Trends in Genetics

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Review

A Matter of Time: Small RNAs Regulate the Duration of Epigenetic Inheritance

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Small RNAs are increasingly emerging as transgenerational carriers of epigenetic information in *Caenorhabditis elegans* and in other organisms. Recent studies have identified factors that are required for the inheritance of small RNAs and for heritable RNAi in worms, which typically persist for a finite number of generations. We examine here recent insights into the mechanisms that control the duration of transgenerational inheritance of small RNAs. We discuss current understanding of two types of regulatory mechanisms: those that prolong RNAi inheritance through amplification and maintenance of heritable small RNAs, and those that limit the persistence of ancestral RNAi by, for example, employing negative feedback loops to reset the transmission of epigenetic information. Collectively, these machineries result in the precise and intricate regulation of small RNA inheritance across generations.

Trends

With a short generation time and a well-studied RNAi machinery, *C. elegans* provides an excellent model system to study the rules that govern transmission of small RNAs across generations.

Recent findings reveal specialized factors and dedicated mechanisms that function in transgenerational transmission of RNAi responses, suggesting that this is a regulated process rather than an epiphenomenon.

Amplification of small RNAs via RNA-dependent RNA polymerases, and competition between different small RNA species, emerge as key processes that determine the potency and duration of heritable RNAi responses.

Transmission of Epigenetic Information across Generations

According to Weismann's germplasm theory, germ cells, which are largely isolated from environmental influences, remain singular in their ability to transmit information to future generations [1]. Over the past several decades it has become increasingly clear that the germ cell's capacity to disseminate information relies not only upon the inheritance of DNA, and its particular epigenetic marks, but also upon the **transgenerational** (see Glossary) propagation of different macromolecules such as small RNAs which, much like canonical epigenetic modifications, are ultimately capable of affecting gene expression. Similarly to chromatin modifications, small RNAs can be created, deleted, modified, targeted, or replicated based upon environmental events, and subsequently persist to modulate gene expression over several generations [2,3].

To become heritable, small RNAs must display several essential characteristics. First, small RNAs must avoid removal by dedicated reprogramming machineries that have evolved specifically to promote the removal of epigenetic markers – allowing subsequent generations to function with a 'clean slate' [4]. For example, in the germline or early embryonic stages of many species, extensive reprogramming events entail the widespread removal of chromatin marks, creating a significant barrier to the transgenerational inheritance of epigenetic information. Importantly, however, reprogramming often remains incomplete – allowing selective transmission of epigenetic marks [5–8]. This selective transmission may actively control the passage of heritable epigenetic information across generations.

A second important requirement for the successful transmission of information transgenerationally is that the epigenetic agent which is transmitted across generations (e.g., small RNAs) must be capable of accurately templating its own synthesis. Biological systems utilize a diverse number of feed-forward loops designed to preserve epigenetic memories over time [9,10]. Currently it is not clear which epigenetic marks can self-replicate with high fidelity – a prerequisite

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for endurance over the course of multiple cell divisions and, in particular, across meiosis [11]. Moreover, specific quality control mechanisms or pathways most likely exist to protect the cells from mistakes in the transfer of epigenetic information.

Thus, while data strongly suggest the existence of transgenerational inheritance of epigenetic information, the principles that guide non-DNA based information transmission across generations remain largely unknown. Moreover, it remains to be determined for how long (or for how many generations) particular epigenetic effects can persist. In this review we discuss the mechanisms that control small RNA-mediated RNAi inheritance and how it achieves its heritability and maintains its fidelity. We specifically focus on the dedicated pathways that determine the persistence of epigenetic responses, and hypothesize why there must be limits on their duration.

RNAi Inheritance in *C. elegans*

Alongside the discovery that double-stranded RNA (dsRNA) was the substrate for RNAi (the timeline is given in Figure 1), it was simultaneously observed that dsRNA-induced silencing spreads robustly both between tissues and from parents to untreated progeny [12]. However, these phenomena seemed mechanistically distinct: some of the proteins required for the processing or utilization of small RNAs in the parent were not required for silencing in the progeny [13]. For example, the dsRNA-binding protein RDE-4 and the Argonaute protein RDE-1, which promotes the maturation of small RNAs from chopped dsRNA molecules, are necessary for establishing RNAi. Surprisingly, however, both RDE-1 and RDE-4 were found to be dispensable for RNAi inheritance in subsequent generations. Thus, different protein components must be required for the initiation and for the maintenance of heritable RNAi responses. Intriguingly, the same study demonstrated that, after the initiation process, worms can transmit the silencing signal to their immediate offspring even when the parents lack a genomic locus that corresponds to the targeted sequence. Recently it has been shown that, even in worms that lack a corresponding genomic locus at the initiation step, extracellular dsRNA can be transmitted to the progeny and initiate silencing at the next generation [14]. While we know today that RNAi also operates in the nucleus [15], and that specific histone modifications play a role in RNAi [15], the dispensability of a genomic template for transmission of silencing to the immediate progeny suggests that heritable small RNAs might be sufficient for **intergenerational** RNAi.

In addition to heritable small RNAs, RNAi also generates a heritable trace of histone methylations [e.g., histone H3 lysine 9 (H3K9) and H3K27 trimethylation] that decorate targeted loci in progeny [16–18]. While the roles for the different histone modifications in the transgenerational silencing of RNAi are still not clear, it has been shown that heritable small RNAs are detectable in the progeny before the appearance of these chromatin marks (i.e., at earlier developmental times) [15]. This suggests that heritable small RNAs may actually direct the deposition of chromatin modifications. Experiments in other organisms suggested that this sequence of events might be conserved and that heritable small RNAs indeed direct the deposition of chromatin modifications. Moreover, in several organisms (such as fission yeast and plants) it has been shown that a feed-forward loop between small RNAs and chromatin marks (in plants, also DNA methylation) is required for maintenance of silencing [9,19–21]. These findings suggest that small RNAs may have the capacity to prime the cell for transgenerational epigenetic chromatin markings.

Importantly, in *C. elegans*, RNAi inheritance is not limited to the immediate progeny. Targeting a germline-expressed gene usually leads to the inheritance of silencing for 3–5 generations. In fact, worms can sustain surprisingly long RNAi responses – lasting over 80 generations – but because most individuals in a lineage eventually lose the heritable silencing, such long-term inheritance is

Glossary

Endogenous small RNAs: small RNAs that are encoded in the genome or are amplified based on genomically encoded small RNAs. In *C. elegans*, there are three main species of endogenous small RNAs: microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), and 'endogenous' small interfering RNAs (endo-siRNAs). Such endogenous small RNAs are abundant and are predicted to regulate the expression of thousands of coding and non-coding transcripts.

Exogenous siRNAs (exo-siRNAs): small interfering RNAs that are generated from an exogenous dsRNA trigger.

Intergenerational versus transgenerational: transgenerational epigenetic inheritance involves changes that persist to affect progeny that were not exposed to the original trigger (neither in the uterus nor as germ cells). When progeny are directly exposed to the environmental effect, the effect is not 'transgenerational' but 'intergenerational'.

***motek* genes:** modified transgenerational epigenetic kinetics genes. Genes that influence the duration of transgenerationally inherited silencing of small RNAs and that are, by themselves, regulated by endo-siRNAs.

Primary and secondary small RNAs: primary small RNAs (either endogenous or an exogenous) can be gnomically encoded or derive from exogenously supplied dsRNA (which can be provided, for example, by feeding the worms with bacteria that express dsRNA). Primary small RNAs can recruit RdRPs to complementary targets, and thus trigger amplification of additional 'secondary' small RNAs. Secondary (or 'amplified') small RNAs are typically 22 nt in length and possess guanosine as their first nucleotide (22G).

RNA-dependent RNA Polymerases (RdRPs): the proteins that synthesize amplified small RNAs based on an RNA template. In *C. elegans*, there are four known RdRPs: EGO-1, RRF-1, RRF-2, and RRF-3. The function of RdRPs is crucial for the process of transgenerational inheritance of small RNAs in worms.

so far limited to a case in which worms that silence the targeted gene were selected for in every generation. Maintenance of such long-term silencing was found to depend upon the activity of genes that affect chromatin [22]. Thus, in some cases, through an interaction with chromatin modifiers, small RNAs can induce semi-stable heritable silencing. However, as indicated above, this dramatic, long-term silencing is relatively rare, and careful quantification of the duration of various RNAi responses suggests that, at the population level, RNAi responses almost inevitably diminish drastically after 3–5 generations. This stereotypic limit on the duration of heritable silencing described by Alcazar *et al.* was dubbed 'the bottleneck to RNAi inheritance' [23].

Second trigger: an additional RNAi-inducing dsRNA trigger which differs in its sequence from the first trigger (that triggered RNAi in a previous generation). Thus, the second trigger is administered to progeny that inherit from their parents a different heritable RNAi response (targeted against a different gene). Second triggers enhance the silencing effect and prolong the duration of the ancestral RNAi responses.

Transgenerational: see intergenerational.

Biogenesis of Amplified Small RNAs

In *C. elegans*, different types of **primary small RNAs** – for example, the **exogenous siRNAs** (exo-siRNAs) that are 'diced' directly from exogenously provided dsRNA by the sole worm Dicer protein, DCR-1 – are capable of guiding **RNA-dependent RNA polymerases** (RdRPs) to targeted RNA molecules. As a result, primary small RNAs can induce the synthesis, or amplification, of the much more abundant **secondary small RNAs**, which are predominantly 22 nt in length and usually possess a guanosine as their first nucleotide (22Gs). These 22G **endogenous small RNAs** in turn carry forward the execution of silencing [24,25]. We have some understanding of the mechanisms and proteins required for 22G synthesis: for example, the *C. elegans* genome encodes four RdRP proteins: EGO-1 and RRF-1, 2, and 3. Of these, RRF-3 acts in the biosynthesis of primary small RNAs [24] and is irrelevant for the amplification step, while the function of RRF-2 has not yet been identified [26]. By contrast, both polymerases RRF-1 and EGO-1 are required for the synthesis of secondary small RNAs [24,26].

22Gs can also be synthesized by alternative mechanisms: PIWI-interacting RNAs (piRNAs; 21Us in *C. elegans*) and primary endogenous siRNAs ('26G endo-siRNAs'), similarly to primary exogenous siRNAs, recruit RdRPs (rrf-1 and ego-1) to different endogenous targets where they also induce the synthesis of 22G secondary siRNAs (the different pathways are summarized in Figure 2).

Through largely unknown mechanisms, specific 22Gs become sorted onto multiple, different Argonaute proteins where they each mediate diverse – or in some cases opposing – cellular responses [27–29]. Intriguingly, the *C. elegans* genome encodes 26 different Argonaute proteins [30], an observation that emphasizes the extent to which interactions of 22Gs with specific species of Argonautes may ensure a tight degree of regulatory control over gene expression patterns.

The RNAi Inheritance Machinery

Genetic screens for factors that are required specifically for the inheritance of dsRNA-induced silencing, but not for RNAi *per se*, have revealed several proteins that function in the nucleus [15,31]. Of particular interest is the Argonaute HRDE-1/WAGO-9 (Heritable RNAi deficient-1 or Worm-specific Argonaute-9), which shuttles between the cytoplasm – the likely site of RdRP activity – and the nucleus, where small RNAs act to affect transcription. HRDE-1 has been found to physically associate with and carry both dsRNA-derived exogenous 22G siRNAs and endogenous 22Gs. Importantly, HRDE-1 is required for carrying amplified 22G siRNAs in the germline across generations [31,32]. Although gene silencing is initiated only in a small number of founders, it is faithfully distributed to a logarithmically growing number of descendants. It is thus not surprising that RNAi inheritance depends on 22Gs, the RdRP-amplified small RNAs, which in theory can be replenished in every generation [16,33,34] (see more below on the limits of RdRP-mediated amplification). In fact, it is possible that only small RNA species that can direct RdRP-mediated amplification reactions, and which are carried over to the next generations by dedicated Argonautes such as HRDE-1, can produce long-term transgenerational RNAi responses. For example, microRNAs typically do not trigger the amplification of secondary

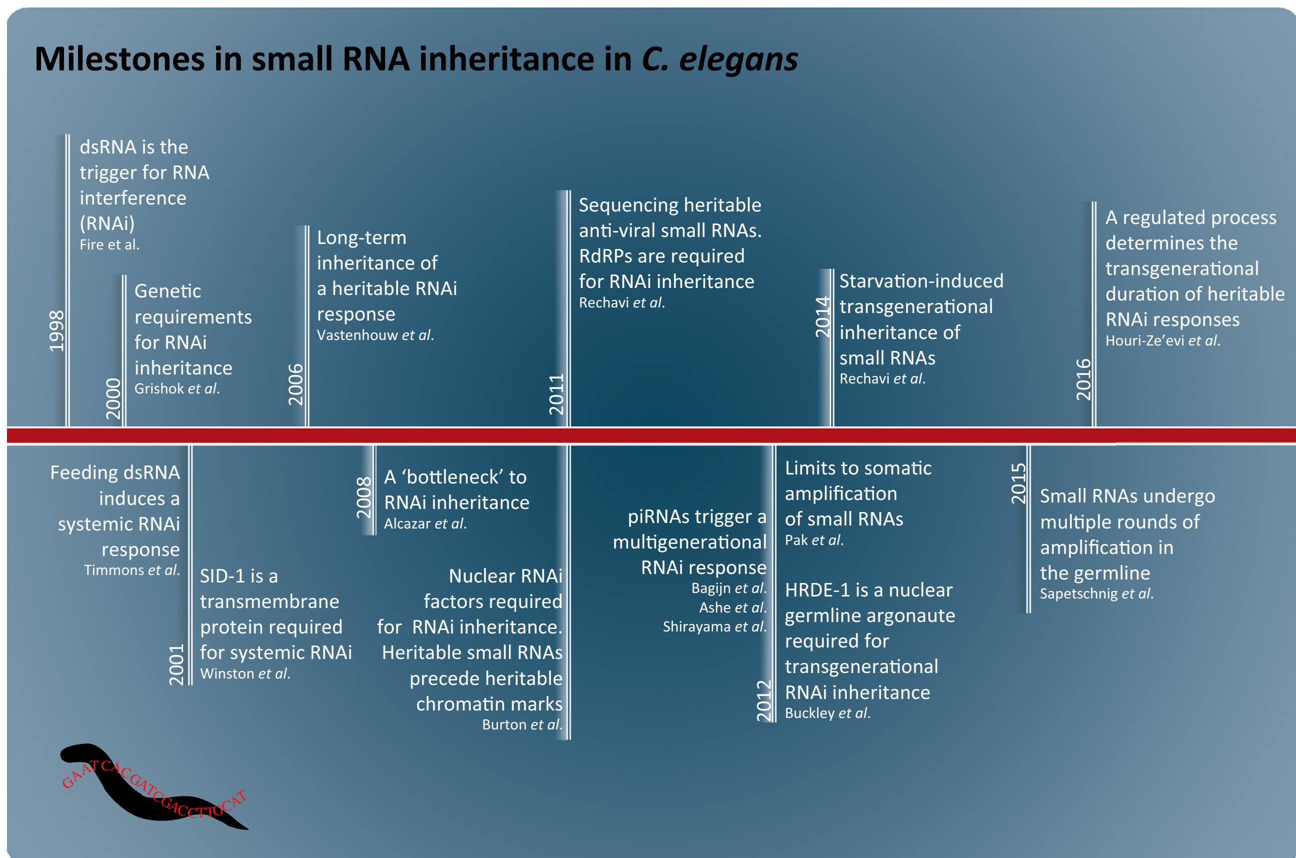

Figure 1. Milestones in Discoveries on Small RNA Inheritance in *C. elegans*. The ease of application of RNAi in *C. elegans* nematodes has opened the way for the study of transgenerational transmission of small RNAs across many organisms. We detail here the major discoveries regarding the transgenerational transmission of small RNAs in *C. elegans*, which remains one of the leading model organisms in the field.

siRNAs, and, perhaps as a consequence, are less likely to produce potent long-term heritable epigenetic responses in worms. There are exceptions to the rule, however: at least one microRNA, miR-243, has been shown to trigger the amplification of 22G endo-siRNAs [35]. Moreover, it is possible that microRNAs (or other types of small RNAs, such as tRNA-derived fragments, which were recently shown to transmit intergenerationally in mice [36,37]), can initiate additional signaling cascades, which can then generate stable silencing through feed-forward dynamics.

Dilution Versus Active Control of Heritable RNAi

Many questions surrounding the dynamics of RNAi inheritance remain unanswered. For example, is the extent of inheritance constrained by an actively regulated process, or does it depend only on passive decay? When RNAi persists for longer times, does it become maladaptive? (see Outstanding Questions). Experiments have consistently shown that, characteristically, exo-RNAi responses at the population level terminate after 3–5 generations [23], leading to the initial hypothesis that parental RNAi effects and the small RNAs that mediate silencing simply become 'diluted' over several generations. Alternatively, because the duration of RNAi affects the fitness and survival of the organism, a limit on the duration of RNAi inheritance responses might represent an actively regulated process. A recent study tested the possibility that a regulated process, rather than passive dilution, controls the dynamics of exogenous RNAi inheritance. This study hypothesized that a mechanism might exist that would enable RNAi inheritance in the

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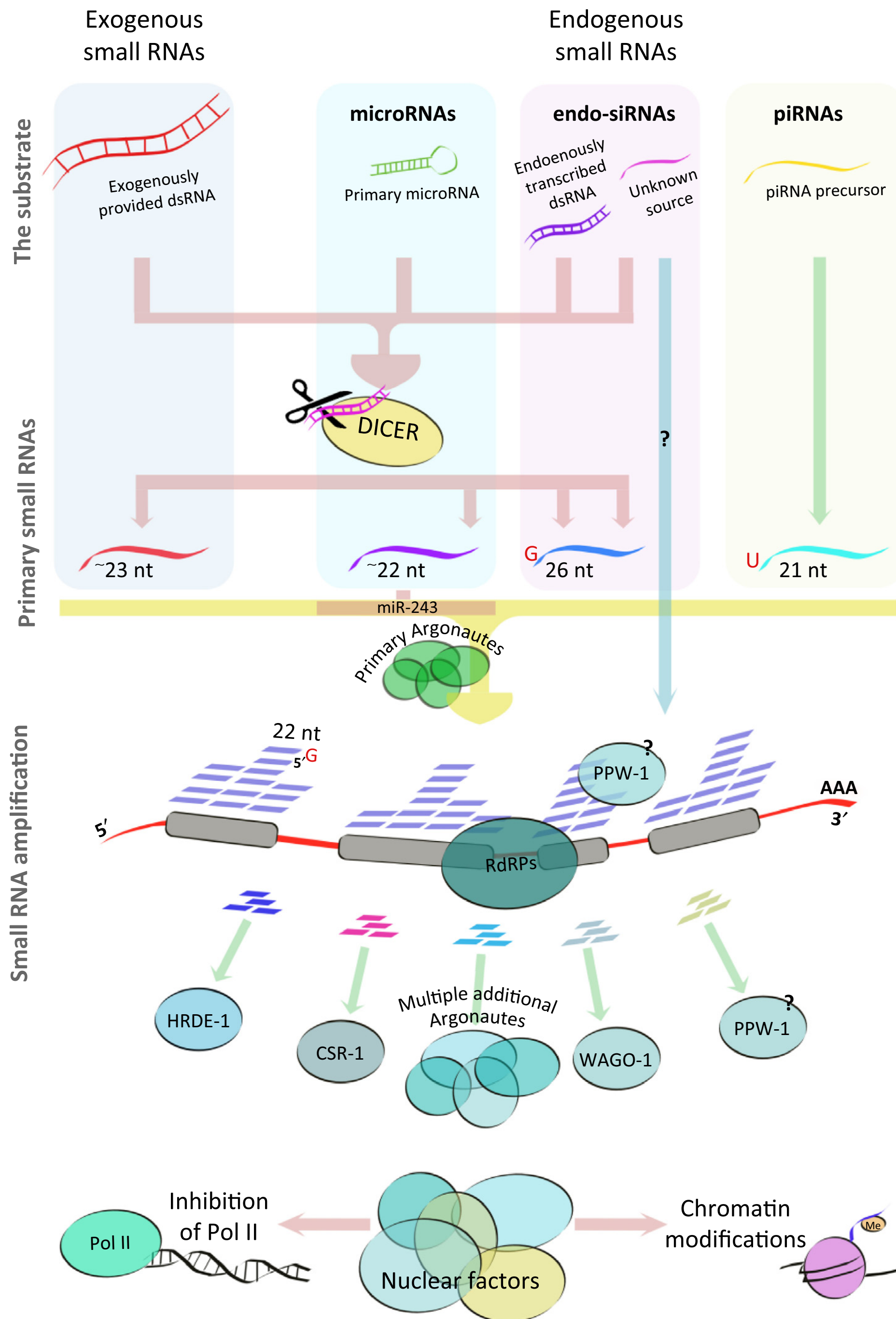

Figure 2. Different Small RNA pathways in *C. elegans.* The exogenous and endogenous pathways of small RNAs converge upon shared protein factors along the course of their processing and ultimately enforce gene silencing. piRNAs, endo-siRNAs and exo-siRNAs (as well as one known miRNA) engage RNA-dependent RNA polymerases (RdRPs) to direct the synthesis of secondary '22G' small RNAs. By guiding diverse Argonaute proteins to their targets, secondary small RNAs mediate RNAi. Secondary small RNAs establish interference by inhibiting elongation by RNA polymerase II (Pol II) and by recruiting chromatin modifiers to the corresponding DNA sequences to instruct the deposition of specific histone marks.

parental generation, but actively terminate it in the progeny. To test this, the authors examined whether activation of the RNAi system in the progeny would extend the transgenerational duration of exo-RNAi responses that originated in previous generations [38]. To this end, they triggered RNAi against *gfp* by exposing transgenic worms that express GFP in the germline to anti-*gfp* dsRNA, and then challenged their F1 progeny with a second, dsRNA trigger matching an unrelated sequence (referred to as the **second trigger**). In these experiments, different dsRNAs, such as anti-*mCherry* dsRNA, were used as second triggers. Although different genes were targeted in the parents and in the progeny, the authors found that exposing the progeny to second triggers significantly extended the duration and the potency of the heritable RNAi responses that were elicited in the parents by the first dsRNA trigger. These experiments indicated that dilution is not the sole or even the main factor that determines the transgenerational duration of heritable RNAi. Furthermore, the authors discovered that constant exposure of the progeny to RNAi triggers, for multiple generations, and against different genes, further prolongs the inherited response. Sequencing of the small RNAs from worms that were exposed to one or two triggers revealed that exogenous RNAi responses change not only the expression of their sequence-specific targets but also the levels of various additional genes.

Intriguingly, it was found that exo-RNAi episodes tilt the balance between the levels of different endogenous small RNA species, which often compete for common protein resources. Specifically, the levels of endogenous small RNAs that target genes functioning in RNAi processes were altered. From these data it was hypothesized that self-regulating small RNAs could constitute a feedback mechanism that potentially affects the dynamics of RNAi inheritance. Indeed, manipulating genes that are targeted by these differentially expressed small RNAs affected the transgenerational duration of RNAi inheritance. The authors named genes with such a phenotype ***motek* genes** (for modified transgenerational epigenetic kinetics). For example, whereas RNAi responses normally last for only 3–5 generations, progeny of *ppw-1* heterozygous animals, a *motek* gene that is affected by endo-siRNAs that change their levels following exo-RNAi induction, inherit RNAi for much longer durations. PPW-1 is one of the many worm Argonautes, and the process by which it functions to affect transgenerational silencing is still unknown.

How could the mechanisms that tune the duration of particular heritable RNAi responses work? Long-term and multigenerational inherited silencing depends on nuclear RNAi genes and correlates with preservation of high levels of 22G small RNAs in the progeny [34]. Thus, factors that affect the balance between forces that decay or amplify specific small RNAs – forces that must abate in strength across generations – may determine the lifespan of heritable RNAi responses.

Overcoming the ‘Reprogramming’ of Small RNAs

Very little is known about events that lead to the ‘erasure’ or reprogramming of parental small RNAs in progeny [4]. This gap in knowledge regarding small RNA reprogramming stands in contrast to our extensive understanding of the mechanisms that remove parental chromatin modifications in the germline. In several organisms, to allow proper development, parental chromatin modifications are removed in the germline by both active and passive processes [39]. In worms, chromatin reprogramming is not required for viability; however, dedicated reprogramming mechanisms do exist. For example, the LSD1 ortholog gene *spr-5* [40] appears to act as a reprogramming agent in *C. elegans*, erasing H3K4 dimethylations in the germline. This reprogramming is not required for immediate viability but, in the absence of *spr-5*, the worms become sterile after several generations [40]. Chromatin reprogramming is achieved also through passive mechanisms because physical bottlenecks such as dilution and histone turnover during DNA replication limit the preservation of ancestral histone marks [41].

It is likewise possible that heritable RNAi responses decline over time in worms because of 'RNA reprogramming' that drives the active degradation of small RNAs. If such mechanism exists, changes to the pathways involved should yield heritable RNAi dynamics that differ from the gradual decrease in small RNA levels that is expected following simple dilution as cell divisions occur. Future screens for mutants that inherit RNAi for longer durations could reveal genes that actively function in heritable siRNA destruction, allowing a distinction to be made between active and passive mechanisms of regulation.

To date, researchers have identified several potential genes that may either promote or protect specific small RNAs from active degradation in *C. elegans*. For example, the enzyme CDE-1 modifies small RNAs that are carried in the germline by the Argonaute CSR-1 (but not by the Argonaute HRDE-1), adding untemplated uridine nucleotides to their 3′ ends, destabilizing the RNA molecules, and shortening their lifespan [42]. By contrast, the 3′ ends of other types of small RNAs are actively protected from these types of untemplated tailing. For example, 26G primary endo-siRNAs and piRNAs can be protected by 3′ methylation catalyzed by the enzyme HENN-1 [43,44]. Importantly, these studies suggest that multiple, distinct mechanisms may have evolved to regulate particular species of heritable small RNAs.

The persistence of heritable small RNAs depends not only upon their degradation and dilution but also upon the rate of their synthesis. The main contributor to this synthesis is the RdRP-mediated amplification process.

Limits to Small RNA Amplification

RdRPs, as are other biological amplification systems, are designed to curb uncontrolled responses. Indeed, it appears that there are true differences in RdRP activities in different tissues, and distinctions in the ability of particular small RNA species, or specific mRNA targets, to support RNAi amplification reactions.

In the Soma

Feed-forward amplification of exogenous, dsRNA-induced silencing is strictly limited in the soma. Primary small RNAs, which do not lead directly to silencing, guide RdRPs to their targets, resulting in the production of secondary small RNAs. In turn, amplified 22Gs (secondary siRNAs), which do enforce silencing, are prevented from triggering additional rounds of amplification themselves and cannot recruit RdRPs to additional mRNA targets (if such a process does occur, it is extremely inefficient [45]). Therefore, by entrusting specific roles to the different RNA molecules in the RNAi cascade, only one round of amplification is allowed for each dsRNA-induced trigger. Provided that both the primary small RNAs and the targeted mRNAs are present, silencing can be maintained; however, depletion of either one will terminate the reaction. This fundamental limitation restricts somatic responses from being preserved over long intervals and prohibits RNAi from affecting the next generations.

It is still not clear how the separation between primary and secondary small RNAs is established, or why only primary small RNAs can direct amplification. DCR-1 cleavage of the dsRNA trigger yields primary small RNAs that are structurally different from RdRP-synthesized 22G secondary small RNAs. In primary small RNAs the 5′ end is mono-phosphorylated, whereas in secondary small RNAs the 5′ end is tri-phosphorylated. This difference, together with other structural differences or distinct associations of particular species of small RNAs with specific proteins during their biosynthesis, leads to differential loading of primary and secondary siRNAs on separate Argonautes. In the soma, primary siRNAs bind to the Argonaute RDE-1, while secondary siRNAs bind to multiple other WAGO (worm-specific) Argonautes [30]. It is possible that the Argonautes that bind to secondary siRNAs in the soma cannot direct RdRPs to new mRNA targets.

In the Germline

Because heritable RNAi responses correlate with the presence of abundant 22G small RNAs across generations, amplified small RNAs must somehow be capable of directing additional rounds of amplifications to maintain a long-term response. Indeed, a recent study found that in contrast to the soma, piRNAs that act in the germline are capable of triggering biosynthesis of secondary 22G RNAs that can lead to spreading of the amplification reaction along mRNA targets. Moreover, these 22Gs can guide additional rounds of amplification. These additional rounds of amplification create 'tertiary' 22G RNAs which trigger amplification on other mRNA molecules *in trans*. While the production and stability of secondary small RNAs within the same generation do not depend on nuclear RNAi factors, tertiary small RNAs, which can continue to trigger more and more rounds of amplification, cannot be detected in nuclear RNAi mutants. Thus, tertiary small RNAs, once synthesized, become independent of the original 21U piRNA that initiated the response, and can persist indefinitely [34].

The same study suggested that, in the germline, dsRNA-derived exogenous siRNAs, can, similarly to piRNAs, induce semi-stable transgenerational silencing by establishing an amplification loop that continues to produce 22Gs in each generation [34]. The dsRNA-induced response that was examined in this particular study was shown to be maintained for four generations. It is not clear whether dsRNA-induced silencing can last longer because, typically, at the population level most exo-RNAi responses do disappear eventually (3–5 generations). Similarly to piRNAs, exogenous dsRNA triggers were shown to initiate amplification of small RNAs that spread along the length of the targeted mRNA – and even between two genes that form part of the same operon. However, while it was convincingly demonstrated that piRNAs can induce amplification of 'secondary' small RNAs that will, in turn, induce the production of 'tertiary' small RNAs in *trans*, it is still unknown whether exogenous, dsRNA-derived, exo-siRNAs can guide RdRPs in *trans* to separate mRNA molecules that do not originate from the same primary transcript. Thus, it remains to be discovered whether the capacity to act in *trans* is unique to piRNA-initiated secondary (and tertiary) small RNAs, or whether other types of 22Gs can also trigger similar amplification responses. In the future, it would be intriguing to learn whether fundamental differences exist between the amplification reactions that different primary small RNAs give rise to. Similarly, the differences in the limits of RdRPs that were observed in different tissues call for further investigation.

Competition between Different Species of Small RNAs

Multiple lines of evidence show that different primary species of small RNAs (exo-siRNAs, 26Gs, piRNAs, and microRNAs) share and compete over common resources and biosynthetic pathways [46,47]. Amplification triggered by piRNAs, endo-siRNAs, and exo-siRNAs also converges on the same limited factors (e.g., RdRPs) that eventually synthesize secondary 22G siRNAs. Because common machineries are used, it is very likely that amplification directed by different primary small RNA species is limited by the production of RNAi responses that are instigated by other competitive primary small RNA triggers. It is thus possible that the kinetics of particular RdRP-mediated amplification responses are context-specific, and are determined by the workload of multiple other RdRP-mediated amplification reactions. The semi-stable silencing that piRNAs trigger, for example, could come at the expense of other heritable RNAi responses, such as transgenerational responses initiated by exogenous dsRNA-derived siRNAs.

A recent study showed that activation of the exogenous RNAi inheritance pathway by administration of dsRNA tilts the balance toward inheritance of exo-siRNAs at the expense of endo-siRNAs [38]. Furthermore, induction of new exo-siRNA responses by different dsRNA 'second triggers' resulted in an overall, non-gene-specific, and RdRP-mediated amplification of ancestral, unrelated heritable exo-siRNAs [38]. One current working model conjectures that heritable exo-RNAi responses terminate, in part, owing to the endo-RNAi machinery gradually regaining

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Key Figure

Maintenance and Termination: Two Opposing Forces Determine the Duration and Potency of Transgenerational Inheritance of Small RNAs in the Worm

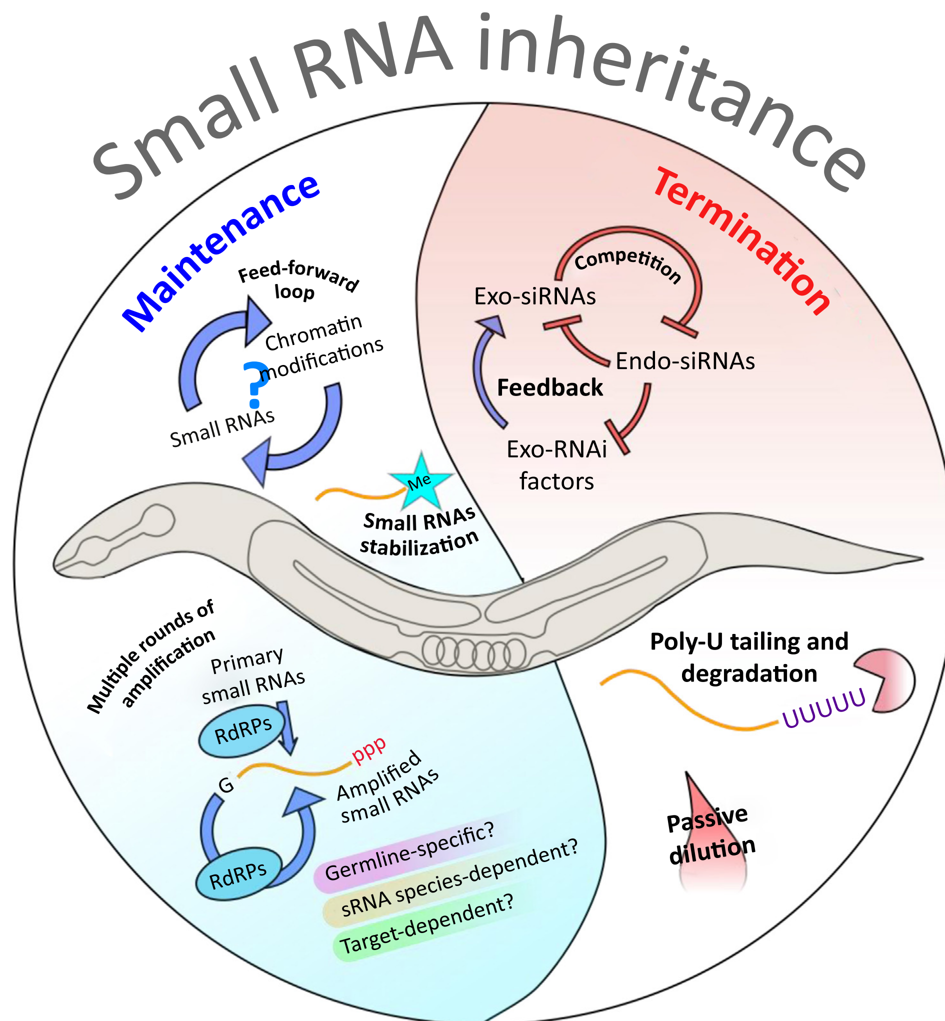

Figure 3. Maintenance of transgenerational small RNA responses involves RNA-dependent RNA polymerase (RdRP)-mediated amplification, a putative feed-forward loop between small RNAs and chromatin modifications, and potentially the stabilization of small RNAs by terminal 2′-*O*-methylation. Termination of the response is mediated by the degradation and passive dilution of the small RNAs, competition between different small RNA species, and feedback regulation of endo-siRNAs on RNAi factors.

its balance across generations, after the external source of dsRNA is depleted, and in the absence of new challenges by exo-siRNAs. According to this model, a new supply of different endogenous small RNAs is constantly provided because endogenous small RNAs are transcribed from the genome and accumulate over time. These endo-siRNAs progressively occupy

the shared protein components, including the amplification systems, and as a consequence exo-siRNAs responses eventually terminate. Alternatively, or in parallel, changes in endo-siRNA levels may directly terminate exogenous RNAi responses after several generations via a negative feedback loop between endogenous small RNAs and RNAi genes.

As described above, the kinetics of this process appear to be regulated by direct manipulation of *motek* genes. These genes affect epigenetic processes and are regulated by endogenous small RNAs that change in numbers following dsRNA-induced silencing. Differentially expressed endo-siRNAs could manipulate the levels of *motek* genes that affect exo-siRNA inheritance, and thus change the duration of the inherited response. Characterizing the exact molecular nature of such 'transgenerational timers' is a major challenge for the future (summarized in Figure 3, Key Figure).

Concluding Remarks. Time is Relative: The Regulation of RNAi Inheritance in Different Animals

Because of the short time interval (about 3 days) that elapses between *C. elegans* generations, naturally-occurring but transient changes in their environment, such as food shortages or seasonal shifts in temperatures, could easily persist across multiple consecutive generations [33,48–50]. Under such circumstances, evolutionary changes that favor the multigenerational transmission of RNAi would often be beneficial. An adaptive change in gene expression would increase overall fitness if detected and initiated in the parental generation and maintained for several successive generations, while the environmental change still persists. However, because such changes are often temporary, it could be necessary to restrict the epigenetic inheritance of the responses of the parents to the appropriate number of generations [49,51]. Unrestricted carry over of every *ad hoc* ancestral response would be harmful because in many instances the environments of the offspring and more distant ancestors will become increasingly incompatible. Experimental designs that aim to address these specific questions directly, by examining the duration of heritable RNAi effects in response to ecologically-relevant environmental challenges, should be invaluable in the future.

Multigenerational RNAi inheritance has been convincingly demonstrated in plants, which also synthesize RdRPs [5]. Plants are sessile organisms and, like worms, can rapidly produce offspring (e.g., ~6 weeks until maturity in *Arabidopsis*). These characteristics predict that many generations of both worms and plants may often experience the same surroundings. This may suggest that multigenerational RNAi will prove limited to animals with a fast reproductive cycle (during which the environment conditions are not likely to change) or with limited migratory capacities. In worms, exogenously triggered RNAi typically lasts for three to five generations, unless the exo-RNAi system is consecutively reactivated in the RNAi-inheriting generations. Perhaps RNAi inheritance in 'higher' organisms, which reproduce slowly, and can travel far, is limited in time. In general, transmission of epigenetic modifications has not yet been shown across multiple generations of mammals. Even so, a search for other feedback loops, analogous to the pathway discovered in worms [38], might reveal mechanisms that enable prolongation of inherited RNAi effects. Similar 'checkpoint' mechanisms could be programmed to extend RNAi inheritance upon sensing related environmental conditions in the progeny signifying that particular ancestral responses could be advantageous.

In this manuscript we have emphasized the importance of RdRPs for the timing of heritable RNAi, and have elaborated on the advanced regulatory processes that evolved to control the amplification reactions that these enzymes perform. The conservation of these mechanisms, however, remains unknown. The canonical RdRPs that are found in nematodes, plants, and fungi have not yet been detected in mammalian genomes. However, even in humans, other polymerases may execute RdRP functions, and therefore may be capable of amplifying RNAi

Outstanding Questions

Which environmental conditions are capable of inducing transgenerational epigenetic changes? In recent years evidence for transgenerational transmission of the memory of environmental conditions in worms has emerged. It would be important to define why particular conditions trigger heritable responses, while others do not. The difference might depend, for example, on the capacity of specific environments to intervene with the function of the RNAi system.

Are there unique types of changes in the heritable pool of small RNAs that arise in response to different types of challenges/stresses? For example, are there specific changes that would be associated with metabolic changes and others that would appear following temperature shifting? Is a common set of genes involved in multiple different transgenerational responses? Alternatively, are there unique epigenetic responses which are environment-specific?

Can heritable small RNAs perpetuate indefinitely, long enough to affect the process of evolution?

What factors restrict or enable inheritance of gene-specific small RNAs? Why are some genes regulated by heritable small RNAs while others are not?

For which factors do the different small RNA pathways compete? Overexpression of relevant factors could shed light on the nature of the competition between the different small RNA pathways, and on the importance of each limiting factor to the overall regulation of heritable RNAi.

What types of regulatory RNAs can elicit transmission of epigenetic effects across generations?

responses [52,53]. Moreover, additional RdRPs could be hidden in mammalian genomes. For example, very recently a bat was found to contain an actively transcribed, intact, endogenous bornavirus-like L (EBLL) element in its genome that encodes a viral RdRP [54]. Likewise, hepatitis delta virus, which infects humans, lacks an RdRP, and instead relies completely on the host for replicating its RNA genome [55]. As an alternative to amplifying heritable RNAs using RdRPs, regulatory effects that are initiated by small RNAs instead might persist by establishing feed-forward cascades that involve non-RNA factors such as chromatin modifications. Regardless of the mechanism, however, any process that evolved to transmit transgenerational epigenetic effects is likely to have an 'off' switch, or some internal self-restricting 'timer', that enables the organism to tune the response and to adapt it to the reality of the offspring's environment.

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