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REVIEW

Small RNAs, RNAi and the Inheritance of Gene Silencing in *Caenorhabditis elegans*

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ABSTRACT

Invasive nucleic acids such as transposons and viruses usually exhibit aberrant characteristics, e.g., unpaired DNA or abnormal double-stranded RNA. Organisms employ a variety of strategies to defend themselves by distinguishing self and nonself substances and disabling these invasive nucleic acids. Furthermore, they have developed ways to remember this exposure to invaders and transmit the experience to their descendants. The mechanism underlying this inheritance has remained elusive. Recent research has shed light on the initiation and maintenance of RNA-mediated inherited gene silencing. Small regulatory RNAs play a variety of crucial roles in organisms, including gene regulation, developmental timing, antiviral defense, and genome integrity, *via* a process termed as RNA interference (RNAi). Recent research has revealed that small RNAs and the RNAi machinery are engaged in establishing and promoting transgenerational gene silencing. Small RNAs direct the RNAi and chromatin modification machinery to the cognate nucleic acids to regulate gene expression and epigenetic alterations. Notably, these acquired small RNAs and epigenetic changes persist and are transmitted from parents to offspring for multiple generations. Thus, RNAi is a vital determinant of the inheritance of gene silencing and acts as a driving force of evolution.

KEYWORDS: RNAi; Small RNA; Inheritance; Gene silencing; *nrde*

INTRODUCTION

In many organisms, exposure to double-stranded RNA (dsRNA) elicits a gene suppression phenomenon through a mechanism known as RNA interference (RNAi) (Fire et al., 1998). dsRNAs are taken into cells and cleaved by the conserved RNase-III-like enzyme Dicer into 21−23 nt small interfering RNAs (siRNAs) (Hammond et al., 2000; Zamore et al., 2000; Bernstein et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Tabara et al., 2002). siRNAs are subsequently loaded onto Argonaute proteins and act together with other factors to form the RNA-induced silencing complex (RISC). The siRNA guides RISC to the targeted nucleic acids carrying complementary sequences and silences gene expression through a variety of mechanisms, including translation inhibition, mRNA degradation, chromatin modification and the inhibition of transcription elongation (Bernstein and Allis, 2005; Matzke and Birchler, 2005; Hock and Meister, 2008; Hutvagner and Simard, 2008; Carthew and Sontheimer, 2009; Winter et al., 2009; Guang et al., 2010; Czech and Hannon, 2011; Ketting, 2011).

A wide array of endogenous small regulatory RNAs have been discovered in eukaryotes (Lee et al., 1993; Pasquinelli et al., 2000; Reinhart et al., 2000). These small RNAs play crucial roles in many biological processes, including but not limited to, the regulation of gene expression and developmental timing, the maintenance of genome integrity and protection from invasive nucleic acids. Small RNAs are categorized according to their length, structure, nucleotide composition and modifications, and the specific Argonaute proteins to which they bind. The best studied categories of

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small RNAs include microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), and endogenous siRNAs (endo-siRNAs) (Ghildiyal and Zamore, 2009).

Previous analyses have demonstrated that gene silencing is heritable and can persist for generations after its initial induction. For example, the injection of dsRNA into the gonad of *Caenorhabditis elegans* induces phenotypic changes that mimic loss-of-function mutations in the animal's descendants (Fire et al., 1998). Although the strongest silencing response is detected in the F_1 progeny, some of the effects can last for three or four generations, followed by reversion to the original phenotype beyond that point (Alcazar et al., 2008). In an extreme case, the injection of dsRNA targeting a germline-expressed green fluorescence protein (GFP) was able to suppress GFP expression for over eighty generations (Vastenhouw et al., 2006). This RNAi-mediated silencing effect can be transmitted *via* either maternal or paternal gametes (Grishok et al., 2000; Alcazar et al., 2008).

INHERITANCE OF RNAi IS MEDIATED BY A DOMINANT EXTRAGENIC AGENT

It was first recognized that some *trans* factors mediate the inheritance of silencing in *C. elegans* (Fire et al., 1998; Grishok et al., 2000). The injection of dsRNAs induces a silencing effect in the same generation (P_0). When the progeny (F_1 generation) of these injected animals are mated with naïve animals that have never been exposed to the dsRNAs, their progeny (F_2 generation) exhibit a silencing phenotype mirroring that of their grandparents. In the F_3 generation, the animals maintain the silencing phenotype, although both chromosomes are naïve (Fig. 1).

Many genes have been identified as indispensable for RNAi (Tabara et al., 1999, 2002). For example, *rde-1* and *rde-4* are required for silencing caused by exogenously provided dsRNA (Liu et al., 2012). Interestingly, *rde-1* and *rde-4* are only required for the formation of the initial interfering agent and are not necessary for the inheritance of silencing in later generations. The direct injection of dsRNA into *rde-1* or *rde-4* homozygous mutants does not elicit silencing in either the parents or their descendants. However, the injection of dsRNA into a mother that is heterozygous for the *rde-1* or *rde-4* gene induces robust interference in both the parental generation and in progeny that are homozygous for the *rde-1* or *rde-4* mutation (Grishok et al., 2000). Thus, the injection of dsRNA into heterozygous hermaphrodites induces an inheritable interference effect that triggers gene silencing in the progeny, independent of the continuous presence of RDE-1 and RDE-4.

The silencing signal is unequally distributed among the population of offspring. Consistent with this observation, the frequency and persistence of heritable silencing depend on the dose of the trigger. A limited amount of the trigger always evokes silencing preferentially in progeny that are born earlier (Alcazar et al., 2008).

TRANSMISSION OF SILENCING BY THE TWO TYPES OF GAMETES

The silencing effect can be transmitted *via* both sperm and oocytes, demonstrating that the inherited signals (e.g., siRNAs) are deposited in both types of gametes. Nevertheless, transmission through the oocyte lineage is less effective than that through the sperm, suggesting the existence of unique features in sperm (Alcazar et al., 2008). This inherited signal is intrinsic to the sperm; it is not present in the seminal fluid that accompanies the sperm as it travels into the hermaphrodite during copulation. In a transmission assay with mixed sperm from an RNAi-exposed parent and unexposed

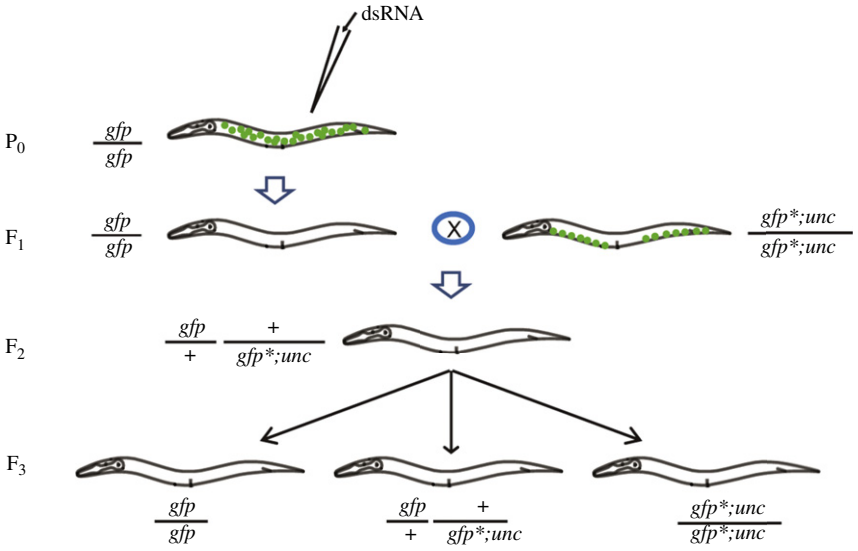

Fig. 1. An extragenic agent mediates RNAi inheritance.

The injection of *gfp* dsRNAs into *C. elegans* triggers a silencing effect in the P_0 generation. Crossing the progeny with another strain expressing an *unc*-marked *gfp* transgene generates F_2 progeny that are silenced as well. In the F_3 generation, the animals that are homozygous for the *unc*-marked *gfp* transgene exhibit a silencing status that mimics that of their heterozygous parents and siblings.

hermaphrodite, researchers demonstrated that the seminal fluid from the parent lacks the ability to convey the silencing signal (Alcazar et al., 2008).

Surprisingly, although males are sensitive to RNAi and can inherit and transmit silencing acquired from their mothers, the direct injection of dsRNA into males does not result in the inheritance of silencing in the progeny, suggesting that the mechanism that establishes the initial transmission is active only in hermaphrodites. The mechanism underlying this phenomenon remains poorly characterized (Grishok et al., 2000).

RNAi AMPLIFICATION AND LONG-TERM SILENCING

The original dsRNA trigger is dramatically diluted over generations. Each adult *C. elegans* produces approximately 250 progeny. Hence, an F_4 progeny may only inherit a $1/250^4$ fraction (1 out of 3.9 billion) of the original dsRNA trigger. Thus, the effective propagation of silencing information must involve an active process that permanently fixes the RNAi information in the chromatin of the progeny or amplifies the RNAi signal for transmission to subsequent generations.

Exogenous dsRNA first associates with RDE-4, which is a dsRNA-binding protein, and then recruits Dicer to cleave the dsRNA into siRNA (Tabara et al., 2002). This siRNA, termed the primary siRNA, recruits RDE-1 to the cognate mRNA template and induces the degradation of the mRNA targets. Meanwhile, the primary siRNA/RDE-1/mRNA complex directs RNA-dependent RNA polymerases (RdRPs) to this mRNA template and primes the *ab initio* synthesis of secondary siRNA (Sijen et al., 2001; Yigit et al., 2006; Pak and Fire, 2007; Sijen et al., 2007). *C. elegans* expresses four RdRPs that function in a tissue-specific manner. Secondary siRNAs are the major force that engages in the actual silencing events in *C. elegans* because they are nearly one hundred-fold more abundant than their cognate primary siRNAs (Sijen et al., 2001, 2007; Pak and Fire, 2007; Gu et al., 2012).

It is speculated that secondary siRNAs are inherited and subsequently amplified by progeny that have not been directly subjected to RNAi (Gu et al., 2012). Therefore, even in the absence of *rde-1* or *rde-4*, these siRNAs have the capability to conduct downstream silencing in the offspring. Consistent with this idea, an RNA-dependent RNA polymerase-1, *rrf-1*, is required for the multigenerational inheritance of an antiviral response (Rechavi et al., 2011).

RdRPs (e.g., RRF-2 and EGO-1) act on mRNA in the germline and generate heritable siRNAs for each generation. These siRNAs elicit gene silencing *via* binding to germline-specific Argonaute proteins. A germline-expressed Argonaute protein, heritable RNAi defective-1 (HRDE-1), has recently been shown to play a role in RNAi inheritance (Buckley et al., 2012). HRDE-1 associates with 22G-siRNAs synthesized by germline RdRP (Vasale et al., 2010). Thus, the transcription of mRNA templates in the germline might be a prerequisite for the long-term inheritance of RNAi. Consistent with this hypothesis, dsRNAs targeting soma-specific genes fail to maintain RNAi for more than one generation (Burton et al., 2011). In contrast, other genes can be easily and heritably silenced (Vastenhouw et al., 2006). For example, the injection of a dose of *ceh-13* dsRNA results in a small and dumpy phenotype that persists for multiple generations in some progeny (Vastenhouw et al., 2006).

However, RdRP is not sufficient to amplify inherited siRNA indefinitely over an infinite number of generations. The existence of a bottleneck that limits most of the silencing effects to fewer than three or four generations suggests an inconsistency between the simple self-renewal of the trigger siRNA and the decrease of silencing efficacy. Two models have been postulated to describe this phenomenon. In the first model, the amplification efficiency is diminished gradually, and there is a threshold detection system to monitor the amount of siRNA present (Alcazar et al., 2008). In the second model, there is another signal that is concurrently inherited but is diluted over generations (Alcazar et al., 2008). Primary siRNAs, which are derived from the original exogenous source, can only be transmitted for a limited number of generations and vanish thereafter. Thus, primary siRNAs could provide a good "timer" for the memory of the initial RNAi (Gu et al., 2012).

NUCLEAR RNAi DEFECTIVE PATHWAY AND HERITABLE GENE SILENCING

dsRNAs silence gene expression in the nucleus through a number of mechanisms, including triggering heterochromatin formation and inhibiting transcription (Buhler and Moazed, 2007; Grewal, 2010). Recently, it was shown that dsRNA can silence nuclear-localized RNAs in *C. elegans* (Fig. 2) (Guang et al., 2008, 2010). This silencing depends on at least four genes, designated as nuclear RNAi defective 1−4 (*nrde-1/2/3/4*) (Guang et al., 2008, 2010; Burkhart et al., 2011). NRDE-3 is an Argonaute protein that escorts RdRP-generated siRNA from the cytoplasm to the nucleus. In the nucleus, siRNA guides NRDE-3 to the targeted pre-mRNA and further recruits NRDE-2 and NRDE-1. NRDE-1 associates with complementary genomic DNA in an NRDE-4-dependent manner (Burkhart et al., 2011). Subsequently, nuclear RNAi halts RNA polymerase II, inhibits transcription elongation, and at the same time, promotes histone 3 lysine 9 trimethylation (H3K9me3) at the genomic loci targeted by siRNAs (Guang et al., 2010).

The Nrde pathway also maintains heritable RNAi silencing and H3K9 trimethylation in the progeny of *C. elegans* exposed to dsRNA (Burton et al., 2011; Buckley et al., 2012). The siRNAs in the progeny, which were induced by RNAi in the parental generation, are bound by the Argonaute protein NRDE-3. Interestingly, nuclear RNAi could promote siRNA perdurance in the progeny. Over the course of development, *nrde*(−) animals, which exhibit impaired nuclear RNAi phenotypes, fail to maintain either RNAi silencing or the level of expression of the siRNA itself (Burton et al., 2011).

It remains unclear why nuclear RNAi is necessary to maintain siRNA expression in the progeny of dsRNA-treated

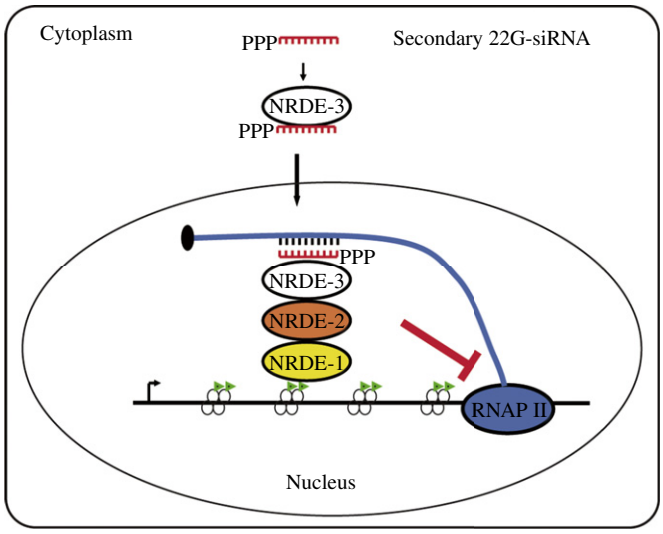

Fig. 2. A working model of the nuclear RNAi defective (Nrde) pathway.
5′-triphosphorylated secondary 22G-siRNA is bound by the shuttling Argonaute protein NRDE-3 in the cytoplasm and translocated into the nucleus. In the nucleus, siRNA guides NRDE-3 to the cognate nascent transcripts that are synthesized by RNA polymerase II. The NRDE-3/siRNA complex further recruits NRDE-2, followed by NRDE-1, to the targeted pre-mRNA. NRDE-1 is deposited on the chromatin and likely recruits unknown histone methyltransferases to the targeted loci to catalyze histone 3 lysine 9 trimethylation. Through an unknown mechanism, the siRNA/NRDE complex inhibits transcription elongation mediated by RNAP II.

animals. The majority of NRDE-3-associated siRNAs in *C. elegans* are generated by RdRP activity upon an mRNA template, which likely takes place in the cytoplasm. NRDE-1 and NRDE-2 localize to the nucleus. The chromatin and RNAi machinery has been postulated to form a self-reinforcing loop that is linked to siRNA expression in the progeny of animals exposed to dsRNA (Burton et al., 2011). In fission yeast, this type of self-reinforcing loop exists to reconcile siRNA expression, H3K9 trimethylation, and gene silencing events (Noma et al., 2004; Sugiyama et al., 2005; Moazed, 2009).

Nevertheless, nuclear RNAi *per se* is not required to transmit the silencing signal from the parent to the progeny. The progeny of *nrde*(−) animals exposed to dsRNA still exhibit inherited RNAi silencing at the embryonic stage (Burton et al., 2011). In later stages of larval development, however, the *nrde*(−)-inheriting animals do not display silencing, indicating that the inherited silencing is not maintained. Thus, nuclear RNAi is likely not required to directly convey the signals from the parents to the progeny, but it appears to be necessary to preserve heritable siRNA expression in the progeny.

HRDE-1 AND RNAi INHERITANCE

A genetic screen conducted in *C. elegans* to search for factors that are specifically required for RNAi inheritance isolated heritable RNAi defective-1, HRDE-1 (Buckley et al., 2012). HRDE-1 is a germline-specific Argonaute protein that localizes to the nucleus. HRDE-1 associates with siRNAs in the germline of progeny for several generations after exposure to RNAi and directs H3K9 trimethylation at targeted genomic loci through the nuclear RNAi pathway. In the germline of *hrde-1* or nuclear RNAi defective animals, both gene silencing and H3K9 trimethylation are progressively lost over several generations (Fig. 3).

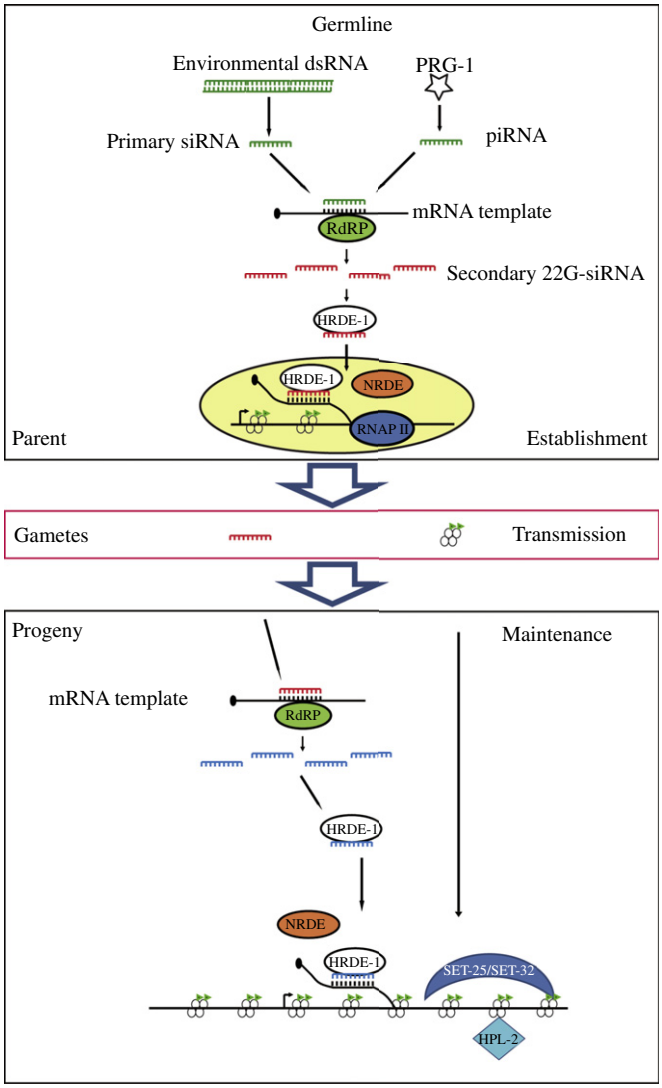

Fig. 3. A model of RNAi inheritance in *C. elegans*.
The inheritance of silencing involves three steps: the establishment of silencing in the parent, the transmission of the signal in the gametes, and the maintenance or re-establishment of silencing in the progeny. In the germline of the parent, exogenously delivered primary siRNAs or endogenous piRNAs associate with the mRNA template, recruit RNA-dependent RNA polymerases (RdRPs), and prime the synthesis of secondary siRNAs. Secondary siRNAs bind to the germline-specific Argonaute protein HRDE-1 and silence gene expression in the nucleus; at the same time, they elicit chromatin modifications (green flags indicate H3K9 trimethylation) by engaging the nuclear RNAi defective (Nrde) pathway. Both secondary siRNAs and chromatin modification marks are deposited in the gametes and transmitted to the progeny. In the progeny, the inherited secondary siRNAs are amplified again and thereafter function with the HRDE-1 and Nrde proteins in the germline. The HP1 ortholog HPL-2 and two histone methyltransferases, SET-25 and SET-32, likely sensitize the inherited chromosome, acting together with the HRDE-1/Nrde pathway to preserve multigenerational gene silencing.

HRDE-1 also associates with endogenous siRNAs and likely promotes gamete formation (Buckley et al., 2012). In *hrde-1*$^{-/-}$ homozygous mutants, a gradual loss of H3K9 trimethylation is observed, and the defects in gamete development become worse as the animals age, which leads to eventual sterility. The *hrde-1*$^{-/-}$ homozygous progeny of *hrde-1*$^{+/-}$ heterozygous parents fail to inherit RNAi silencing. Conversely, animals that lack HRDE-1 in the RNAi generation but express HRDE-1 in the progeny generation are still able to inherit RNAi silencing. Thus, instead of acting as a *bona fide* carrier to transmit the silencing signal *per se*, HRDE-1 likely acts in the germline of inheriting progeny to facilitate the memory or re-establishment of the RNAi silencing events that occurred in the previous generations. Consistent with this model, *hrde-1* is also required for H3K9 trimethylation in the germline of the generation subjected to the initial RNAi. Both male and female gametes can inherit RNAi, while HRDE-1 localizes to the nuclei of oocytes, but not sperm, which further suggests that HRDE-1 is unlikely to directly participate in this transmission of the inheritance signal from the parents to the progeny.

piRNAs AND MULTIGENERATIONAL MEMORY OF GENE SILENCING

The transgenerational inheritance of RNAi has a vital influence on biological adaptation, evolution, and human health. Recently, several groups have reported that piRNAs play critical roles in recognizing nonself RNA in the genome, activating the genome surveillance system, and promoting multigenerational epigenetic memory in the germline of *C. elegans* (Ashe et al., 2012; Bagijn et al., 2012; Lee et al., 2012; Shirayama et al., 2012).

piRNAs in *C. elegans* are small RNAs of 21 nt in length with a 5′ uracil (21U-RNAs). piRNAs are mainly generated from specific clusters on chromosome IV but may also be produced from several other loci in the genome (Batista et al., 2008). They are essential for germline development and genome integrity. Two PIWI proteins, PRG-1 and PRG-2, have been identified as required for piRNA production. Both *prg-1* and piRNA exhibit restricted expression in the germline.

piRNA exhibits imperfect base pairing with potential targets and likely recruits RdRP to generate 22G-siRNA (Batista et al., 2008; Gu et al., 2009; Bagijn et al., 2012; Lee et al., 2012). Therefore, PRG-1 is required to generate a subset of 22G-RNAs and thus fine-tunes endogenous gene expression. The genomic loci targeted by piRNA preferentially overlap with the start and end regions of transposons. Furthermore, transposons and targets of 22G-RNAs accumulate in the germline of *prg-1* mutants.

piRNA is essential for initiating multigenerational RNAi inheritance. A piRNA sensor strain has been generated by inserting a sequence complementary to an endogenous piRNA (21U-RNA) into a GFP-H2B fusion gene, which is then integrated into the genome (Ashe et al., 2012; Shirayama et al., 2012). This transgene is silenced in the germline in a *prg-1*-dependent manner. Consistent with this finding, certain *trans* factors (e.g., piRNA) are involved in this silencing, and the silenced transgene can act in a dominant manner to silence other naïve, homologous transgenes. Interestingly, this silencing is highly stable and can be perpetuated for at least 20 generations.

However, PRG-1 is not required to maintain the silencing status. Once established, this long-term silencing is independent of the piRNA trigger and instead relies on the nuclear RNAi pathway and chromatin modification machinery (Lee et al., 2012). When a *prg-1* mutation is crossed into a previously silenced reporter strain, the transgene does not recover its expression status, even after propagation for many generations (Ashe et al., 2012). Conversely, when a transgene that is active in the *prg-1* mutants is outcrossed into wild-type animals, the transgene is immediately silenced in the F_1 cross progeny. Furthermore, this silenced transgene remains silent in the F_2 generation, which includes *prg-1*$^{+/+}$, *prg-1*$^{+/-}$, and *prg-1*$^{-/-}$ animals. RDE-3 is a nucleotidyltransferase that likely functions in a late step of the RNAi pathway (Chen et al., 2005). The introduction of an *rde-3* mutation can fully rescue the silencing of the piRNA sensor transgene, suggesting that *rde-3* acts downstream of piRNA and is required for the maintenance of silencing (Lee et al., 2012).

The nuclear RNAi pathway and chromatin modification factors are involved in propagating this piRNA-induced silencing. The germline-specific Worm ArGOnaute (WAGO) protein HRDE-1, the nuclear RNAi defective genes *nrde-1/2/4*, the HP1 ortholog HPL-2, and the two histone modification enzymes SET-25 and SET-32 have been identified as necessary for this process (Ashe et al., 2012). Nevertheless, the functional mechanism of these genes remains unclear.

It has been postulated that piRNA acts to distinguish self from nonself nucleic acids by comparing foreign sequences to RNAs that were previously expressed in the germline (Shirayama et al., 2012). Transgenes containing a germline-expressed sequence are recognized as self and are protected from piRNA-mediated silencing. Conversely, an activated transgene is able to reactivate a silent copy of that transgene by hindering piRNA-mediated silencing.

Interestingly, piRNA has also been found to facilitate transgenerational paramutation in *Drosophila* (de Vanssay et al., 2012; Grentzinger et al., 2012). Paramutation is an epigenetic interaction between a paramutable allele and the paramutagenic form of a locus in heterozygotes that elicits a heritable epigenetic change in the phenotype of the paramutable allele (Chandler, 2010; Erhard and Hollick, 2011). Paramutation is meiotically stable and is inherited in the absence of the inducing allele. Strikingly, the newly silenced allele itself becomes paramutagenic in subsequent generations, even after the previous paramutagenic allele is segregated away. In the *Drosophila* germline (de Vanssay et al., 2012), a P-element-derived transgene can induce a strong *trans*-silencing effect (TSE) by generating piRNAs, which thereafter convert other homologous transgene clusters that are incapable of TSE into strong silencers. This paramutation can last for up to 50 generations. A loss-of-function mutation in the

aubergine gene, which is required for piRNA biogenesis, abolishes this multigenerational paramutation effect.

CHROMATIN MODIFICATION, EPIGENETICS, AND RNAi INHERITANCE

In addition to *trans* factors, *cis* signals have also been shown to participate in RNAi inheritance. RNAi-directed chromatin modification is linked to gene silencing in a variety of organisms. For example, small RNAs initiate H3K9 trimethylation and gene silencing in *Schizosaccharomyces pombe* (Moazed, 2009). In this yeast, the siRNA/Ago complex associates with nascent transcripts, recruits the H3K9 methyltransferase Clr4 to the genomic loci targeted by RNAi, elicits H3K9 trimethylation, and facilitates transcriptional gene silencing (Moazed, 2009). In *Arabidopsis thaliana*, small RNAs direct DNA methylation at targeted loci (Chan et al., 2004; Henderson et al., 2006), and in *C. elegans*, RNAi-induced H3K9me3 is also correlated with transcriptional gene silencing (Guang et al., 2010; Burkhart et al., 2011).

In *C. elegans*, the exposure of a parental strain to dsRNA has been shown to silence a naïve *gfp* locus in F_1 inheriting animals, as expected, suggesting that diffusible signals, likely siRNAs, act in *trans* to silence a *gfp* locus that has not encountered dsRNA previously. Interestingly, in later generations, the inherited gene silencing is more pronounced at loci that have been exposed to *gfp* dsRNA previously, suggesting that a *cis* signal that associates with the parental locus is indeed inherited in the progeny (Buckley et al., 2012). Studies of the chromatin-binding protein HPL-2 and histone-modification enzymes SET-25 and SET-32 have suggested that chromatin modification can play a role in the establishment or maintenance of this *cis* signal (Ashe et al., 2012).

It is speculated that nuclear RNAi pathway-directed H3K9me3 contributes to long-term silencing in *C. elegans*. NRDE-1, which is guided by siRNA to complementary genomic loci, is required for RNAi inheritance (Burton et al., 2011). HRDE-1-mediated H3K9me3 marks, which are triggered by both exogenously provided dsRNA and endogenous siRNAs, are heritable as well (Buckley et al., 2012). The requirement of both NRDE-1 and HRDE-1 to preserve heritable silencing suggests that nuclear RNAi and chromatin modifications act together to promote the multigenerational maintenance of RNAi.

However, it is unclear whether this chromatin mark is directly inherited or re-established in the progeny. A time-course experiment revealed that siRNAs are detectable before the appearance of H3K9 trimethylation in the progeny, suggesting that the H3K9me3 mark can be re-established by inherited siRNAs. Interestingly, the germline transmission of the silencing signal may enhance the efficiency of siRNA-directed H3K9 trimethylation. The H3K9me3 marks are more pronounced in the progeny than in the parents that were directly exposed to the dsRNAs (Burton et al., 2011).

Moreover, the inheritance of heterochromatin might be stochastic due to the segregation of parental histones to sister chromatids. Parental histone marks and associated proteins alone are unlikely to provide sufficient chromatin modification to recapitulate the parental chromatin state in the descendants. Instead, small RNAs likely act together with the chromatin modification machinery to re-establish the heterochromatin status. The fact that small RNAs are amplified again in subsequent generations supports this idea (Gu et al., 2012).

The direct role of chromatin modification in RNAi inheritance is obscure. One hypothesis is that RNAi triggers H3K9me3 at a targeted sequence, which sensitizes this locus to inherited siRNAs and facilitates more pronounced silencing (Buckley et al., 2012). Consistent with this model, in *S. pombe*, the RNAi machinery induces H3K9me3 at genomic loci that are targeted by siRNAs. Meanwhile, the H3K9me3 mark aids in recruiting the RNAi machinery to these genomic loci to further assist the silencing process in a self-reinforcing loop (Buhler and Moazed, 2007).

Other chromatin modification marks and chromatin-associated factors might also be involved in RNAi inheritance (Vastenhouw et al., 2006). A candidate-based RNAi screen has been conducted to identify genes that are required for RNAi inheritance in *C. elegans*. The identified genes include *hda-4* (a class II histone deacetylase), *K03D10.3* (a histone acetyltransferase), *isw-1* (a chromatin-remodeling ATPase), and *mrg-1* (a chromo-domain protein) (Vastenhouw et al., 2006). Treating *C. elegans* with the histone deacetylase inhibitor trichostatin A (TSA) can alleviate RNAi silencing, suggesting that histone acetylation might also participate in RNAi inheritance (Grishok et al., 2005). Nevertheless, the precise functions of these chromatin modifications are unknown (Ashe et al., 2012).

Interestingly, epigenetic inheritance is also guided by small RNAs to direct genome reprogramming in plant pollen (Calarco et al., 2012). Unlike mammals, plants do not have a system that can erase epigenetic information in the germline. In vegetative nuclei and early embryos, CHH (where H = A, T or C) methylation is lost in microspores and sperm cells but restored by *de novo* DNA methylation mediated by 24 nt siRNAs. In sperm, targets of Demeter (DME) and Repressor of Silencing 1 (ROS1) are premethylated and accumulate small RNAs.

PERSPECTIVES

Why does the inheritance of acquired silencing exist? Environmental factors such as nutrition or supplementation can have dramatic impacts on gene expression and development. Although these impacts may not directly alter the DNA sequence of a gene, they could favor survival under specific circumstances and aid in their selection during the course of evolution. However, these alterations must be inherited in the progeny and propagate in their descendants to provide an evolutionary advantage over other populations or species. For example, a small RNA-based antiviral response can be inherited for multiple generations in *C. elegans* (Rechavi et al., 2011).

Chromatin modification has been widely accepted as a mechanism for the inheritance of epigenetic information. However, small RNAs and RNAi machinery also make

important contributions to epigenetic inheritance. The propagation of small RNA-mediated gene silencing in nematodes, maize and mice strongly suggests that this mechanism is prevalent in a variety of species. Nevertheless, it is unclear to what degree chromatin modification and the small RNA-mediated inheritance of gene silencing are connected to each other. We speculate that these two pathways reconcile to efficiently maintain a memory of environmental exposure and promote long-term, multigenerational changes in gene expression. *C. elegans* is an ideal model organism for studying the inheritance of silencing and testing these hypotheses due to its short generation time (approximately 3 days) and its ease of culture and genetic manipulation in the laboratory.

The nature of the actual silencing signal that is transmitted from parents to their progeny is still unknown. The maintenance of the multigenerational inheritance of acquired silencing requires several steps: the initial establishment of the heritable state in parents, the transmission of the silencing signal from the parents to the progeny through gametes, and the maintenance or re-establishment of the silenced state in the offspring. Although many lines of evidence have demonstrated that RNAi and small RNAs are involved in the initial establishment and ultimate maintenance of silencing, the actual signal that is transmitted between parents and their progeny is still unknown. Small RNAs are good candidates for conveying this silencing information (Buckley et al., 2012).

It is also unclear why most transgenerational silencing appears to be restricted to three or four generations (Alcazar et al., 2008). Small RNAs are not sufficient to mediate paramutation in maize (Erhard and Hollick, 2011), suggesting that both small RNAs and chromatin modifications likely work together to maintain the transgenerational memory of silencing. Alternatively, other signals arising from the initial dsRNA exposure may be conveyed to the progeny to determine the number of generations over which silencing persists.

Another important question is whether inherited silencing information can be stably integrated into the genome. For example, some genes can be permanently shut off by the accumulation of chromatin methylation over multiple generations. Alternatively, small RNAs might direct a DNA repair process under certain conditions such that genomic sequences are perpetually altered according to the small RNA sequences present (Chalker and Yao, 2011; Wei et al., 2012).

Transgenerational epigenetic inheritance is also involved in phenomena other than gene silencing. For example, the multigenerational longevity of *C. elegans* is mediated by the histone 3 lysine 4 trimethylation (H3K4me3) complex, composed of ASH-2, WDR-5 and the histone methyltransferase SET-2 (Greer et al., 2011). Whether small RNAs are involved in these processes remains to be investigated.

In summary, the discovery of the small RNA-mediated inheritance of gene silencing has redefined our views on the mechanism of inheritance and evolution. The suppression of potentially deleterious nucleic acids by RNAi has evolved into a powerful regulatory means for protecting genomic integrity and control of chromosomal functions. Further investigations into the mechanism of the inheritance of gene silencing, in particular the interaction between small RNAs and chromatin modifications in this process, will shed light on the epigenetic control of eukaryotic genomes.

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