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Review

Principles of Transgenerational Small RNA Inheritance in *Caenorhabditis elegans*

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Examples of transgenerational inheritance of environmental responses are rapidly accumulating. In *Caenorhabditis elegans* nematodes, such heritable information transmits across generations in the form of RNA-dependent RNA polymerase-amplified small RNAs. Regulatory small RNAs enable sequence-specific gene regulation, and unlike chromatin modifications, can move between tissues, and escape from immediate germline reprogramming. In this review, we discuss the path that small RNAs take from the soma to the germline, and elaborate on the mechanisms that maintain or erase parental small RNA responses after a specific number of generations. We focus on the intricate interactions between heritable small RNAs and histone modifications, deposited on specific loci. A trace of heritable chromatin marks, in particular trimethylation of histone H3 lysine 9, is deposited on RNAi-targeted loci. However, how these modifications regulate RNAi or small RNA inheritance was until recently unclear. Integrating the very latest literature, we suggest that changes to histone marks may instigate transgenerational gene regulation indirectly, by affecting the biogenesis of heritable small RNAs. Inheritance of small RNAs could spread adaptive ancestral responses.

Introduction

"Who controls the past controls the future"
(George Orwell, 1984)

The segregation of the germline from the soma, envisioned by August Weismann many years ago [1], should in theory prevent ancestral responses from being inherited to generations to come. While direct exposure of the germline to environmental effects could shape heritable materials [2] such as chromatin, modifications to chromatin are known to be largely 'reprogrammed' in the germline and embryo, to ensure that the progeny will have a clean state, allowing stereotypic, species-appropriate development [3,4]. In spite of these conceptual barriers, many studies have documented inheritance of physiological responses to environmental challenges in various organisms, including, for example, bacteria [5], fungi [6], ciliates [7], plants [8], nematodes [9,10], insects [11], and rodents [12–14].

Different heritable effects are often referred to as 'epigenetic'. However, many misconceptions arise regarding this term [15], and therefore we suggest to use the expression 'heritable responses' instead. We will use this term to refer to the transmission of transient, parental physiological or gene expression changes to the progeny.

Two major themes for heritable responses emerge across kingdoms. First, memorization of parental 'immune' responses protects the progeny. For example, in water fleas, helmet structures that form in the parents in response to predators persist and protect the next generations from the same predators [16]. In worms, RNA interference (RNAi) responses that are mounted against viruses and transposable elements are carried over for multiple generations and 'vaccinate' the progeny [17]. In the ciliate *Oxytricha trifallax,* heritable small RNAs specify DNA regions that are retained in the soma macronucleus. Transposon sequences, which are not protected by these small RNAs, are excised during the development of the somatic macronucleus [18]. Second, harsh environmental challenges affecting the organisms' metabolism, such as changes to the diet, have been demonstrated in many organisms to leave lasting physiological changes [19,20]. For example, in *Caenorhabditis elegans*, the progeny of animals that experience starvation-induced developmental arrest inherit small RNAs that regulate nutrition-related genes, live longer, and survive starvation that is otherwise lethal [19,20]. Therefore, in some cases at least, inheritance of parental responses could be adaptive. In other cases, however, such heritable effects could be epiphenomena, resulting from 'carryover' of damage accumulated across generations.

It is pertinent to define the mechanisms that enable such non-standard transmission of parental information to the progeny. The goals of this review are to clarify how somatic responses can be communicated to the germline, and to elucidate the nature of the mechanisms that perpetuate such changes in the descendants. Specifically, we will elaborate on recent mechanistic insights that ascribe a pivotal role for small RNAs in transgenerational heritable responses.

How Somatic Small RNAs Get to the Germline

Almost 20 years ago it was shown that induction of RNAi in *C. elegans* by injection of double stranded RNA (dsRNA) also affects non-treated progeny [21]. More specifically, it was demonstrated that triggering RNAi in the soma, for example by ingestion of bacteria that express dsRNA, also leads to silencing in the germline [21]. When germline-expressed genes were targeted by RNAi, the silencing responses were found to perpetuate transgenerationally for at least three generations (until the F3). These effects qualify as *transgenerational* heritable responses, since no cell of the F3 animal was in direct contact with the dsRNA trigger, and an active mechanism was required in order

Box 1. Defining the different types of heritable responses.

Heritable responses to environmental cues can persist for many generations after exposure to the original trigger, or peter out after one generation. Specific terminology is used to differentiate between the different types of effects.

Intergenerational Inheritance (or 'parental effects'): The effect is observed in the F1 or F2 progeny of the animals exposed to the environmental trigger. In these cases, the embryo (F1) or its germ cells (F2) might be directly exposed to the environmental cue *in utero*.

Transgenerational Inheritance: Progeny which were not exposed directly to the environmental challenge are nevertheless affected, and therefore an inheritance mechanism which amplifies the original response is likely in place. To ensure that an effect could be considered transgenerational, one must examine the F2 progeny of exposed fathers (to make sure that the effects do not stem from exposure of the father's sperm), or the F3 of exposed females (since exposure of the embryo *in utero* also exposes the germline, which will give rise to the F2).

Multigenerational Inheritance: The heritable effect becomes stable.

to maintain the memory across generations, to avoid dilution of the RNA. In certain cases, dsRNA-derived small interfering RNAs (siRNAs), or piwi-associated RNAs (piRNAs), can also trigger stable or multigenerational responses that are maintained for tens of generations [9,22–26]. RNAi targeting of genes expressed exclusively in the soma, however, mostly affects the F1 progeny (rare exceptions to this rule have been reported nevertheless; see [26] for a recent example). In such *intergenerational* heritable responses, it is very possible that the progeny is also directly exposed to the original RNAi trigger (see Box 1).

Systemic silencing by exogenously provided dsRNA ('exogenous RNAi responses', or 'exo-RNAi') is abrogated in a number of *Sid* mutants (Systemic Interference Deficient), and most notably in mutants defective in the conserved dsRNA transporter *sid-1* [27]. The vast majority of the studies in which systemic RNAi was examined focused on the transmission of RNAi effects between different somatic tissues [27–29]. Recently the mechanism for intergenerational transmission of somatically derived exogenous dsRNA to the F1 progeny has also been explored [30].

Inheritance of RNAi by the F1 progeny, regardless of whether germline or somatic genes are targeted by dsRNA, depends on factors of the nuclear RNAi pathway (Nuclear RNAi Deficient genes *nrde-1, 2,* and *4*), that enforce transcriptional silencing in the nucleus. In the soma, transfer of small RNAs from the cytoplasm to the nucleus depends on the nuclear argonaute NRDE-3 [31]. Accordingly, the expression of *nrde-3* in the developing progeny is essential for intergenerational inheritance [31].

A recent study suggested that intergenerational silencing of somatic genes depends on transmission of dsRNAs to the progeny [30]. Fluorescently labeled dsRNA molecules were found to move from the extracellular space, through the intestine, into oocytes together with vitellogenin [30]. The RME-2 low density lipoprotein receptor is also required for transport of the dsRNA together with the yolk to the oocytes [30]. It was hypothesized that the dsRNA is carried in vesicles; however, this hypothesis remains to be proven. The transferred dsRNA was shown to be released via the SID-1 transporter from these vesicles to the cytosol of the developing embryos' cells, where the dsRNA could enforce RNAi [30]. Thus, in contrast to earlier models, this hypothesis suggests that the SID-1 transporter functions not in transmission of dsRNA between cells, but in the release of the acquired mobile dsRNA from intercellular compartments that 'trap' it. In support of this model, it was found that dsRNA is efficiently transmitted to *sid-1(-/-)* progeny from *sid-1(+)* parents; however, despite localizing to the oocytes, the inherited dsRNA cannot induce silencing [30] (Figure 1).

Unlike *exogenous* dsRNA-derived small RNAs, to date, no *endogenous* small RNAs have been shown to move between the worm's soma and germline, or in general to be exchanged between other tissues. The microRNA *lin-4*, for example, was explicitly shown to be restricted to the cells in which it is expressed [32]. However, a systematic investigation of the capacity of all other microRNAs to transmit between cells, or whether other types of small RNAs act non-cell autonomously, has not been tested yet. It is possible that only particular small RNAs, with specific molecular characteristics, can transit through selective channels or vesicles from the soma to the germline. In mammals, however, diverse types of small RNAs (including microRNAs and tRNA-fragments) can move between different cell types [33,34]. tRNA fragments, for example, are present in secreted vesicles of different types, including epididymosomes that are secreted from the epididymis (a somatic tissue) to nearby sperm cells [35].

Inheritance of Small RNAs in the Germline

In the *C. elegans* germline heritable small RNAs avoid transgenerational dilution owing to an RNA amplification mechanism. Not only dsRNA-derived exo-siRNAs, but also endogenous small RNAs, such as primary endo-siRNAs, and piRNAs, function by guiding RNA-dependent RNA polymerases (RdRPs) to mRNA molecules, which are used as templates for the synthesis of much more abundant secondary small RNAs termed 22G RNAs [36,37]. The recently discovered small antisense ribosomal siRNAs (risiRNAs) also depend on RdRPs for their production [38]. Although not known to be typical of other microRNAs, mir-243 has been shown to trigger amplification of secondary small RNAs as well [39]. Small RNA species which are amplified by RdRPs are perhaps more likely to produce transgenerational RNAi effects, since this mechanism prevents their dilution across generations. However, it is possible that other non-RdRP-mediated types of feedback interactions preserve long-term, heritable silencing. For example, piRNA-mediated gene silencing in flies, fish and mice involves amplification of piRNAs via a 'ping-pong' cycle mechanism [37,40]. In plants, microRNAs can direct chromatin modifications, which recruit factors that initiate additional rounds of small RNA synthesis [41]. In *Paramecium tetraurelia*, small RNAs (termed iesRNAs)

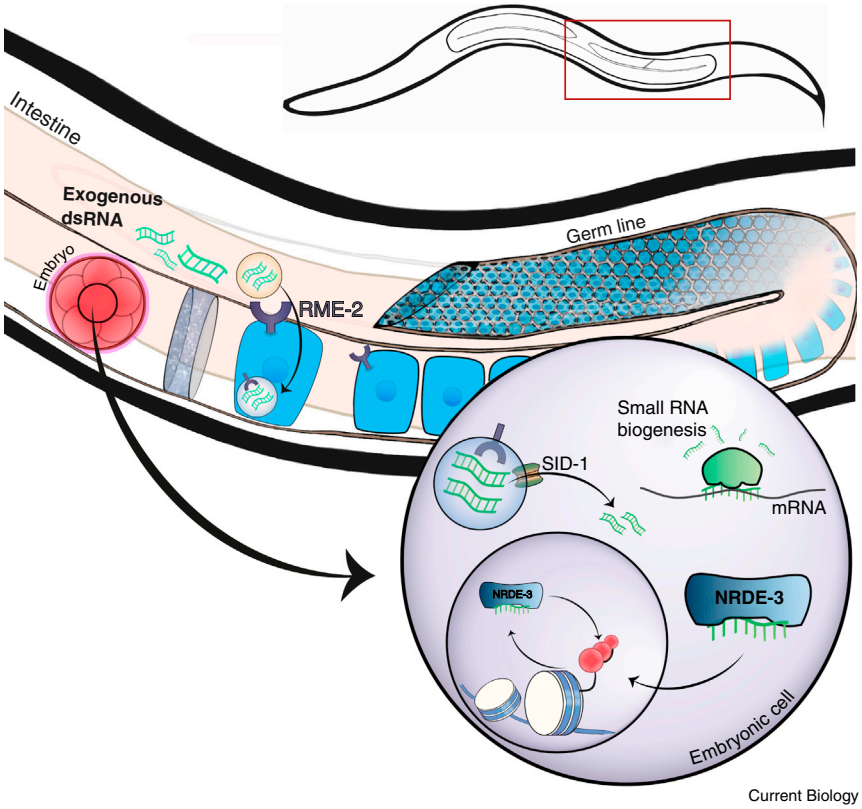

Figure 1. Soma to germline transmission of RNAi.
Uptake of dsRNA results in spreading of the dsRNA throughout the body of the worm. The dsRNA is transferred with vitellogenin from the intestine to the oocyte via the RME-2 receptor. In the developing embryo cells, the dsRNA is released to the cytosol by the SID-1 channel to initiate the production of primary and secondary small RNAs. The secondary small RNAs enter the nucleus by the nuclear argonaute NRDE-3 to establish nuclear gene silencing and H3K9me3 of the targeted locus.

target specific DNA sequences for deletion. The deleted DNA pieces are later ligated to form circles of concatenated DNA. The DNA circles allow bi-directional transcription of dsRNAs from which a second round of iesRNA production is initiated [42].

RdRP-amplified nuclear secondary endo-siRNAs usually 22nt long with a 5' guanosine bias, or 22Gs, are carried by argonautes in the worm germline and guide transgenerational responses [43]. In the germline, but apparently not in the soma [44], secondary small RNAs can further direct efficient amplification of 'tertiary' small RNAs, and therefore set in motion a feed-forward process that could theoretically preserve transgenerational inheritance *ad infinitum* [45]. Amplified 22G small RNAs that originate in responses that were primed by different 'primary' small RNAs, such as primary exo-siRNAs, primary endo-siRNAs ('26G'), and piRNAs ('21U'), eventually converge in the germline on common effector proteins [23]. Heritable 22G small RNAs were found to be carried in the germline by three nuclear argonautes: CSR-1 (Chromosome-Segregation and RNAi deficient [46]), HRDE-1 (Heritable RNAi Deficient 1 [47]), and WAGO-1 (Worm ArGOnaute protein 1 [48]). 22G small RNAs bound by CSR-1, a *C. elegans* argonaute that possess 'slicing' activity [36], complement primarily germline-expressed genes, and were suggested to promote the expression of their targets [46], and also to 'tune' gene expression levels by using CSR-1's slicing activity [49]. HRDE-1 is essential for transgenerational inheritance of dsRNA-induced silencing responses, and its associated 22G endo-siRNAs are thought to inhibit the transcription of their cognate target genes in the nucleus [47]. Importantly, HRDE-1 is required for secondary small RNA-mediated amplification of the aforementioned tertiary small RNAs. The synthesis of RdRP-amplified tertiary small RNAs, which spread across the targeted gene's transcript, also requires the nuclear RNAi pathway proteins NRDE-1, NRDE-2 and NRDE-4 [45].

Transgenerational RNAi 'Timers'

In principle, RdRP-mediated amplification of heritable small RNAs could maintain the inheritance of small RNAs indefinitely. However, a dedicated regulatory program including an active feedback process between heritable endo-siRNAs and small RNA inheritance genes restricts the duration of dsRNA-induced RNAi responses. This pathway generates RNAi inheritance patterns that are stereotypic in their length, so that silencing typically endures for 3–5 generations (this system has been reviewed lately; for details see [24,50]). Mutants defective in specific 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 [24] and, in certain cases, exhibit unusually long RNAi inheritance durations [24].

Chromatin modifiers are likewise required for long-term RNAi inheritance [22]. In particular, the histone H3 lysine-9 (H3K9) methyltransferases SET-25 and SET-32, and the histone methylation-binding protein HPL-2 (Heterochromatin Protein 1-Like 2) were shown to be required for transgenerational RNAi inheritance [23,51]. However, how these factors affect the production of heritable small RNAs or change the length of RNAi responses was unknown until recently.

Feedback between H3K9me and Small RNAs

Interactions between small RNAs and histone modifications have been described in various organisms. For example, nuclear-acting small RNAs were found to induce H3K9 methylation in *Arabidopsis thaliana*, *Schizosaccharomyces pombe* [52], *Drosophila melanogaster* [53], and even in human tissue cultures [54]. In *A. thaliana* and *S. pombe*, nuclear small RNAs and trimethylation of H3K9 (H3K9me3) form a self-reinforcing feed-forward loop — nuclear small RNAs direct the deposition of H3K9me3 at peri-centromeric regions, and in turn H3K9me3 recruits the small RNA machinery to synthesize additional nuclear small RNAs [52]. Recently, a similar

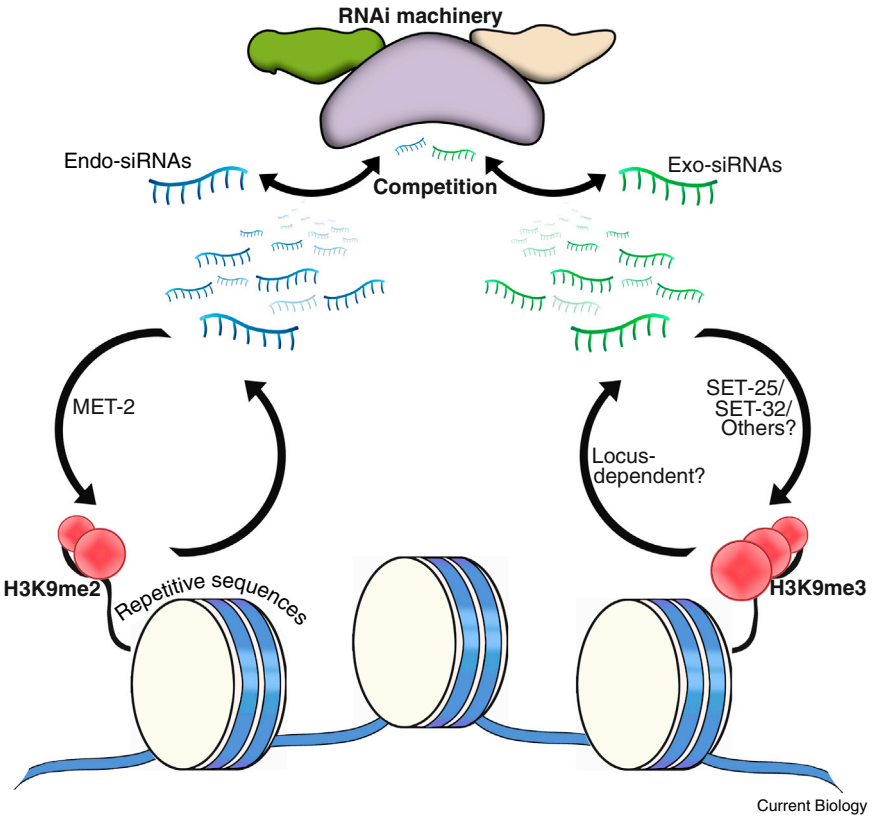

Figure 2. Endogenous and exogenous nuclear siRNAs interact with H3K9me and compete over shared RNAi machinery.
MET-2-dependent H3K9me1/2 and endo-siRNAs cooperate, possibility through a reinforcing feedback loop, to silence repetitive sequences and other genomic elements. SET-25, SET-32 and potentially other uncharacterized methyltransferases collaborate with dsRNA-derived exo-siRNAs to silence the targeted gene. Exo-siRNAs and endo-siRNAs compete over shared RNAi machinery, and accordingly inhibition of particular nuclear endo-siRNA pathways affects the intensity of exo-siRNA-mediated heritable RNAi silencing.

self-reinforcing loop was described between piRNAs and H3K9me3 in *D. melanogaster* [55,56]. The dependency of small RNAs on H3K9me3 in the abovementioned species, and the involvement of SET-25 and SET-32 in heritable RNAi in *C. elegans* suggested that H3K9me3 would promote small RNA biogenesis also in worms. We will now elaborate on new findings that show that in worms the relationship between histone marks and heritable small RNAs is more complex.

Small RNA Memory of Histone Modifications

In *C. elegans*, following RNAi induction, a small RNA-induced footprint of the 'repressive' histone modifications H3K9me3 and H3K27me3 can persist in the absence of the original dsRNA trigger for a few generations [57,58]. This observation suggested that, as seen in other organisms, chromatin modifications promote transgenerational inheritance of RNAi, and that the deposition of H3K9me3 could be responsible for the heritable transcriptional silencing. Still, a casual role for these histone marks was not established, and the specific functions that the marks might play in the inherited response remained unclear. Several studies have shown that the transmission of RNAi-induced silencing to the F1 progeny can occur even when the genomic locus that was exposed to the original RNAi responses is lost, implying that the chromatin marks on the locus that was silenced in the P0 generation are not pivotal for short-term inheritance [17,30,59,60]. However, it remained possible that inheritance of histone modifications is required especially for transgenerational RNAi.

The Role of H3K9me1/2 in RNAi Inheritance

Methylation of H3K9 is achieved in two consecutive steps in *C. elegans*. First, the MET-2 methyltransferase deposits mono- and di-methyl on H3K9, and then SET-25 adds the third methyl to create H3K9me3. In addition, careful mass spectrometry analysis has shown that in *met-2* animals SET-25 has some capacity to deposit residual H3K9me3 in the soma [61], and antibody staining showed that some levels of H3K9me3 were also found in the adult *met-2* germline [62,63]. Recent work examined how H3K9me affects the biogenesis of heritable small RNAs that target particular genomic loci.

We recently examined different H3K9 methyltransferase mutants that express *gfp* in the germline. The dynamics of RNAi inheritance after exposure of the parental generation to anti-*gfp* dsRNA were monitored. Surprisingly, *met-2* mutants, which are deficient in H3K9me1/2, exhibited enhanced multigenerational heritable silencing (lasting more than 30 generations), as well as stable inheritance of high levels of anti-*gfp* small RNAs, and H3K9me3 modifications on the *gfp* locus [25]. Stable transmission of RNAi to *met-2* mutant progeny depended on heritable small RNAs, as inheritance was terminated upon CRISPR-induced deletion of the germline-expressed nuclear argonaute HRDE-1. In addition, in *met-2* mutants a dramatic, genome-wide, and transgenerationally accumulating decrease in endogenous small RNAs targeting different genomic elements, such as repetitive elements, pseudogenes and specific protein-coding genes was detected. These findings suggested that *met-2* mutants are in general defective in endogenous small RNA inheritance. Interestingly, repetitive elements are enriched for H3K9me2 [64,65], and lose this chromatin mark in *ego-1* mutants, which are defective in the production of germline small RNAs [66]. Accordingly, in *met-2* mutants, repetitive elements are expressed [65,67]. Altogether, accumulative evidence supports the existence of a positive feedback loop between MET-2-mediated H3K9me1/2 and small RNAs, which could be essential for silencing of repetitive elements (Figure 2, Table 1). More research is required to uncover the identity and elucidate the function of specific effector proteins which are recruited to

Table 1. A list of factors involved in transgenerational small RNA inheritance.

Protein	Function	The type of heritable response influenced	Effect on inheritance	Mortal germline phenotype?
RRF-1	RNA-dependent RNA polymerase [104]	dsRNA-induced RNAi [24,45,57,104], anti-viral [9] and transposon heritable immunity [45]	Supports inheritance, could be involved in both the initiation and maintenance stages, contributes also to the synthesis of tertiary small RNAs [45]: exhibits a Motek phenotype [24]	N/A
EGO-1	RNA-dependent RNA polymerase [105]	dsRNA-induced RNAi [105], and transposon heritable immunity [45]	Supports inheritance, could be involved in both the initiation [105] and maintenance [45] stages of the small RNA responses, contributes also to synthesis of tertiary small RNAs [45]	Sterile [105]
RDE-4	dsRNA binding protein [106]	dsRNA-induced RNAi [59] and starvation-induced inheritance [10]	Required for initiation of heritable endo-siRNA and exo-siRNA responses [9,10,59]	N/A
PRG-1	Argonaute [107]	Heritable immunity against transposons [51,107,108]	Required for initiation of piRNA-induced heritable small RNAs [44]	Mortal germline phenotype [93]
HRDE-1	Nuclear Argonaute [47]	dsRNA-induced RNAi [47], starvation- [20] and high temperature- [109] induced inheritance, heritable immunity against transposons [23,51]	Required for maintenance of heritable small RNAs [47], and tertiary small RNAs [45], competes with CSR-1 [110,111]	Mortal germline phenotype in high temperatures [47]
CSR-1	Argonaute [46]	Self vs non-self-discrimination [103], tuning of maternal mRNA levels [49]	Required for inheritance of CSR-1-bound small RNAs [103], competes with HRDE-1 [110,111]	Sterile [103], or defective sperm in high temperatures [112]
PPW-1	Argonaute [113]	dsRNA-induced RNAi inheritance [113,114], rescues the Mortal germline phenotype of PRG-1 mutants [93]	Required for initiation of heritable RNAi [113,114], controls the duration of already initiated RNAi inheritance (exhibits a Motek phenotype) [24]	N/A
NRDE-1, 2, 4	Nuclear RNAi factors [47]	dsRNA-induced RNAi [47], transposon heritable immunity [23], and transgene silencing [23]	Maintenance of heritable small RNAs [47], and tertiary small RNAs [45]	Mortal germline phenotype at high temperatures [47]
MET-2	H3K9me 1/2 methyltransferase [61]	dsRNA-induced RNAi [25], heritable regulation of transposons [25], and transgene silencing [25]	Terminates heritable RNAi responses induced by dsRNA, and required for endo-siRNA inheritance [25]	Mrt phenotype [96]
SET-25	H3K9me3 methyltransferase [61]	dsRNA-induced RNAi [23,25], heritable regulation of transposons [23] and transgene silencing [71]	Required for maintenance of piRNA-induced RNAi [23], promotes some, but not all dsRNA-induced RNAi responses [23,25,72]	N/A
SET-32	H3K9me3 methyltransferase [23]	Heritable regulation of transposons [23]	Required for piRNA-induced heritable small RNAs [23]	N/A
HPL-2	Heterochromatin protein 1 homologue [115], binds methylated H3K9 [65,116] and H3K27 [117]	dsRNA-induced RNAi [51], heritable regulation of transposons [23]	Required for piRNA-induced heritable small RNAs [23]	Sterile [115]

Listed are proteins which function in either initiation, maintenance, or timing of small RNA inheritance.

H3K9me2, and participate in this positive feedback loop. The decrease in the overall levels of endogenous small RNAs that was observed in *met-2* mutants was hypothesized to make more protein resources, for example HRDE-1, available for exogenous small RNAs (Figure 2). A competition between the different arms of the RNAi system has been described in many cases [68–70]. In accordance with the 'competition hypothesis', *met-2* mutants were found to be hypersensitive to RNAi overall, regardless of the identity of the RNAi-targeted gene [25]. This shift in the balance in favor of the exo-RNAi pathway could explain the strong and stable RNAi inheritance observed in *met-2* mutants [25]. Importantly, RNAi responses were potentiated in *met-2* mutants also when genes were targeted for silencing in the cytoplasm, obviously independently of the chromatin status of the targeted locus [25].

The Effects of H3K9me3 on RNAi Inheritance Are Locus-Specific

While in *met-2* mutants every tested heritable RNAi response was prolonged, the duration of the inherited silencing in *set-25* mutants (defective in H3K9me3) depended on the identity of the RNAi-targeted gene. In *met-2;set-25* mutants, regardless of the characteristics of the target, SET-25 was dispensable for RNAi inheritance and RNAi-induced H3K9me3 methylation [25]. However, when foreign transgenes were targeted by RNAi in *set-25* single mutants, inheritance was weak, and no heritable small RNAs could be detected in the F3 generation [23,25]. Stochastic silencing of repetitive transgenes [61,71] and piRNA-guided transgene silencing [23] were also found to depend on SET-25. In contrast, dsRNA-induced heritable silencing of *oma-1*, an endogenous gene, was independent or even potentiated in *set-25* single mutants [25,72]. Even in *met-2;set-25;set-32* triple mutants, where no H3K9me3 could be found on the RNAi-targeted *oma-1* locus, heritable silencing of *oma-1* was observed [72]. Unlike *met-2* mutants, *set-25* mutants were found to have only minor alterations in the global pools of small RNAs targeting protein-coding genes [25]. It therefore appears that H3K9me3 affects heritable silencing of foreign and endogenous genes differently. Indeed the correlation between H3K9me3, small RNAs, and transcription is complex — only a fraction of the genome was found to lose H3K9me3 in *hrde-1* mutants, and only a small fraction of the loci that lost H3K9me3 in *hrde-1* mutants were also de-silenced [73]. Interestingly, also in *D. melanogaster* it was recently shown that knocking down of the linker histone H1 allows piRNA-targeted transposons to escape silencing, even when there is no reduction in the levels of H3K9me3 on the transposons [74]. These results suggested that H3K9me3 is not sufficient for repression, and that other factors which compact the chromatin play a central role in these gene silencing effects.

The distinct effects of H3K9me3 on heritable silencing of different genes could stem from specific genomic features that distinguish the different targeted loci. In *S. pombe* and *D. melanogaster*, particular DNA sequences were found to be important for inheritance of repressive epigenetic marks across cell divisions (not across generations) [75–77]. Similarly, in *C. elegans*, poly-A/T sequences that reside in introns shield from stochastic targeting [78]. In addition, SET-25-mediated and heat-induced heritable de-silencing preferentially affected pseudogenes and repetitive elements [71]. More research is required to elucidate how specific DNA sequences affect the transgenerational regulation of particular loci.

A Chicken and Egg Question

How are histone modifications affecting transgenerational silencing? Are chromatin marks the 'cog' or the 'cause'? [79]. The reigning model assumes that repressive chromatin marks mediate the silencing guided by heritable small RNAs. In *C. elegans* small RNAs were shown to bind to the nascent transcript and control gene expression by inhibiting Pol II elongation [80], and recently H3K9me3-independent transcriptional regulation of RNAi- and endo-siRNA-induced silencing was reported [25,72]. In addition, after exposure to dsRNA, in the progeny, heritable nuclear small RNAs are detectable before H3K9me3 is deposited on the RNAi-targeted locus [81]. Therefore, future studies are required to elucidate if the RNAi-induced heritable H3K9me3 and H3K27me3 footprints observed on the targeted locus initiate silencing directly, or alternatively, arise in response to the small RNA-induced inhibition of Pol II. A direct interaction between HRDE-1, a main factor in the nuclear small RNA machinery, and the histone-modifying enzymes, has not been found to date [82]. Several studies support a model whereby in certain cases transcription can dictate the chromatin status, and not vice versa. For example, inhibition of transcription by knockdown of *ama-1*, a Pol II component, results in loss of MET-1-dependent H3K36me3 methylation (a mark associated with gene expression) [83]. As H3K9me3 contributes to the biogenesis of certain small RNAs [25], the heritable effects observed in mutants that reduce H3K9me could arise indirectly, as a consequence of small RNA-induced inhibition of Pol II; the role of H3K9me3 in the process could be to regulate the positive feed-back loop that promotes additional rounds of heritable small RNA synthesis.

What Happens in Chromatin Stays in Chromatin?

In *S. pombe* it was shown that H3K9me3 marks deposited through site-specific tethering of Clr4, an H3K9 methyltransferase, can be stably maintained across cell divisions in the absence of Epe1, a putative histone H3K9 demethylase [84]. The maintenance of this induced H3K9me3 mark was found to be independent of *ago1* and *dcr1* [84,85]. RNAi-independent cis-inheritance of H3K9me3 was also described for endogenous loci [84,86]. It remains to be seen whether similar RNAi-independent inheritance of H3K9me3 can also occur in multicellular organisms, and in particular in animals which segregate their germline. In *A. thaliana*, the memory of a different type of epigenetic mark, CHH cytosine methylations on retrotransposons, was found to be memorized via mobile small RNAs. This DNA methylation mark is removed during reprograming, but is reintroduced later in development, guided by small RNAs that transfer to the sperm nucleus from the supporting vegetative nucleus [87,88]. Similarly, it is possible that the memory of parental histone modifications is transmitted transgenerationally by heritable small RNAs. One of the main scientific findings of the 20th century was that genetic inheritance is mediated by DNA, and not proteins [89,90]. As elaborated above, several lines of evidence indicate that small RNAs can be inherited transgenerationally in *C. elegans* — can chromatin marks direct their own

Box 2. Outstanding questions.

1. Which environmental challenges can initiate the synthesis of heritable small RNAs?
2. Which small RNA species are transferred from the soma to the germline?
3. Why do specific genes become targets of heritable endogenous small RNAs?
4. Which DNA features of the RNAi-targeted gene affect the dynamics of the heritable RNAi response?
5. Can histone modifications be inherited independently of small RNAs in *C. elegans*?

inheritance independently of small RNAs in this organism? More research is needed to establish if both chromatin modifications and small RNAs carry transgenerational information.

Which Mechanisms Maintain Transgenerationally Inherited Responses?

Inheritance of small RNAs and histone modifications were both proposed to give rise to the same observed transgenerational phenomena; for example, both small RNAs and histone modifications were suggested to be the agents that transmit heritable effects that change the progeny's life span and fertility [20,91–93]. Mutants defective in the production of certain small RNAs (for example, *prg-1* [93], *hrde-1* [47], *rsd-2/6* [94], *csr-1* [46], and *alg-3/4* [95]) and mutants in which histone modifications are disrupted (such as *spr-5* [92], *met-2* [96,97], *wdr-5.1* [91]) exhibit a mortal germline (Mrt) phenotype. Transient perturbations to the histone modification landscape, e.g. mutations in genes encoding the H3K4 tri-methylation enzymes, were shown to induce transgenerational inheritance of extended longevity that lasts for at least three generations in descendent wild-type progeny [91]. Starvation-induced developmental arrest [20] and fasting [98] were shown to extend the lifespan of the progeny, and its resistance to stress, respectively. Starvation-induced developmental arrest led to changes in the heritable pools of small RNAs targeting various metabolic genes [20]. Recently it was shown that the transgenerational Mrt phenotype of *met-2* mutants is rescued (the mutants become fertile again) if the mutant's transgenerationally accumulated disruptions in small RNAs are reset [25]. Thus, at least in this case, the heritable phenotype of the chromatin mutant was shown to depend on small RNA inheritance.

The Gene Targets of Heritable Small RNAs

Different environmental stresses give rise to changes in heritable small RNAs, potentially regulating specific genes that in turn improve the progeny's chances to cope with similar stresses [9,20]. For example, expression of a transgene-derived virus [17], or infection with a replicating vesicular stomatitis virus (VSV) [99] triggers the production of heritable small RNAs that protect the next generations of worms from similar viral sequences. The capacity to regulate viruses transgenerationally probably depends on the intensity of expression of the viral genes, or on the tissues in which the viral genes are transcribed (especially the capacity of the virus to replicate in the germline) [100,101]. Similarly, starvation-induced developmental arrest leads to transgenerational inheritance of small RNAs that regulate nutrition-related genes [20]. Recently, an intergenerational heritable response to osmotic stress was described. Parental worms exposed to osmotic stress were found to protect their immediate F1 progeny from osmotic stress by increasing the expression of the glycerol biosynthetic enzyme GPDH-2 [102]. It would be intriguing to elucidate the mechanism that regulates *gpdh-2* in the F1 progeny (any secreted factor might be involved). While in the cases of the above-mentioned heritable responses the production of heritable small RNAs could be adaptive, in other cases (perhaps when it comes to temperature-induced heritable responses) the adaptive value of the inherited small RNAs is less obvious. It is possible that the environmental stress disrupts the pool of heritable small RNAs, and such damage may be carried over to the next generation, exerting physiological effects that are not necessarily adaptive, and could even be detrimental.

In contrast to the gene-specific silencing induced by dsRNA, exposure to environmental challenges leads to complex heritable responses. Such inheritance involves both downregulation and upregulation of multiple genes, presumably via both direct and indirect effects. Heritable small RNAs can directly enforce both up- and down-regulation of genes, as heritable endo-siRNAs that associate with HRDE-1 induce gene silencing [47], while CSR-1-bound heritable small RNAs were suggested to promote transcription [103]. However, indirect, or secondary effects of heritable small RNAs compound the interpretation of inherited gene expression changes. Changes in the levels of specific small RNA species affect the levels of other types of endogenous small RNAs, since the different pathways compete over shared small RNA biogenesis components [70]. Moreover, sequencing of small RNAs collected from entire animals masks tissue-specific or cell-specific effects.

In summary, we predict that in the near future additional important findings will improve our understanding of the principles that govern heritable responses (see Box 2 for outstanding questions). The use of isogenic model animals such as *C. elegans* nematodes will be instrumental for distinguishing heritable information that is carried by the DNA from memory kept in other inherited molecules.

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