that should be defective in this feedback regulation, display altered RNAi inheritance dynamics. Our screen revealed genes that we dubbed "MOTEK" genes (for MOdified Transgenerational Epigenetic Kinetics) that significantly extend or shorten heritable RNAi responses and display altered responsiveness to second triggers. For example, animals that do not express a functional copy of the argonaute PPW-1 cannot initiate germline RNAi. However, we found that initiation of RNAi responses in *ppw-1*/+ heterozygous animals produces heritable RNAi responses that last more than 2 times longer than responses in wild-type animals, and that the silencing effects continue also in homozygous *ppw-1 (−/−)* progeny. It will be fascinating to learn in the future the mechanisms by which genes that lead to a MOTEK phenotype affect RNAi inheritance dynamics, and the specific kinetics of small RNAs-mediated regulation of RNAi genes.

What dictates the duration of the reprogramming process?

The reprogramming of DNA methylations and histone modifications occurs in mammals in every generation during specific periods of development. In contrast, the process of small RNA reprogramming in *C. elegans* is gradual and takes multiple generations to complete. According to our model, the erasure of small RNA memories is affected not only by passive decay of the original response, but also, and more interestingly, by the accumulation of self-regulating endo-siRNAs that re-establish the balance between the exogenous and endogenous RNAi pathways. We speculate that small RNA reprogramming might also takes place in defined

periods of development, similarly to chromatin reprogramming, perhaps at particular stages during the germline cycle when certain endogenous small RNA species are transcribed.[47,48] If such "check points" indeed exist, intervention in the small RNA reprogramming process by administration of second triggers might be efficient only when properly timed. For example, we observed that second triggers pushed back the germline reprogramming of the ancestral RNAi responses when introduced in the generation following the initial dsRNA trigger, but not when introduced 2 generations after the initial dsRNA trigger. Thus, transgenerational "coupling" between the different RNAi responses must occur. The critical period during which second triggers are effective might be the window during which the RNAi inheritance machinery normally restores the balance between the endo- and exo-RNAi systems. In this time window, reprogramming of heritable RNAi is still flexible, and the process responds to exposure to additional dsRNA triggers. It is possible that other environmental conditions, such as changes in temperature or changes to the feeding state (e.g. starvation), which were shown to alter the pool of heritable endogenous small RNAs,[49,51] could affect the transgenerational duration of "coupled" RNAi responses.

Summary

Recent studies documented heritable alterations in the endogenous pool of small RNAs in response to specific perturbations, such as activation of the exogenous RNAi pathway,[2,50] starvation-induced developmental arrest,[49] and growth at high temperatures.[51] It is possible that, similarly to exo-RNAi responses, the RNAi-related heritable effects that follow exposure to other environmental conditions have programmed "expiration dates." In fact, this must be the case, since heritable epigenetic effects in response to stress are observed in the lab, even though every worm culture has been starved, contaminated, or grown at high temperatures at some point in history. For this reason, it is crucial to cultivate worms for multiple generations in very defined conditions before starting an experiment in which the heritable effects of particular environmental challenges are examined. While "remembering" ancestral environments could be adaptive, it is certainly crucial to "forget" most past experiences: retaining every heritable small RNA response, even when it is unrelated to the current environmental reality, would be a huge burden and therefore detrimental. Such regulation upon the transmission of "memories" between generations can help the animal to adapt better to changing environments and thus, in theory, might have a role in the process of evolution. Studying the interactions between specific types of heritable RNAi responses and the factors that determine whether particular species of small RNAs are "reprogrammed" or "remembered" is crucial for understanding how the epigenetic landscape is constructed in every generation.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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