Seminars in Cell and Developmental Biology 127 (2022) 142–154

Contents lists available at ScienceDirect

Seminars in Cell and Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

Mechanisms of epigenetic regulation by *C. elegans* nuclear RNA interference pathways

Uri Seroussi [a,1], Chengyin Li [b,1], Adam E. Sundby [a], Tammy L. Lee [b], Julie M. Claycomb [a,*], Arneet L. Saltzman [b,*]

[a] Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada
[b] Department of Cell and Systems Biology, University of Toronto, Toronto, ON, Canada

ARTICLE INFO

Keywords:
Nuclear RNAi
Transcriptional gene silencing
Chromatin modification
Epigenetic inheritance
Argonaute
Small RNA

ABSTRACT

RNA interference (RNAi) is a highly conserved gene regulatory phenomenon whereby Argonaute/small RNA (AGO/sRNA) complexes target transcripts by antisense complementarity to modulate gene expression. While initially appreciated as a cytoplasmic process, RNAi can also occur in the nucleus where AGO/sRNA complexes are recruited to nascent transcripts. Nuclear AGO/sRNA complexes recruit co-factors that regulate transcription by inhibiting RNA Polymerase II, modifying histones, compacting chromatin and, in some organisms, methylating DNA. *C. elegans* has a longstanding history in unveiling the mechanisms of RNAi and has become an outstanding model to delineate the mechanisms underlying nuclear RNAi. In this review we highlight recent discoveries in the field of nuclear RNAi in *C. elegans* and the roles of nuclear RNAi in the regulation of gene expression, chromatin organization, genome stability, and transgenerational epigenetic inheritance.

1. Introduction

RNA interference (RNAi) pathways, minimally consisting of Argonaute proteins (AGO) and small RNAs (sRNA) that provide sequence specificity by antisense complementarity, play key roles in gene regulation across all domains of life. Initially discovered in plants, and later recognized in other organisms, nuclear RNAi pathways are important regulators of genome stability and gene expression [1]. Nuclear AGO/sRNAs mediate a variety of functions across organisms. These activities include repression of repetitive elements such as transposons (e.g. *Drosophila*, mice, *C. elegans*) [2], regulation of transcription and splicing (e.g. fungi, plants, animals) [3], directing chromatin modifications (e.g. *C. elegans*, *Drosophila*, mice, fungi, plants) and DNA methylation (e.g. mice, plants) [4,5], coordinating genome rearrangement and DNA elimination (e.g. ciliated protozoans) [6], and facilitating epigenetic inheritance (e.g. *C. elegans*, *Drosophila*, plants) [7,8]. One key organism which has contributed tremendously to our understanding of RNAi, and nuclear RNAi in particular, is the nematode *C. elegans*. In this review we synthesize our current understanding of the mechanisms of nuclear RNAi in the worm, emphasizing discoveries from the past five years. We provide an overview of the functions of *C. elegans* nuclear AGOs and explore the relationships between nuclear RNAi and other gene regulatory pathways, highlighting their roles in Transgenerational Epigenetic Inheritance (TEI).

2. Overview of *C. elegans* nuclear RNAi

RNAi in *C. elegans*, whether initiated by an exogenous (exo-RNAi) or endogenous (endo-RNAi) source, follows a similar process that leads to the amplification of secondary small RNAs (sRNAs) that are 22 nucleotides (nt) in length and begin with a guanine (22G-RNAs) (Fig. 1**A**). In exo-RNAi, dsRNA is processed by the endoribonuclease Dicer into primary sRNAs that are loaded into the primary AGO RDE-1 [9]. In endo-RNAi, several primary AGOs recognize targets when bound to

Abbreviations: RNAi, RNA interference; AGO, Argonaute; sRNA, small RNA; dsRNA, double stranded RNA; 22G-RNA, endogenous small RNAs produced by RNA dependent RNA polymerases that are predominantly 22 nt long and begin with a 5′ guanosine triphosphate; 26G-RNA, endogenous small RNAs produced by RNA dependent RNA polymerases that are predominantly 26 nt long and begin with a 5′ guanosine monophosphate; piRNA, piwi-interacting RNA; siRNA, small interfering RNA; HMT, histone methyltransferase; NRDE, nuclear RNAi defective; HRDE, heritable RNAi deficient; CSR, chromosome segregation and RNAi deficient; RdRP, RNA dependent RNA polymerase; TEI, transgenerational epigenetic inheritance.

* Corresponding authors.
E-mail addresses: julie.claycomb@utoronto.ca (J.M. Claycomb), arneet.saltzman@utoronto.ca (A.L. Saltzman).

[1] These authors contributed equally.

https://doi.org/10.1016/j.semcdb.2021.11.018
Received 19 July 2021; Received in revised form 17 October 2021; Accepted 17 November 2021
Available online 6 December 2021

specific classes of primary endo-siRNAs: PRG-1, the only PIWI-type AGO in *C. elegans*, binds 21U-RNAs (the *C. elegans* Piwi-interacting RNAs, or piRNAs), whereas ERGO-1, ALG-3 and ALG-4 bind 26G-endo-siRNAs (small interfering RNAs). Targeting of transcripts by primary AGOs/sRNA complexes leads to the recruitment of RNA-dependent RNA polymerases (RdRPs) that generate secondary 22G-RNAs antisense to the target gene. These amplified 22G-RNAs are loaded into the secondary worm-specific AGOs (WAGOs), which elicit post-transcriptional or (co-)transcriptional silencing (nuclear RNAi), depending on their localization to the cytoplasm or nucleus, respectively [10]. Please refer to Ref. [11] for a recent thorough review of all *C. elegans* AGOs and RNAi pathways.

Among the five studied 22G-RNA binding WAGOs in *C. elegans*, at least three are known to be present in the nucleus: HRDE-1, NRDE-3, and CSR-1 [12–14]. Of these three AGOs, only CSR-1 possesses four key residues in the RNase H-like PIWI domain that render it capable of endonucleolytic cleavage (slicing) of its RNA targets [15,16]. It is thought that the slicer activity of CSR-1 is important for cleavage of exo-RNAi targets, histone tail maturation, elimination of maternal mRNAs in the embryo, and further recruitment of the RNA-dependent RNA polymerase EGO-1 to CSR-1 targets [17–20]. In contrast, HRDE-1 and NRDE-3 do not possess slicer activity, and likely rely solely on nuclear co-factors to exert their regulatory functions.

Upon target recognition, both HRDE-1 and NRDE-3 can engage the nuclear RNAi factors NRDE-1, NRDE-2, and NRDE-4 to direct silencing by inhibiting RNA Pol II elongation and facilitating the deposition of repressive chromatin marks (termed the NRDE pathway) [14,21–24]. Thus, a generalized model for nuclear RNAi in *C. elegans* involves the

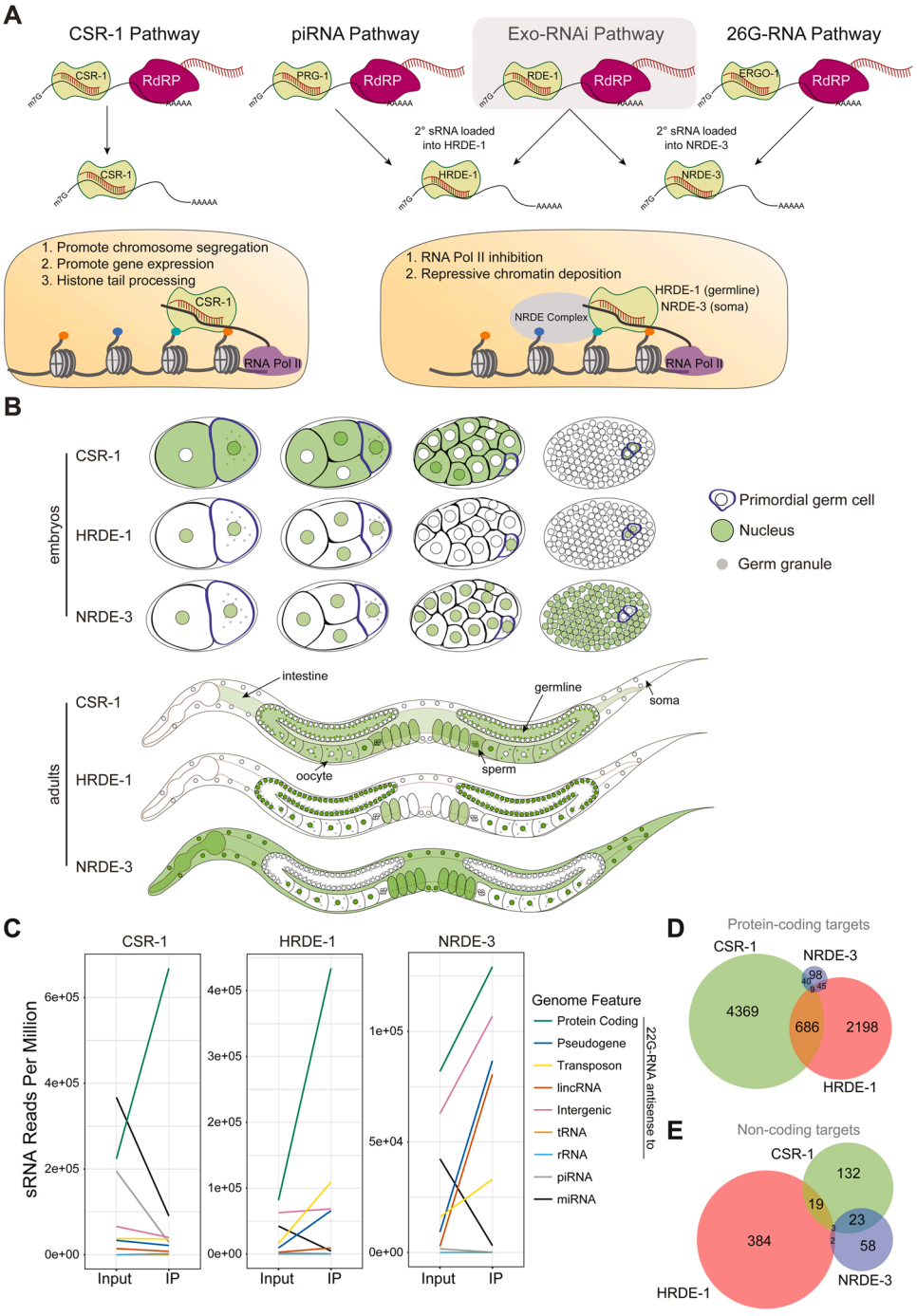

Fig. 1. Overview of the nuclear AGOs in *C. elegans*. **A.** A schematic of the AGO/sRNA pathways that can lead to nuclear RNAi. Primary sRNAs are bound by primary AGOs which recruit RdRPs for the generation of 22G-RNAs. 22G-RNAs are loaded into the WAGOs. Primary AGOs that feed into nuclear RNAi pathways include: RDE-1, binds primary sRNAs derived from Dicer products; PRG-1, binds piRNAs; and ERGO-1, binds 26G-RNAs. Nuclear WAGOs translocate to the nucleus (likely dependent on 22G-RNA binding) where they can engage nascent transcripts and recruit nuclear co-factors such as the NRDE complex and HMTs. **B.** An illustration of the different expression patterns of CSR-1, HRDE-1 and NRDE-3 throughout *C. elegans* development. **C.** sRNA data from published libraries of CSR-1 [13], HRDE-1 [119] and NRDE-3 [29] immunoprecipitations (IPs). Levels of sRNA reads corresponding to the indicated genomic feature are shown in input (total RNA) and AGO-IP samples. Antisense sRNA reads are 22G-RNAs. **D, E.** Venn diagrams showing proportions of distinct and overlapping protein-coding **(D)** or non-protein-coding **(E)** targets of 22G-RNAs associated with CSR-1, HRDE-1 or NRDE-3.

translocation of a nuclear WAGO into the nucleus, where it engages nuclear co-factors at nascent transcripts to regulate gene expression (Fig. 1**A**). Additional nuclear RNAi factors will be described below in Section 3.1 .

2.1. *Argonautes in the nucleus – expression patterns and targets*

HRDE-1 and NRDE-3 robustly localize to nuclei of germline and somatic cells, respectively [12,14], while a pool of CSR-1 localizes to the nucleus in oocytes and in a cell-cycle dependent manner during early embryogenesis (Fig. 1**B**) [13]. A separate pool of CSR-1 is present in the cytoplasm, where it is enriched in germ granules throughout germline development, and is present in puncta within somatic cells of the embryo [25,26]. The nuclear localization of NRDE-3 requires small RNA binding, upon which NRDE-3 translocates from the cytoplasm to the nucleus [14]. It is currently unknown whether HRDE-1 and CSR-1 require small RNA loading to translocate to the nucleus. Indeed, the mechanisms regulating the loading and nuclear localization of these AGOs are active areas of investigation (see Section 4.2).

Although a single unified study of AGO expression patterns is lacking, examining the developmental expression profiles described to date provides insight into their functions (Fig. 1**B**). In the early embryo, HRDE-1 localizes to the nucleus in all cells, but, by the ~ 28 cell stage, its expression is restricted to the primordial germ cells (PGCs), which will give rise to the germline [26]. In larval and adult stages, HRDE-1 shows constitutive nuclear germline expression. CSR-1 also shows broad expression during early embryogenesis whereas expression is restricted to the PGCs by the ~ 100 cell stage [19,25]. Throughout larval and adult stages, CSR-1 is constitutively expressed in the germline where it is present in germ granules, and becomes enriched in oocyte nuclei before fertilization. NRDE-3 shows robust and broad nuclear localization in embryos by the ~80 cell stage and is expressed in most somatic cells in larvae and adults [14,27]. Although transgenes expressing NRDE-3 were not detected in the early embryo, an endogenously CRISPR-tagged NRDE-3 shows nuclear localization during oogenesis and embryogenesis (Seroussi, unpublished). Together, these localization studies point to HRDE-1 and NRDE-3 as the main nuclear AGOs in the germline and soma, respectively. CSR-1, on the other hand, displays a more restricted nuclear localization pattern, that appears particularly important during early embryogenesis.

What portions of the genome do the nuclear AGOs regulate? Sequencing of the small RNA populations co-immunoprecipitated with AGOs revealed that CSR-1 associated 22G-RNAs are complementary to germline expressed genes encompassing almost 25% of the coding

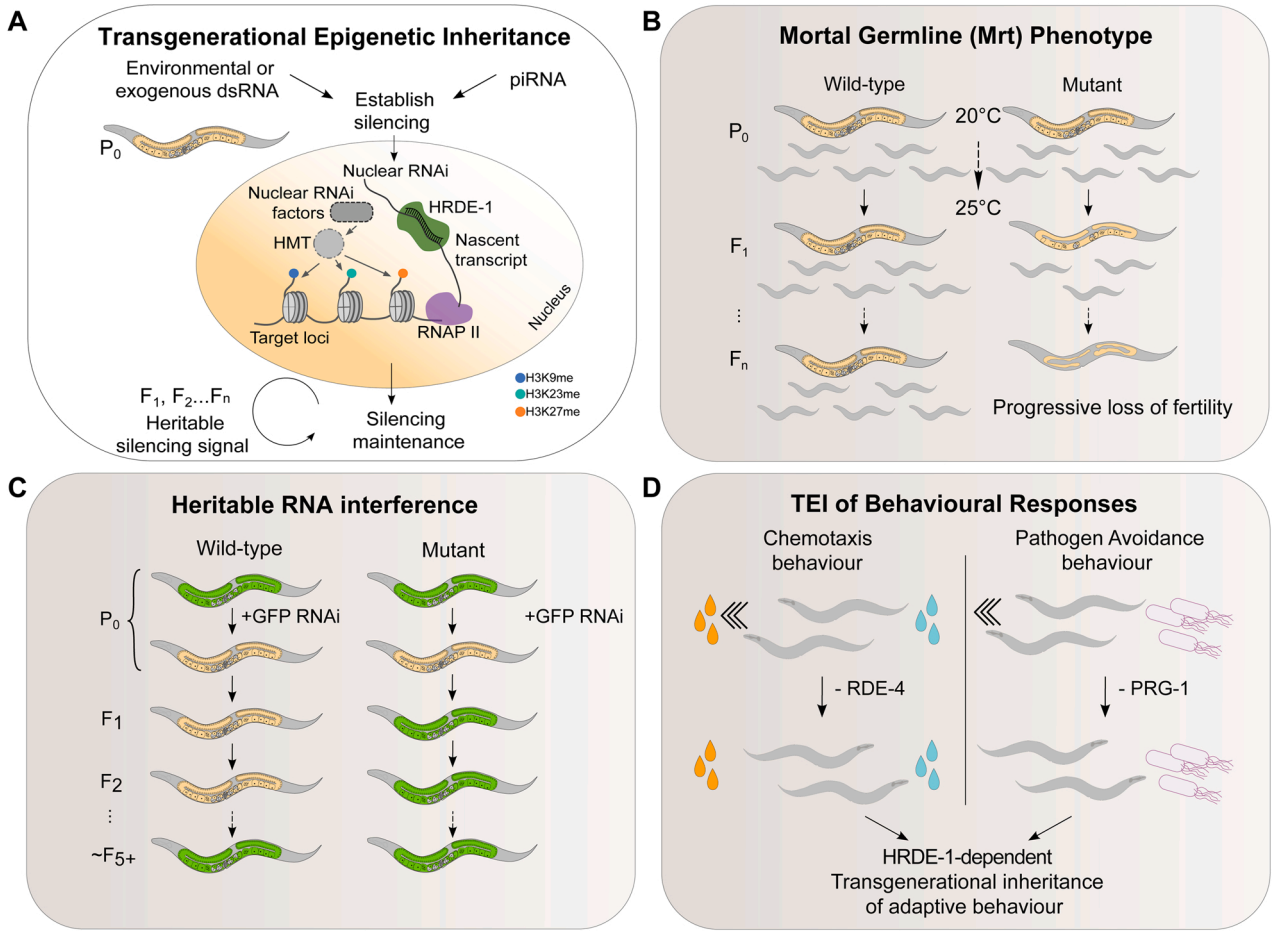

Fig. 2. Examples of TEI phenomena involving nuclear RNAi. A. A heritable silencing signal can be established in the P0 generation upon exposure to environmental/exogenous dsRNA or a piRNA trigger. Long-term maintenance of the heritable silencing signal in subsequent generations requires the germline nuclear AGO HRDE-1 and nuclear RNAi factors. Nuclear RNAi is involved in and required for the inheritance of RNAi-mediated gene silencing **(B)**, maintaining germline immortality **(C)**, and adaptive behavior **(D)**. **B.** Chromatin mis-regulation and loss of nuclear RNAi pathways in the germline lead to a mortal germline phenotype (Mrt), or the progressive loss of fertility over generations. **C.** In RNAi inheritance, silencing of germline-expressed *gfp* transgenes can be maintained for several generations after exposure to exo-dsRNA. In heritable nuclear RNAi mutants, the transgene is silenced in the P_0 generation, but silencing is not inherited in progeny. **D.** Chemotaxis and pathogen avoidance are examples of behaviors that require sRNA pathways. Neuronally-expressed RDE-4 is required for the transgenerational inheritance of chemotaxis behavior. The inheritance of pathogen avoidance behavior is dependent on PRG-1, and pathogenic learning in P0 animals requires proper functioning of the nuclear RNAi pathway. Inheritance of both behaviors requires nuclear RNAi pathways.

genome (Fig. 1**C-E**) [13]. In contrast, HRDE-1 associated 22G-RNAs target germline repressed genes (14% of the coding genome), cryptic loci, and repetitive elements (Fig. 1**C–E**) [12,28]. NRDE-3 associated 22G-RNAs target protein coding genes (1% of the coding genome), pseudogenes, lincRNAs, DNA and RNA transposons, and intergenic sequences (Fig. 1**C–E**) [14,29,30]. These three AGOs have largely distinct targets. However, there is significant overlap of HRDE-1 and NRDE-3 protein-coding targets (Fisher's Exact Test, $p = 5.3 * 10^{-5}$), and of CSR-1 and HRDE-1 ($p = 4.6 * 10^{-3}$) and CSR-1 and NRDE-3 ($p = 5.4 * 10^{-20}$) non-coding targets (Fig. 1**D–E**). Thus, nuclear AGOs target thousands of loci and are important for maintaining genome integrity as well as regulating gene expression.

2.2. Overview of transgenerational phenotypes associated with nuclear RNAi

2.2.1. Fertility, germline immortality and RNAi inheritance

Nuclear RNAi can lead to inherited phenotypes (Fig. 2**A**). A striking phenotype of germline nuclear AGOs and associated nuclear RNAi factors is their effects on fertility. Mutation of *csr-1* leads to chromosome segregation defects and sterility [13,16,31]. In contrast, mutations of *hrde-1* or the associated nuclear RNAi factors, *nrde-2*, *nrde-1* or *nrde-4*, do not show overt fertility defects under normal growth conditions, but display a progressive loss of fertility across generations, known as a mortal germline (Mrt) phenotype, particularly at elevated temperatures (25 °C) for *hrde-1* and *nrde-2* (Fig. 2**B**) [12]. Moreover, loss of germline nuclear RNAi factors also leads to a progressive depletion of histone H3K9me3 at endogenous target loci across generations [12,32]. These studies highlight that nuclear RNAi connects sRNAs and chromatin modifications to mediate epigenetic memory that can be inherited across multiple generations, referred to as Transgenerational Epigenetic Inheritance (TEI).

Several experimental paradigms have provided insight into the mechanisms of nuclear RNAi in TEI in *C. elegans* (Reviewed in Ref. [33]). Here we will focus on the workhorse model, RNAi inheritance, in which silencing initiated by exogenous dsRNA can be inherited for several generations in the absence of the initial trigger. This TEI phenomenon has been demonstrated for a germline-expressed single copy *gfp* transgene (Fig. 2**C**) [12,34] and for the endogenous gene *oma-1* [35]. RNAi inheritance is related to a form of TEI directed at transgenes that is initiated by piRNAs/PRG-1 recognizing sequences within transgenes as "non-self". Both transgene TEI and RNAi inheritance rely on chromatin factors, 22G-RNAs, and nuclear RNAi as downstream effectors (Fig. 2**A**) [12,34,36,37]. One particular strength of the RNAi inheritance model is that focusing on a single target gene/locus provides a simpler context for understanding the roles of chromatin modifications and sRNAs in establishing and maintaining the transfer of epigenetic information between generations, as well as how the characteristics of the target locus influence this process (see Section 3). Moreover, being able to trigger silencing in a specific generation by the addition of dsRNA is also a key feature of this system. Importantly, genes required for RNAi inheritance identified by genetic screens also exhibit a temperature-dependent Mrt phenotype, further emphasizing the link between factors involved in TEI and fertility [12,38].

2.2.2. Behavior, adaptation and TEI

Nuclear RNAi also plays a role in the response of *C. elegans* to its environment, through regulation of behavioral responses, including olfactory adaptation, chemotaxis and pathogen avoidance (e.g. [39–41]). Moreover, recent studies provide two fascinating examples of nuclear RNAi in the TEI of chemotaxis behavior and pathogen avoidance (Fig. 2**D**) [42–44]. Animals mutant for *rde-4* (RNAi-deficient 4), a dsRNA-binding protein required for endo-siRNA biogenesis [45,46], are defective in chemotaxis to benzaldehyde [44]. However, expressing RDE-4 only in neurons led to a partial rescue of chemotaxis behavior, not only in the animals expressing RDE-4, but in their F3 *rde-4* (-/-) mutant progeny, which did not express RDE-4. This transgenerational behavior rescue was dependent on the germline nuclear AGO *hrde-1*. Moreover, neuronal RDE-4 expression led to heritable changes in sRNAs and gene expression in the germline of progeny [44]. This study raises the possibility that neuronal endo-siRNA activity can be communicated to the germline and propagated across generations by nuclear RNAi to affect the chemotaxis behavior of offspring.

Inherited information from parents can be a form of adaptive response, where progeny display increased fitness in response to a stress their parents experienced. *C. elegans* are attracted to the pathogenic bacteria *Pseudomonas aeruginosa* (PA14) as a food source, but quickly learn to avoid it within hours of exposure [47]. Remarkably, this information can be transmitted to naïve progeny and persists through four generations (Fig. 2**D**) [43]. Whereas some nuclear RNAi and chromatin factors were required for the initial PA14 attraction or avoidance (including *hrde-1*), the Piwi AGO *prg-1*, the RdRP *rrf-1,* along with downstream histone methyl transferases (HMTs; e.g. *set-25*) and H3K9me-binding proteins (*hpl-2*) were specifically required for the transgenerational inheritance of avoidance behavior [43]. While additional studies will be needed to determine the tissue where *prg-1* acts and the significance of changes in sRNA levels in this paradigm, it is a striking example of how sRNAs and chromatin regulate TEI to influence organism fitness.

3. Interplay of nuclear RNAi with genome and chromatin organization

In *C. elegans*, nuclear RNAi directs the deposition of histone marks at the target gene locus, including heterochromatic H3K9me3 [22,22,23], H3K27me3 [24] and the less well-studied but abundant H3K23me3 [48–50] (Fig. 3**A**). In this section, we highlight recent insights into the roles of these modifications, and the SET (Su(var)3–9, Enhancer-of-zeste and Trithorax)-domain-containing histone methyltransferases (HMTs) that deposit them, in the establishment and maintenance of transgenerational nuclear RNAi responses. We also discuss the relationships between nuclear RNAi and other chromatin regulatory machinery in the silencing of endogenous genes and repetitive elements.

3.1. Histone methyl transferases (HMTs) in the establishment and maintenance of silencing by nuclear RNAi (SET-25, SET-32, MET-2)

The roles of H3K9me3 and H3K23me3 HMTs in TEI have been most extensively studied using RNAi inheritance assays (Fig. 2**C**). In this context, exo-RNAi leads to a histone H3 methylation 'footprint' at the target locus that lags at least 20 hours behind the accumulation of 22G-RNAs and persists for at least 3 generations after the exo-RNAi trigger has ceased [23,48]. While these findings implicated histone modifications in TEI, subsequent analysis of HMT mutants revealed three interrelated principles: (*1*) H3K9me3/H3K23me3 can be decoupled from silencing, (*2*) genetic requirements for silencing may be sequence- or locus context-specific, and (*3*) HMTs play a more prominent role in the establishment than maintenance of silencing.

In embryos, two HMTs contribute to H3K9me3 – MET-2, which deposits H3K9me1/2, and SET-25, which deposits H3K9me3 [51] through both *met-2*-dependent and independent mechanisms [52] (see Section 3.3). However, in adults, *met-2 set-25* mutants retain some H3K9me3, whereas *set-32; met-2 set-25* triple mutants have undetectable H3K9me3 [38,53,54]. Therefore, it was initially thought that SET-32 may be another H3K9me3 HMT, but recent evidence identified SET-32 as a H3K23me3 HMT, and that this activity may promote H3K9me3 [48]. H3K27me3 is also deposited at nuclear RNAi targets [24], however mutants of the H3K27me3 HMT, MES-2, have a maternal effect sterile phenotype, complicating the analysis of TEI.

RNAi inheritance assays (Fig. 2**C**) revealed locus-dependent effects of HMTs in TEI: *set-25, set-32* and *set-32*; *met-2 set-25* triple mutants show defective transgenerational silencing inheritance when a GFP transgene,

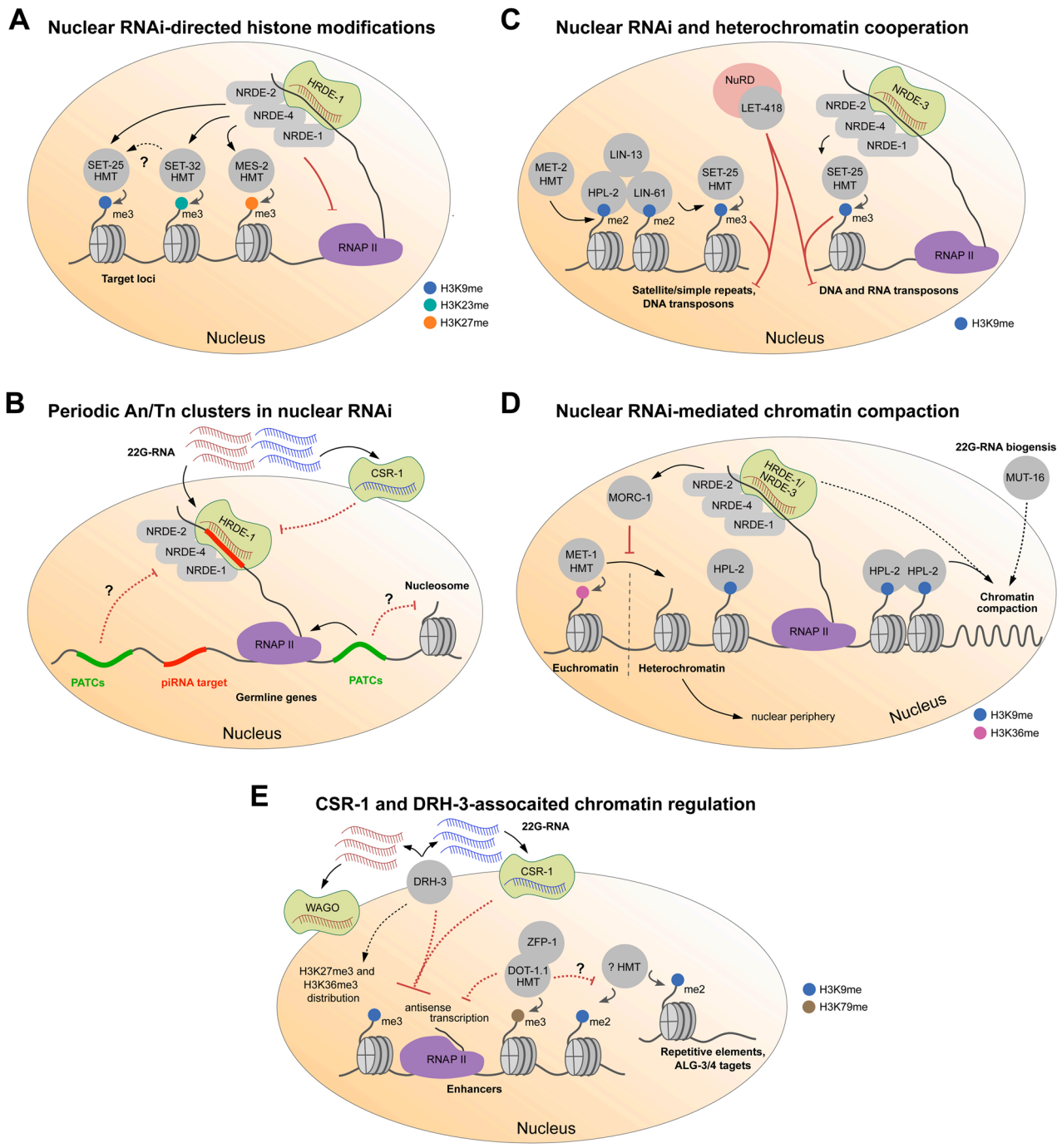

Fig. 3. Nuclear RNAi influences genome and chromatin organization. **A.** The nuclear RNAi machinery assembles in a hierarchical manner. HRDE-1 (or NRDE-3), in complex with 22G-RNA, engages nascent transcripts, leading to the recruitment of NRDE-2, which in turn recruits NRDE-4 and then NRDE-1. This leads to deposition of heterochromatic marks by histone methyltransferases (HMTs) as well as inhibition of RNA Pol II elongation. **B.** Periodic An/Tn Clusters (PATCs) are enriched at germline genes and promote gene expression by inhibiting piRNA-directed silencing and potentially regulating nucleosome positioning. PATCs may function synergistically with CSR-1 activity to antagonize piRNA-dependent silencing by the nuclear RNAi pathway. **C.** Nuclear RNAi pathways interact with H3K9me-associated heterochromatin factors. The H3K9 histone methyltransferase SET-25 can be recruited through nuclear RNAi targeting or through LIN-61 interactions. Nuclear RNAi and heterochromatin proteins could play redundant roles in transcription repression, particularly at repetitive elements. **D.** Nuclear RNAi pathways recruit MORC-1, which inhibits MET-1-dependent H3K36me encroachment at silenced loci. Nuclear RNAi-dependent compaction also depends on MORC-1 and HPL-2. Secondary 22G-RNA biogenesis, dependent on MUT-16, also promotes chromatin compaction, particularly during heat stress. **E.** At certain enhancers, DRH-3 and CSR-1 inhibit antisense transcription and the acquisition of heterochromatin-associated histone modifications. The H3K79me HMT DOT-1.1 also antagonizes H3K9me at enhancers, repetitive elements, and ALG-3/4 targets.

but not the endogenous gene, *oma-1*, was targeted [38,53–55]. Along similar lines, an analysis of endogenous sRNA target loci, which show *hrde-1* dependent transcriptional silencing and/or *hrde-1*-dependent H3K9me3, revealed little change in RNA-seq or RNA Pol II ChIP profiles at these loci in *set-32*; *met-2 set-25* triple mutants when compared to wild-type animals [28,53]. Therefore, for the majority of endogenous targets, nuclear RNAi-dependent silencing can be maintained in HMT mutants devoid of H3K9/23me3, likely through other *hrde-1*-dependent

mechanisms. However, HMTs may play a role in maintaining silencing for a small subset of these endogenous *hrde-1* targets, such as the Cer3 LTR retrotransposon, for which combining *hrde-1* and *set-32; met-2 set-25* HMT mutations did enhance de-silencing in comparison to *hrde-1* mutants [56]. Together, these findings underscore the complex and locus-dependent relationship between nuclear RNAi-induced histone methylation and transcriptional silencing.

Further analysis of RNAi inheritance targeting GFP transgenes revealed that *set-25* and *set-32* mutants are only defective for RNAi inheritance to the F1 generation. When the small minority of F1 progeny of *set-25* or *set-32* mutants which did manage to silence GFP were selected and propagated, these animals inherited the silencing to the F2 and F3 generations comparably to wild-type animals [57]. This raised the question whether these HMTs are required for the establishment of silencing in the parental (P0) generation. By establishing an exo-RNAi response in either homozygous or heterozygous *set-25* or *set-32* mutants, and examining the inheritance of silencing to the homozygous F1 generation, it was demonstrated that only the P0 homozygous mutants were deficient in the inheritance of silencing [57]. Thus, these HMTs promote the establishment of transgenerational silencing but are dispensable for its maintenance [58]. This finding is consistent with previous studies showing that RNAi is still inherited across generations even after the removal of the original targeted DNA locus (and consequently, the histone marks on it) [59–63]. Another study evaluated silencing establishment at endogenous nuclear RNAi targets using a *hrde-1* mutant background, which is silencing-defective [56]. The timing of re-establishment of silencing was then assayed by RT-qPCR and RNA-seq after repairing *hrde-1* using CRISPR/Cas9. Comparing the timing of re-silencing in wild-type and HMT mutant animals across several generations revealed that re-silencing of a subset of *hrde-1* targets was delayed in *set-32* mutants, some for up to 20 generations. This assay further implicates *set-32* in promoting the transgenerational establishment of silencing by nuclear RNAi.

3.2. Role of chromatin and sequence features in nuclear RNAi targeting – HMTs and Periodic An/Tn Clusters (PATCs)

The different roles of HMTs in the establishment and maintenance of silencing at transgenes versus endogenous genes (e.g. *oma-1*) may reflect underlying sequence and/or chromatin features of these target loci, which may promote or counteract silencing mechanisms. One important sequence feature is Periodic An/Tn Clusters (PATCs), endogenous non-coding sequences that act in *cis* to promote germline gene expression in *C. elegans* (Fig. 3**B**). PATCs makeup ~ 10% of the *C. elegans* genome [64] and are anti-correlated with heterochromatin-associated H3K9me3 [65]. PATCs are enriched in germline-expressed genes, particularly when located within heterochromatic regions of the genome, and these PATC-enriched genes show reduced 22G-RNA accumulation targeting their potential piRNA target sites [66,67]. In addition, the incorporation of PATC-enriched introns into "foreign" transgenes provides resistance to piRNA-mediated silencing, suggesting that PATCs enable these transgenes to be recognized as "self" and escape piRNA/sRNA-mediated surveillance [66–68]. PATCs likely work alongside other mechanisms that counteract piRNA-mediated surveillance, such as licensing by the CSR-1–22G-RNA pathway [37,69–71]. Although the mechanisms through which PATCs may oppose nuclear RNAi-mediated silencing are still unclear, one possibility is that they affect nucleosome density, which has been observed for AT-rich sequences in yeast [72,73]. In addition to PATCs, additional sequence features such as introns contribute to locus-specific effects of nuclear RNAi (Section 3.1).

Nuclear RNAi mediates heterochromatic histone modifications, but can histone modifications influence small RNAs? Such a 'feedback' mechanism has been described in other organisms – for example in fission yeast, where nuclear RNAi recruits RdRP complexes to reinforce silencing [74]. However, the potential inhibition of heterochromatin formation by PATCs suggests that genomic loci and their chromatin state may, directly or indirectly, influence the biogenesis of 22G-RNAs [67]. To examine the potential roles of HMTs in this feedback, the effects of *set-25* or *set-32* mutation on global sRNA profiles revealed that these mutants are depleted of specific subsets of sRNAs that are normally associated with HRDE-1 and a cytoplasmic 22G-RNA binding WAGO, WAGO-1, but not CSR-1 [55]. The targets of these sRNAs are enriched for "newly evolved" genes, defined as genes without orthologs outside of *C. elegans*, and have relatively low PATC density. This observation supports the notion that foreign sequences or "newly evolved" genes, but not endogenous germline-expressed/CSR-1-targeted genes such as *oma-1*, are recognized as non-self-elements. At these targets, H3K9me3/H3K23me3 may contribute to the maintenance of heritable sRNA populations. However, since evidence to date indicates that 22G-RNA biogenesis occurs outside the nucleus in *C. elegans*, histone methylation may influence this process indirectly, for example by affecting the sorting of transcripts to 22G-RNA biogenesis pathways.

3.3. Cooperation between nuclear RNAi and H3K9me heterochromatin regulation

How does nuclear RNAi cooperate with other machinery regulating heterochromatin? By comparing the effects of *nrde-2* mutation with mutation of several heterochromatin factors, a partial functional redundancy in repetitive element silencing was uncovered [75]. Heterochromatin factors previously known to have reduced fertility and pleiotropic developmental defects, including the H3K9me readers HPL-2 and LIN-61, H3K9me1/2 HMT MET-2, zinc-finger protein LIN-13, and nucleosome remodeler LET-418, were examined in this study. These chromatin factors are enriched throughout the genome at heterochromatic loci, particularly H3K9me2-marked repetitive elements (Fig. 3**C**). A small proportion of these repetitive elements, in particular DNA transposons, are de-repressed in the single heterochromatin factor mutants and *met-2 set-25* double mutants [75]. In contrast, repetitive elements de-repressed in *nrde-2* mutants were mostly LTR retrotransposons, which are enriched for H3K9me3 [28,75,76]. Interestingly, *let-418;nrde-2* double mutants showed synergistic effects on fertility and repetitive element de-repression, suggesting partial functional redundancy. It will be of interest to determine the mechanisms through which *let-418*, an Mi-2 homolog and component of the nucleosome remodeling and deacetylase complex (NuRD), cooperates with nuclear RNAi in repetitive element silencing and whether there are similar effects at protein-coding genes.

Genetic and genomic studies have further revealed the relationships between nuclear RNAi and histone H3K9 HMTs *met-2* and *set-25* [52]. In embryos, the majority of H3K9 methylation is deposited in a step-wise manner, where MET-2 deposits H3K9me1/2 and SET-25 subsequently deposits H3K9me3 [51]. This pathway also involves the H3K9me-binding protein LIN-61 [52]. However, ~ 13% of H3K9me3 in embryos is deposited independently of *met-2*. Instead, these loci, which are enriched in intact DNA and RNA transposons, rely on targeting by the somatic nuclear 22G-sRNA-binding AGO, *nrde-3* (Fig. 3**C**). Loss of both H3K9 deposition pathways by *met-2* and *nrde-3* mutation resulted in de-silencing of repetitive elements, reduced H3K9me3 at transposons, and developmental defects that are worse than in the respective single mutants, indicating that somatic nuclear RNAi and the *met-2-lin-61* pathway work in a partially redundant manner [52]. In addition, even in *met-2;nrde-3* mutants, H3K9me3 remains on some transposons, indicating that additional pathways, such as *set-32*-dependent H3K23me (Section 3.1), may also participate in the targeting of H3K9me3 HMTs. Notably, *nrde-3*-dependent recruitment of SET-25 was required to initiate silencing of a multicopy reporter transgene [52], suggesting that the *met-2*-independent pathway is more important for *de novo* silencing at endogenous loci, such as developmentally regulated genes or new transposons. This finding is also consistent with the role of *set-25* in the establishment of heritable silencing by exo-RNAi and the targeting of "newly evolved" genes by *set-25*-dependent sRNAs (Sections 3.1 and

3.2). Future studies should focus on distinguishing unique and synergistic roles and feedback mechanisms between sRNA and chromatin pathways in genome regulation.

3.4. Nuclear RNAi-mediated chromatin compaction (MORC-1, HPL-2)

While it is established that nuclear RNAi leads to deposition of heterochromatin marks, additional mechanisms are needed downstream for the maintenance of silencing. One such mechanism is chromatin compaction. Microscopy approaches revealed that nuclear RNAi factors (*hrde-1*, *nrde-2*, *nrde-3*) are required for compaction of germ cell nuclei at elevated temperatures (25 °C), the X chromosome in intestine cells, and exo-RNAi-directed compaction of repetitive transgene arrays [32,77]. Two key conserved compaction factors are the HP1-like histone methylation reader HPL-2 and the GHKL family ATPase MORC-1 (mouse microrchidia family CW-type zinc finger protein) (Fig. 3**D**). HPL-2 is required for nuclear RNAi-directed transgene array compaction and for maintenance of transgenerational silencing initiated by the piRNA pathway [34,37,75]. Although HPL-2 can associate with both H3K9me and H3K27me [78,79], its genomic binding is correlated with, although not entirely dependent upon, H3K9me1/2 [80]. The mechanisms of HPL-2-mediated compaction have not been fully described, but, like HP1 homologs in other species, HPL-2 self-dimerization [81] likely plays a role.

The MORC-1 ATPase functions downstream of nuclear RNAi and is required for RNAi inheritance and localization of heterochromatic regions to the nuclear periphery [32,38]. *morc-1* mutants also display a temperature-sensitive Mrt phenotype, which corresponds to progressive loss of H3K9me3 and gain of H3K36me3 across generations, at a subset of *hrde-1*/ 22G-RNA targets [32]. A mutagenesis screen in *morc-1* mutants identified *met-1*, a H3K36 histone methyltransferase, as a suppressor of germline mortality in *morc-1* and *hrde-1* mutants [32]. However, the loss of H3K9me3 in *morc-1* mutant is not restored by *met-1* mutation [32], suggesting that the compaction activity of MORC-1 [82] maintains H3K9me3 and germline immortality by inhibiting the spread of euchromatin (Fig. 3**D**).

Loss of *hrde-1* or *mut-16*, a 22G (and 26G) RNA biogenesis factor, leads to fertility defects at elevated temperatures that are correlated with upregulation of genes normally expressed in the soma [83,84]. These mis-regulated genes also displayed increased chromatin accessibility in *mut-16* mutant germ cells, however most were not known sRNA targets and did not display significant changes in their mapped sRNAs [84]. Overall, these findings suggest that disruptions to nuclear RNAi/22G-RNA pathways can regulate gene expression through altered global chromatin compaction at both sRNA target and indirectly at non-target loci.

3.5. Regulation of chromatin landscape at CSR-1 targets and enhancers

The impact of nuclear RNAi/22G-RNA pathways on genome-wide chromatin landscape has also been examined in mutants of the Dicer-related helicase *drh-3*, an RdRP-associated sRNA biogenesis factor [85,86], and in *csr-1* 'partially rescued' animals, where the sterility phenotype of a *csr-1* null mutation is partially rescued by a germline-expressed *csr-1* transgene [13,87]. Hypomorphic *drh-3* mutants, which have reduced WAGO- and CSR-1-associated 22G-RNAs, display increased H3K27me3, decreased H3K36me3, and reduced expression at actively transcribed genes, including CSR-1 targets, while exhibiting the opposite regulatory effects at transcriptionally inactive genes [87,88]. However, the mechanisms of this redistribution and its dependence on nuclear RNAi have not been fully explored.

In addition, although global H3K9me3 is reduced in *drh-3* mutants, both *drh-3* and *csr-1* mutants show a similar impact on enhancer regions – increased H3K9me3 as well as increased antisense transcription and sRNAs mapping to these enhancers [88]. Thus CSR-1–22G-RNA pathways may directly or indirectly regulate enhancers and enhancer-associated RNAs (Fig. 3**E**). Enhancer transcription and accumulation of heterochromatic H3K9me2 at enhancers are also counteracted by the conserved euchromatic H3K79me-HMT DOT-1.1 and its partner protein, zinc-finger protein ZFP-1 [89–91]. In addition to enhancers, loss of DOT-1.1 also resulted in increased H3K9me2 enrichment at a subset of repetitive elements, and ALG-3/4 26G-RNA targets [90]. Together with genetic interactions between *dot-1.1* and sRNA biogenesis factors (*rde-1* and *rde-4*) [91], these findings suggest additional cooperation between endo-RNAi and chromatin pathways influence chromatin state.

4. *Trans*-acting factors and regulation of nuclear RNAi

Forward genetic screens and proteomics approaches have proven effective in identifying nuclear RNAi factors. The nuclear AGO *nrde-3* and the nuclear RNAi co-factors *nrde-1*, *nrde-2* and *nrde-4* were identified in a screen for animals resistant to exo-RNAi against *lir-1* [14,21,22,92]. Although *lir-1* null mutants are viable, exo-RNAi targeting *lir-1* leads to developmental arrest as a result of the co-targeting of *lin-26*, a gene located in an operon with *lir-1*. Because the polycistronic *lir-1/-lin-26* operon pre-mRNA is processed into separate mRNAs in the nucleus, co-targeting of *lin-26* by *lir-1* RNAi was one of the first indications that RNAi could target nuclear transcripts in *C. elegans* [93]. Likewise, the GFP RNAi inheritance paradigm described above (see Section 2.2.1) was a fruitful tool for the screening and candidate approaches that identified the nuclear AGO *hrde-1*, histone methyltransferases, and other key nuclear RNAi factors [12,34,36–38]. More recently, proteomics approaches have complemented genetic approaches, identifying new interactors of the core nuclear RNAi factors and revealing connections to RNA processing and AGO loading. Additional genetic and genomic approaches have also revealed factors that are required for limiting the duration and extent of TEI.

4.1. Connections to RNA processing: MTR-4, EMB-4

A recent immunoprecipitation-mass spectrometry (IP-MS) approach identified the nuclear DExD box RNA helicase MTR-4 as an NRDE-2 interactor in *C. elegans* [94] (Fig. 4**A**). MTR-4 associated with nascent pre-mRNA of nuclear RNAi targets in a *nrde-2* and *hrde-1* dependent manner, and this association was required to trigger co-transcriptional silencing (Fig. 1**A**). However, it is still unclear whether MTR-4 might directly bind mRNA or indirectly associate with mRNA as part of the larger complex [94]. Null mutations of *mtr-4* were lethal in *C. elegans*, and selective degradation in the germline or soma using the degron system caused sterility and larval arrest, respectively, indicating that MTR-4 is broadly important [94]. In human cells, NRDE-2 is essential and required for proper cell cycle progression [95]. Although the mechanisms have not been fully elucidated, these phenotypes may be in part attributed to the role of NRDE-2 in promoting the splicing of 'weak' introns in human cells [95]. In addition to the role of MTR-4 in splicing, the NRDE-2:MTR-4 interaction is conserved with homologs in mammals and fungi, where it is implicated in regulating mRNA quality control by the nuclear exosome [96–98]. Further studies in *C. elegans,* such as examining splicing changes and the stability of nuclear RNAi target transcripts in *nrde-2* and *mtr-4* mutants may illuminate the mechanisms through which NRDE-2 and MTR-4 link co-transcriptional silencing with splicing and nuclear export.

Using similar proteomics approaches with HRDE-1 or CSR-1 as baits, the nuclear RNA helicase and splicing factor EMB-4 (Aquarius (AQR) in humans) was identified as an interactor of both AGOs [99,100] (Fig. 4**B**). Sequencing EMB-4 associated transcripts revealed that while EMB-4 associated with both introns and exons of nearly 8000 genes, it was more highly enriched in the introns of CSR-1 targets. The differential association of EMB-4 with CSR-1 vs. HRDE-1 targets points to a potential mechanism for routing specific transcripts into either the CSR-1 or HRDE-1 pathway [99,100]. Mutation of *emb-4* leads to

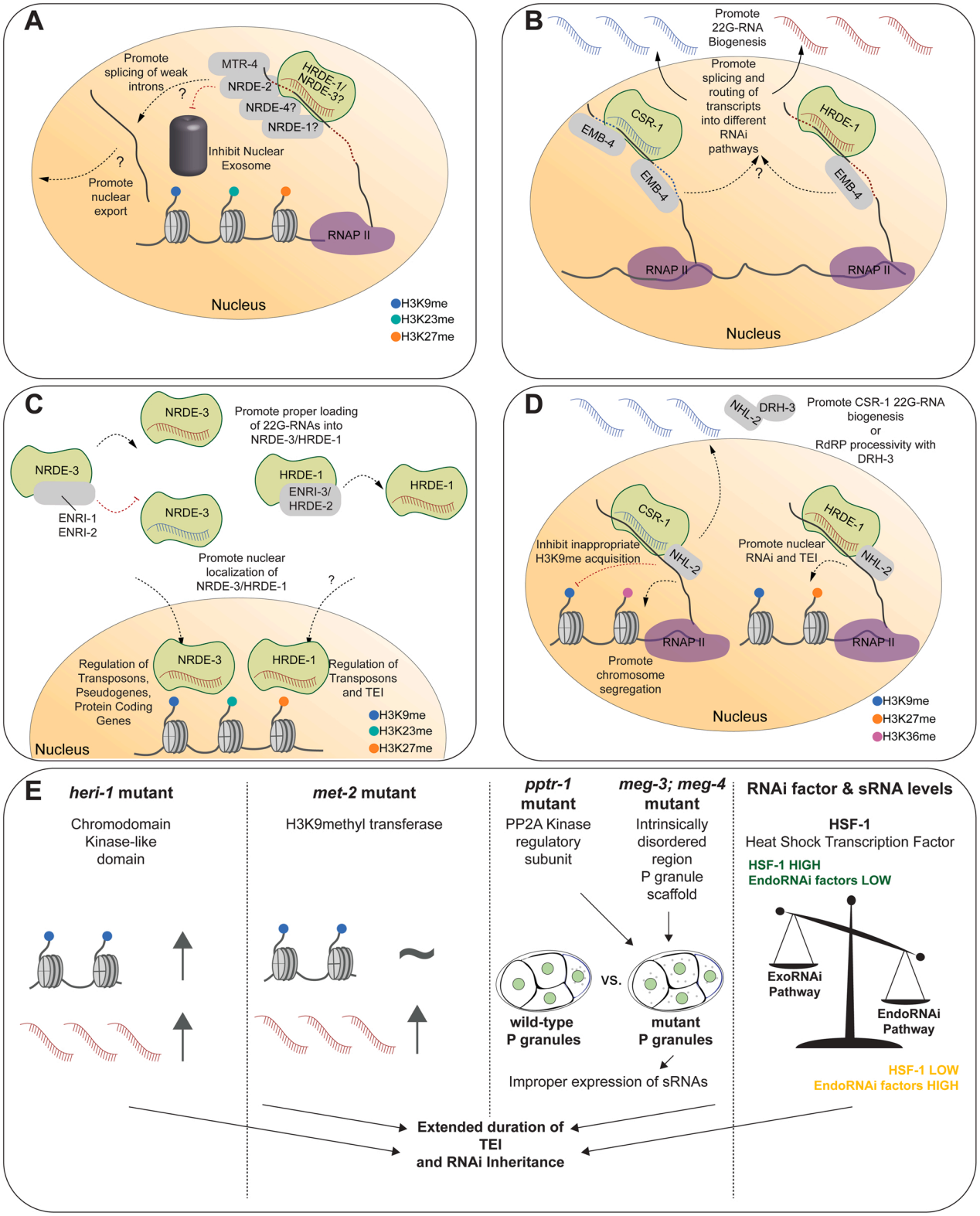

Fig. 4. Factors that modulate nuclear RNAi and TEI. A. NRDE-2 physically interacts with the nuclear exosome component MTR-4. This may inhibit nuclear exosome activity, promote the splicing of weak introns, and facilitate the nuclear export of target transcripts. **B.** The intron binding protein EMB-4 binds both CSR-1 and HRDE-1 target transcripts. This may promote the splicing, export, and sorting of these transcripts into different sRNA pathways, and facilitates the production of sRNAs. **C.** The ENRI proteins interact with unloaded nuclear AGOs to facilitate the proper loading of sRNAs. In turn, this facilitates nuclear AGO translocation to the nucleus and proper regulation of targets. **D.** The RNA binding TRIM-NHL protein NHL-2 physically associates with CSR-1 and HRDE-1. This may facilitate the proper acquisition of histone modifications, promoting proper gene regulation. NHL-2 also associates with DRH-3, which may enable RdRP processivity along CSR-1 target transcripts. **E.** Multiple factors limit the duration and penetrance of RNAi inheritance, including HERI-1, MET-2, PPTR-1, MEG-3 and MEG-4, and HSF-1, along with sRNA and RNAi protein levels.

decreased transcription of CSR-1 target genes and decreased levels of CSR-1 22G-RNAs. In contrast, transposable elements normally targeted by HRDE-1 22G-RNAs are upregulated in *emb-4* mutants. Using a piRNA-reporter transgene that is targeted by HRDE-1 22G-RNAs acting downstream of PRG-1, Akay et al. showed that loss of *emb-4* led to a decrease in 22G-RNAs specifically across intronic regions. It is currently unclear whether EMB-4 interacts with RdRP complexes to synthesize 22G-RNAs (either within the nucleus or cytoplasm), or if EMB-4 forms the basis of a ribonucleoprotein (RNP) complex that serves to direct transcripts into different AGO pathways [99,100]. Further study will be required to disentangle the specific functions of EMB-4 in the CSR-1 and HRDE-1 pathways.

The roles of *emb-4* and *mtr-4* highlight the connection between splicing regulation and nuclear RNAi. Indeed, mutations of three core small nuclear ribonucleoprotein (snRNP)-associated factors, U1A (*rnp-2*), U2B″ (*rnp-3*) and U2A′ (*mog-2*), also impact endo-RNAi [101]. Mutation of *rnp-2* resulted in decreased 26G-RNAs against ERGO-1 targets, but this defect was most likely attributed to the mis-splicing of *eri-6/7*, which is required for 26G-RNA biogenesis [101,102]. In contrast, mutations of *rnp-3* and *mog-2* disrupted RNAi inheritance, suggesting a role in nuclear RNAi. Many endo-RNAi targets have weak splicing signals, stalled splicing is associated with RNAi targeting [101–103], and *nrde-2* regulates splicing of weak introns in mammals cells [95]. However, whether the splicing machinery plays a direct role in *C. elegans* nuclear RNAi will require further investigation.

4.2. Regulation of AGO loading and 22G-RNA biogenesis: ENRI-1/2, HRDE-2, NHL-2

Proteomic studies of the AGO NRDE-3 led to the identification and characterization of a family of proteins, ENRI-1 and -2 (Enhanced nuclear RNAi) that regulate the loading of 22G-RNAs into AGOs [104] (Fig. 4**C**). These proteins contain several predicted disordered regions and a region with resemblance to a P loop-containing nucleoside triphosphate hydrolase motif [104]. In *enri-1/2* mutant embryos, NRDE-3 bound more sRNAs (including classes of sRNAs it would not bind in a wild-type context) and translocated to the nucleus earlier in embryogenesis than wild-type. Because nuclear translocation of NRDE-3 requires sRNA loading, and ENRI-1/2 bound unloaded NRDE-3, it appears that ENRI-1/2 block inappropriate 22G-RNA loading of NRDE-3 [104]. Another member of the ENRI family, ENRI-3, was also identified as HRDE-2 in a genetic screen for RNAi inheritance factors [38]. Like several other RNAi inheritance factors, loss of *hrde-2* results in a temperature-sensitive Mrt phenotype. HRDE-2 facilitated the association of 22G-RNAs with HRDE-1, but not another WAGO, PPW-3, although it was not determined whether this was through a direct or indirect mechanism [38]. HRDE-2 was also required for maintaining proper 22G-RNA profiles and transposon silencing [104]. Together these studies indicate that ENRI-1/2 and HRDE-2 (ENRI-3) are part of the elusive mechanisms that regulate the specificity of WAGOs for different subsets of 22G-RNAs.

The identification of 22G-RNA pathway factors, including *csr-1* and *drh-3*, in an RNAi screen for genetic interactions with the TRIM-NHL family *nhl-2* led to the characterization of *nhl-2* as a new cofactor in 22G-RNA biogenesis [105] (Fig. 4**D**). As an RNA binding protein [105] with previously known roles in microRNA regulation [106], NHL-2 likely has multiple sRNA-dependent as well as independent effects on gene regulation. However, mutation of *nhl-2* led to defects that mirror nuclear RNAi pathways, including a Mrt phenotype, resistance to *lir-1* exo-RNAi, and defective RNAi inheritance, as well as the CSR-1 pathway, including germline chromosome organization and segregation defects [105]. There was also a decrease in abundance of a subset of CSR-1 and WAGO-associated 22G-RNAs in *nhl-2* mutants, and a notable change in the distribution of 22G-RNAs across CSR-1 target genes, where the abundance at the 5′ end was reduced in comparison to wild-type. Furthermore, co-IP of NHL-2 associated with HRDE-1, CSR-1, or the RdRP-associated helicase DRH-3, led to a model that NHL-2 may influence the activity or processivity of the RdRPs that generate 22G-RNAs, or may regulate the loading of AGOs with 22G-RNAs [105].

4.3. Factors that influence the duration and penetrance of TEI and RNAi inheritance

While TEI can be beneficial (e.g. pathogen avoidance, Section 2.2.2), it may also be deleterious in changing environmental conditions. Therefore, it seems prudent for the worm to have methods for halting and re-setting epigenetic inheritance mechanisms. In fact, *C. elegans* has evolved such "brakes," and TEI phenomena including RNAi inheritance typically perdure for approximately five generations [33]. This observation provides a useful experimental context to genetically identify factors that limit the number of generations TEI persists. One such screen identified a mutant that displayed RNAi inheritance for >20 generations: the chromodomain and kinase-like domain containing protein HERI-1 (Heritable Enhancer of RNAi) [107] (Fig. 4**E**). Both repressive histone marks and sRNA levels are increased in *heri-1* mutants which could explain why TEI perdures. This extended TEI phenotype along with others, including sperm defects, can be suppressed by mutation of *hrde-1*, solidifying a role for HERI-1 in the nuclear RNAi pathway. HERI-1 is recruited to chromatin in a HRDE-1 and SET-32 (HRDE-3) dependent manner to a genomic locus that is silenced by RNAi [107]. While the specific molecular role(s) of HERI-1 on chromatin are not understood, it is possible that HERI-1 associates with modified histones via its chromodomain to recruit negative regulators such as chromatin remodelers that remove the repressive histone marks deposited by the nuclear RNAi pathway.

Similarly, loss of the HMT, MET-2, leads to the RNAi inheritance-mediated silencing of a *gfp* transgene for >30 generations [54] (Fig. 4**E**). In this background, prolonged RNAi inheritance does not appear to be due to drastic changes in the histone methylation landscape surrounding the silenced locus, but correlates with an increase in 22G-RNAs targeting the silenced locus over multiple generations [54]. *met-2* mutants also displayed a global mis-regulation of sRNAs, which may shift the balance between endogenous sRNA pathways and liberate shared endogenous/exogenous RNAi components to increase their activity in exogenous RNAi. Loss of *hrde-1* in the *met-2* mutant suppressed the RNAi inheritance phenotype, leading to a resumption of GFP expression. Loss of HRDE-1 leads to a loss of associated 22G-RNAs, thereby implicating 22G-RNA levels as important modulators of RNAi inheritance duration.

Germ granules are phase separated, non-membrane bound organelles present in germ cells that harbor various RNAi proteins and play multiple roles in endogenous and exogenous sRNA pathways (Fig. 4**E**). While space limitations preclude a full discussion of the role of germ granules in TEI, the reader is referred to recent reviews for more detail [108,109]. Important here is the observation that in embryos, double mutants of the germ granule scaffold proteins *meg-3;meg-4* and mutants the PP2A phosphatase regulatory subunit *pptr-1* disrupt germ granules called P granules yet display RNAi inheritance for tens of generations [110]. This suggests that germ granule integrity is not required for sRNA inheritance, but may limit the duration of RNAi inheritance.

While the levels of 22G-RNAs are clearly important for determining the strength and duration of RNAi inheritance, so are the levels of RNAi pathway proteins (Fig. 4**E**). Upon exposure to dsRNA (e.g. for *gfp*), Houri-Ze'evi et al. observed a change in the levels of sRNAs targeting genes encoding RNAi factors, including *rde-1, rde-2, mut-7,* and *nrde-4*. These particular genes are the targets of CSR-1, and are therefore expected to be licensed for expression during RNAi. However, with increasing generations, overall sRNA levels decrease, contributing to a finite duration and decreased penetrance of RNAi inheritance. Notably, the same study also found that exposure to a second and unrelated dsRNA trigger was sufficient to enhance the strength of the original RNAi effect, collectively demonstrating the presence of a feedback loop

between sRNAs and RNAi factors that regulates RNAi inheritance [60]. In a related and extensive study, Houri-Ze'evi et al. defined three key principles of RNAi inheritance, and identified the conserved transcription factor HSF-1 as a negative regulator of endogenous sRNA factors. By suppressing competing endogenous sRNA pathways, HSF-1 enables RNAi to be inherited with greater penetrance and for additional generations [111]. Collectively, these studies highlight the importance of multiple types of feedback regulation of sRNA levels, RNAi factors, and chromatin modifications in determining the extent and duration of TEI and RNAi inheritance.

5. Conclusions

In this review, we have synthesized key recent findings regarding the mechanisms of nuclear RNAi, TEI, and RNAi inheritance in *C. elegans*, yet many questions remain. For instance, it seems clear that both chromatin modifications and sRNAs can serve as epigenetic couriers, however the relationships between sRNAs and chromatin, including whether histone modifications might regulate the biogenesis of sRNAs within the nucleus, require further examination. While studies so far place RdRPs in perinuclear germ granules or the cytoplasm, their localization has not been sufficiently examined to make a definitive conclusion. A related question is how chromatin might contribute to the communication between the nucleus and germ granules, which harbor the RNAi inheritance machinery. Heterochromatic modifications help anchor DNA to the nuclear periphery, suggesting that spatial (3D) chromatin architecture regulation could be involved [112]. Indeed, recent evidence indicates that RNAi-induced histone methylation can re-localize the DNA locus to the nuclear periphery [113]. In this way, heritable silencing may be initiated in the nuclear-adjacent germ granules. Developing approaches in which histone modification and sRNA biogenesis can be uncoupled will be important for understanding the relationship between histone modification and sRNA biogenesis. Such approaches may involve tethering HMTs to particular loci, engineering specific point mutants in HMT and sRNA biogenesis machinery via CRISPR genome editing, and causing the temporally-regulated degradation of HMTs and sRNA biogenesis factors.

Another critical question is: how are transcripts selected for targeting by particular sRNA pathways, and how does this lead to TEI? It seems that the molecular characteristics of a gene/transcript, including sequence context (e.g., PATC, repetitive sequences, self/non-self sequences), strength of regulatory elements (promoters, enhancers) and transcript(ion) levels, intron number and composition, splicing regulatory sequences, and transcript localization (e.g. to germ granules vs. the cytoplasm) are some of the many features that contribute to a molecular signature. For transcripts targeted by TEI, a recent study revealed that stretches of non-templated alternating uridine (U) and guanosine (G), termed polyUG or pUG tails, added to a transcript by the ribonucleotidyl-transferase RDE-3/MUT-2 are sufficient to initiate heritable RNAi silencing, which lasted for 3–5 generations [114]. The mechanism for this phenomenon seems to rely on a cycling model of pUG tail addition and sRNA amplification from these specified templates [114]. Notably, RDE-3/MUT-2 localizes to germ granules, again invoking the concept of routing transcripts to the germ granules for sRNA biogenesis.

Finally, with regard to the transgenerational inheritance of behaviors (e.g. pathogen avoidance, chemotaxis), the information transferred between the soma and germline (and vice-versa), and how this information moves or is communicated, remain enigmatic. Is this communication direct, via sRNAs, which could transit as dsRNA, bound to AGOs, in vesicles or virus-like particles [115–118], or via other epigenetic couriers? Or, is it indirect, through gene expression changes that influence the overall physiology of the worm and/or the germline, and in turn influence the development of progeny? Clearly, the implications of intercellular and transgenerational RNA-based communication are far-reaching and this will undoubtedly be a topic of intense future investigation.

Armed with an extensive cadre of genetic, genomic, and cell biological approaches, worms have clearly hit their stride as a premier model for nuclear RNAi and TEI. It is an exciting moment for the field, with the contributions of many more AGOs, HMTs, and protein and nucleic acid modifications remaining to be discovered.

5.1. Key outstanding questions

- What mechanisms determine the targeting of different genomic loci by different AGO/sRNA pathways?
- How is sRNA pathway-based communication and coordination between the nucleus and cytoplasm achieved?
- What are the key couriers of epigenetic information over generations?
- How are different AGOs recruited to the nucleus in different cell types?
- Are there additional AGOs, histone modifications, or nucleic acid modifications that contribute to nuclear RNAi and TEI?
- How does "information" move between soma and germline in learning paradigms that lead to adaptive behaviors?

Conflict of Interest Statement

All authors participated in the drafting of this manuscript and have approved the final version.

All authors confirm that there are no known conflicts of interest associated with this publication. The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

Acknowledgements

We apologize to authors whose work we were unable to cite due to space constraints. This work was supported by Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to J.M.C. (RGPIN-2020-06235) and A.L.S. (RGPIN-2019-06843), and Project grants from the Canadian Institutes of Health Research (CIHR) to J.M.C. (PJT-156083 and PJG-175378) and A.L.S. (PJT-175245). J.M.C. is supported by a Tier II Canada Research Chair in Small RNA Biology. C.L. was supported by an NSERC-CGS-M.

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