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Review

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Small RNAs and chromatin in the multigenerational epigenetic landscape of *Caenorhabditis elegans*

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For decades, it was thought that the only heritable information transmitted from one individual to another was that encoded in the DNA sequence. However, it has become increasingly clear that this is not the case and that the transmission of molecules from within the cytoplasm of the gamete also plays a significant role in heritability. The roundworm, *Caenorhabditis elegans*, has emerged as one of the leading model organisms in which to study the mechanisms of transgenerational epigenetic inheritance (TEI). Collaborative efforts over the past few years have revealed that RNA molecules play a critical role in transmitting transgenerational responses, but precisely how they do so is as yet uncertain. In addition, the role of histone modifications in epigenetic inheritance is increasingly apparent, and RNA and histones interact in a way that we do not yet fully understand. Furthermore, both exogenous and endogenous RNA molecules, as well as other environmental triggers, are able to induce heritable epigenetic changes that affect transcription across the genome. In most cases, these epigenetic changes last only for a handful of generations, but occasionally can be maintained much longer: perhaps indefinitely. In this review, we discuss the current understanding of the role of RNA and histones in TEI, as well as making clear the gaps in our knowledge. We also speculate on the evolutionary implications of epigenetic inheritance, particularly in the context of a short-lived, clonally propagating species.

This article is part of the theme issue 'How does epigenetics influence the course of evolution?'

1. Introduction

Gametes are highly specialized cell types whose primary role is reproduction—the generation of a new individual. They contain all the hereditary material (DNA) required to generate a new organism, but oocytes in particular have long been known to contain much more than just DNA sequence. They also host an abundance of proteins and RNA, and maternal provisioning is well established as being essential for the survival of the embryo. However, in recent years, it has become increasingly apparent that maternal provisioning is not the sole mechanism by which a parent can influence its offspring. Indeed, it has become clear that parental influences can alter gene expression in offspring in a long-lasting fashion, sometimes leading to changes that last for generations. This phenomenon is termed epigenetic inheritance, meaning inheritance 'on top of' genetics. More specifically, the term is typically used to describe the roles of epimutations such as DNA methylation, histone modifications and non-coding RNAs in the inheritance of phenotypic traits.

There are three classes of epigenetic inheritance, largely defined by their longevity in the absence of a trigger. These are parental effects, intergenerational effects and transgenerational epigenetic inheritance (TEI) (table 1). Parental and intergenerational effects describe effects observed in individuals exposed to a trigger and their immediate offspring. This can include things such as parental

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Table 1. Glossary.

Abbreviations	
TEI	transgenerational epigenetic inheritance
RISC	RNA-induced silencing complex
ncRNA	non-coding RNA
siRNA	small interfering RNA
piRNA	PIWI-interacting RNA
tsRNA	tRNA-derived small RNA
Terms	
RNAi	gene silencing triggered by exposure to small RNA molecules complementary to the target gene
RNAe	RNA-induced epigenetic silencing initially triggered by piRNAs that persists indefinitely
germ granules	non-membrane-bound organelles that self-assemble via liquid–liquid phase separations. Types of germ granules include Mutator foci, P granules and Z granules
epigenetic inheritance	non-DNA sequence-based inheritance—used in this review to specifically refer to genome-associated mechanisms such as DNA methylation, histone modifications and non-coding RNAs
intergenerational	heritable effects that result from direct exposure to the trigger and last for only one generation after the trigger
transgenerational	heritable effects that persist beyond the direct effect of a trigger, for at least two generations after exposure for paternal transmission and three generations for maternal transmission
multigenerational	encompasses both intergenerational and transgenerational effects

provisioning, or nutrition *in utero* altering the epigenome of an embryo: but these are generally not long-lasting phenomena. TEI, on the other hand, describes a long-lasting epigenetic change, persisting beyond any direct effect of a trigger. This usually means at least two generations after the trigger for paternal transmission and at least three for maternal transmission in organisms in which embryonic development occurs *in utero*.

Within the past decade, the nematode *Caenorhabditis elegans* has emerged as a leading organism for the study of TEI. This is in a large part because of their fast generation time—3–7 days—and genetic tractability. Robust paradigms exist that have been used for mechanistic studies in order to determine the molecular pathways involved in TEI. These studies have shown that both RNA molecules and histone modifications are essential for TEI and have identified numerous genes required for the production and/or maintenance of epigenetic marks over generations. Other studies have sought to determine how broad-ranging TEI is and what environmental triggers may be able to give rise to heritable epigenetic signals. However, there is still much that remains unclear both at a mechanistic level and an organismal level.

There are many different paradigms that have been shown to cause multigenerational epigenetic inheritance in *C. elegans*, and small RNA molecules are an essential component of all the mechanistic pathways. Here, we discuss some of the major heritable RNA-based gene silencing pathways that play a role in TEI (table 1).

RNA interference (RNAi) is a process that causes gene silencing in response to exogenous double-stranded RNA (dsRNA) [1]. This process generates small interfering RNA (siRNA) molecules that primarily mediate gene silencing through the degradation of target mRNA molecules. In some cases, these siRNAs can also direct gene silencing through interactions with effector complexes that alter the chromatin states and/or inhibit elongation of their target mRNA molecules. While RNAi usually stops when the dsRNA trigger is removed, sometimes gene silencing is maintained for many generations after the removal of this trigger [2–7]. This system has been the most widely used method to study RNA-related TEI as it gives clear insights into the processes governing this inheritance.

RNA-induced epigenetic silencing (RNAe) is another mechanism of RNA-based TEI. RNAe is a completely penetrant, extremely stable form of gene silencing that is triggered by PIWI-interacting RNA (piRNA) molecules produced from the transcription of endogenous loci. This process relies on much of the same machinery as RNAi but has a far greater duration and often appears to be permanent [8,9], suggesting that it is one of the sturdiest forms of epigenetic silencing and is akin to paramutation [10]. Though these mechanisms have been reported as distinct phenomena and seem to have different origins and requirements, their overlapping maintenance machinery suggests the possibility that RNAi and RNAe could be two branches of a single overarching pathway.

Both of these heritable silencing paradigms rely on small RNAs. Currently, we know of four distinct populations of small RNAs involved in heritable silencing: piRNAs; 22G RNAs, separated into the WAGO-class and CSR-1-class (named for their 22 nucleotide length and 5′ guanosine bias [11]); and 26Gs [12,13] (again, named for their length and 5′ nucleotide). 26Gs are expressed in the oogenic germline and embryos and also play a role in spermatogenesis. They have not yet been clearly linked to TEI and so are not discussed in detail in this review (but are discussed in detail in references [12] and [14]).

Small RNA populations can be separated into primary small RNAs—piRNAs, CSR-1-22Gs and 26Gs—and secondary small RNAs—WAGO-22Gs—so named as they require a primary small RNA for their biogenesis. These pathways share many common features and machinery. Small RNAs

function in association with an argonaute protein, though the exact argonaute varies depending on the pathway. In the gene silencing pathways, the argonaute protein acts as the RNA binding component of the RNA-induced silencing complex (RISC). RISC-induced silencing of the target gene occurs systemically throughout most nematode tissues, including the germline where some of these effector molecules may then be passed onto the next generation.

2. The role of WAGO-22Gs in epigenetic inheritance

WAGO-22Gs are the major effector siRNAs of heritable silencing and are defined by their binding to the worm-specific argonaute (WAGO) class of proteins. The biogenesis of these secondary RNA molecules is triggered by the binding of a RISC-bound primary RNA to the target mRNA. The target mRNA then acts as a template for RNA-dependent RNA polymerases (RdRPs) to produce 22G siRNAs, which are antisense to the target mRNA [15]. Most of these molecules are generated in germ granules such as the P granules and Mutator foci, RNA–protein droplets that exist on the outer nuclear membrane where many 22G biogenesis factors are concentrated [16,17] (discussed below).

The biogenesis of WAGO-class 22Gs is the critical amplification step common to most of the RNA-based gene silencing pathways. WAGO-22Gs can be generated from the targets of 26G RNAs, piRNAs and exogenously derived small RNA molecules [17–21]. This commonality causes all these silencing pathways to rely on many of the same factors, but exactly how the specific secondary siRNAs manage to mediate diverse responses is still under debate. WAGO-22Gs bind the WAGO-class argonaute proteins, a group of 12 partially redundant proteins that facilitate the formation of the nuclear RISC [11]. These small RNA molecules act as guides for nuclear RISC allowing it to target transcripts through base complementarity. Nuclear RISC mediates transcript silencing by binding to the nascent mRNA precursors and inducing stalling of RNA Pol II [22,23] (figure 1). This is followed by the recruitment of histone modifiers to the targeted genomic loci, which deposit repressive histone marks such as H3K9 tri-methylation (H3K9me3) [2,8,22,24] and create a heterochromatic environment [25].

One of the core proteins required for multigenerational epigenetic inheritance is a WAGO-class argonaute: 'heritable RNAi-defective' HRDE-1(WAGO-9). This protein, along with non-argonaute components of nuclear RISC (NRDE-1, NRDE-2 and NRDE-4), is essential for heritable RNAi [2,22,23], strongly implicating WAGO-22Gs in multigenerational epigenetic inheritance. Many studies have shown a correlation between silencing by both RNAi and RNAe and an abundance of targeting 22Gs [2,9,10,26], but these 22Gs are also found in strains that cannot pass silencing onto their offspring [27]. WAGO-22G molecules also rely on the target mRNA—and hence the target DNA locus—for their biogenesis, but RNAi silencing can be inherited in the absence of this DNA locus [28,29]. These findings suggest that although WAGO-22Gs are crucial to the heritable silencing process, they may not be the heritable mark directly transferred to offspring over multiple generations. dsRNA molecules themselves can be transmitted to offspring and may contribute to heritability [29,30] or other processed primary RNA molecules could be the heritable mark [30,31].

One such potential heritable molecule is a recently discovered type of RNA called poly-UG (pUG) RNA. These are RNA molecules with a long, non-templated poly-UG tail appended to their 3′ end by RDE-3 (MUT-2) [32], a protein originally discovered in a screen for RNAi-defective mutants [33,34]. RDE-3 is required for the production of WAGO-22Gs initiated by both the RNAi and piRNA pathways [9,11,35]. Injection of pUG RNAs is sufficient to cause multigenerational gene silencing [36]. pUG RNAs localize to Mutator foci and may be primary RNA molecules that are transmitted in low levels between generations that then get amplified in these granules to form 22Gs. pUG RNAs are a new discovery and there is still much that remains unknown about these molecules.

3. The role of piRNAs in epigenetic inheritance

Piwi-interacting RNA molecules (piRNAs) characterized by their 21-nucleotide length and 5′ uridine bias in *C. elegans* [8,37,38] represent a Dicer-independent class of primary small RNAs. This distinguishes them from the other classes of silencing small RNAs that rely on Dicer cleavage of dsRNA for their biogenesis. piRNAs play a key role in transposon repression [20,37,39], in the maintenance of germline health [40–44], and are able to establish RNAe: a permanent, heritable form of transgene silencing that demonstrates the role of piRNAs in protecting the genome from foreign gene expression [8,9].

piRNA precursors are transcribed from genomic loci in the germline. After export and processing, mature piRNAs bind to the Piwi clade argonaute, PRG-1, for loading into the PRG-1-RISC (piRISC) (figure 1). The piRISC has a high mismatch tolerance and is able to bind a wide variety of both protein-coding genes and transposable elements [19,20], and sequencing has found that it is able to bind almost all germline transcripts [35]. As silencing of the whole germline would obviously be detrimental, this clearly shows that there must be regulatory mechanisms in place, guiding the piRISC away from necessary self-transcripts. The CSR-1-22G pathway (discussed below) is a likely contender for this piRNA pathway regulation. The absence of piRNAs causes the mis-localization of downstream piRNA pathway components and causes them to erroneously target many essential genes including replicative histone genes and many CSR-1 targets [40–43], causing progressive fertility loss over generations. The evolutionary utility of the piRNA pathway is extremely clear. Both transposon repression and correct gene expression are crucial for germline health and PRG-1 mutants exhibit germline mortality [44]. This pathway has also been linked to inherited pathogen avoidance behaviours [45,46] (see below), indicating that piRNAs may have a role in immunity.

In *C. elegans*, piRNAs only initiate RNAe target silencing: subsequently most of the silencing actions are carried out and maintained by WAGO-22Gs [8]. RNAe causes gene silencing though interactions with varied nuclear transcriptional machinery and chromatin-regulatory elements. Both target pre-mRNA and mRNA are downregulated during RNAe, indicating that it acts both transcriptionally and post-transcriptionally [9]. H3K9me3, a repressive histone modification, is also enriched on RNAe-targeted genomic regions [9,47].

PRG-1 is required for the initiation of RNAe, and acts within the piRISC to bind the piRNAs required for the biogenesis of secondary 22G RNAs that localize close to the piRNA recognition site on the targeted mRNA [8,19]. The stable maintenance of

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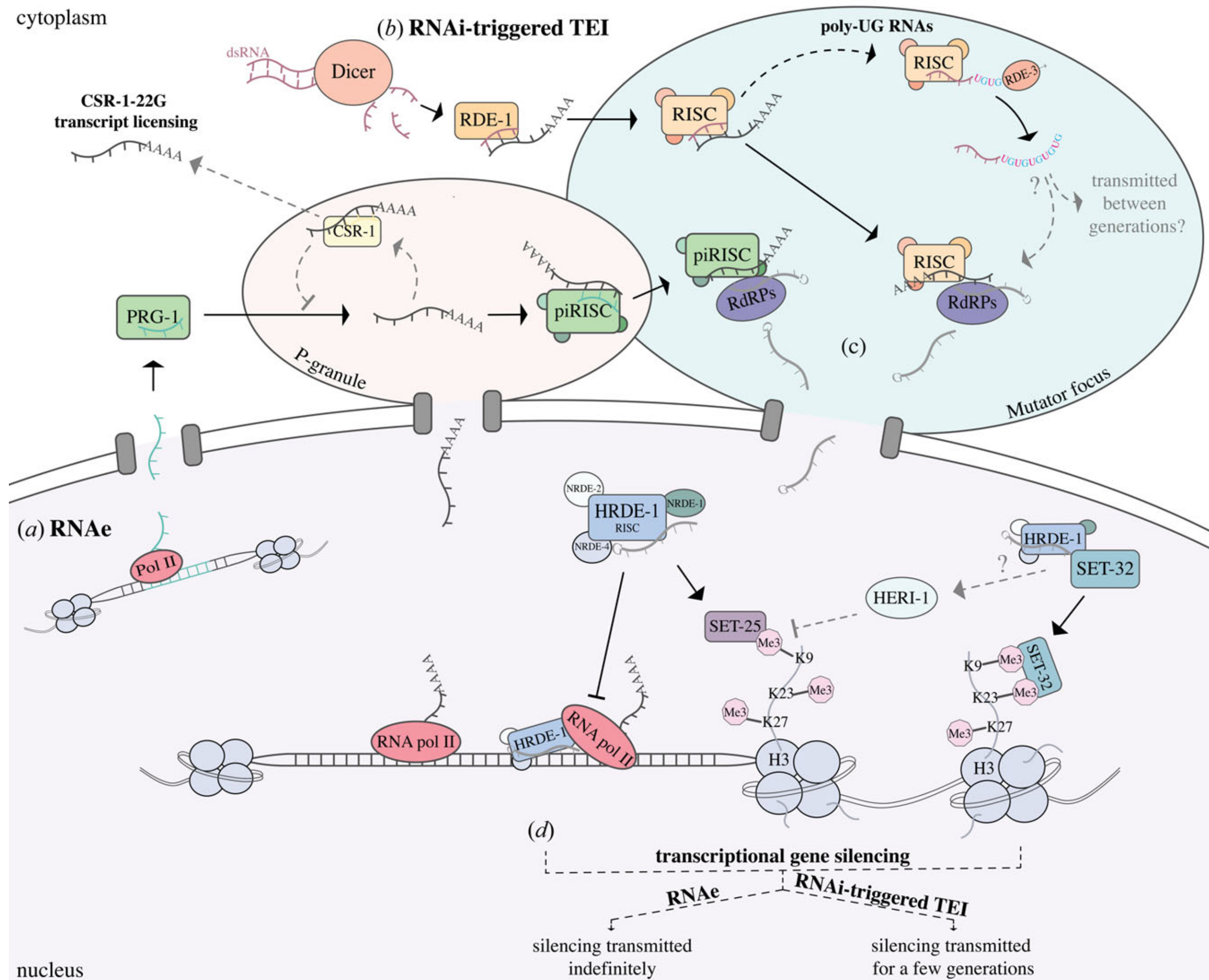

Figure 1. Overview of the RNAi-triggered TEI and RNAe pathways. (*a*) RNAe starts with piRNAs, synthesized from endogenous loci, being loaded onto the Piwi clade argonaute, PRG-1, to allow formation of the PRG-1-RISC (piRISC). The piRISC then competes for transcript binding with the CSR-1-22G regulatory complex in P granules, causing transcripts to be sorted for silencing or licensing. piRISC-bound transcripts are shuttled to the Mutator foci for secondary siRNA amplification, while CSR-1-22G licensed transcripts are released for expression. (*b*) RNAi-triggered TEI begins with Dicer slicing of dsRNA, which is then bound by RDE-1 and the RISC and shuttled to the Mutator foci for secondary siRNA amplification. This pathway also creates poly-UG RNAs, which may be the heritable molecules transmitted in this pathway. (*c*) Both pathways converge on the amplification of WAGO-22Gs, which are able to translocate to the nucleus and form the nuclear RISC with the HRDE-1 argonaute. This complex can then cause gene silencing by stalling RNA pol II and calling histone modifiers (such as SET-25 and SET-32) to deposit histone marks. (*d*) These pathways then diverge once again to transmit these signals to their offspring. RNAi-triggered TEI can be transmitted in the absence of the target locus and so its heritable molecule must be upstream of locus-dependent factors such as histone modifications or secondary siRNA molecules. It not yet clear if this is also the case for RNAe.

RNAe over generations requires 22G amplification in each generation, but interestingly does not require PRG-1 [8,9]. 22Gs carry out this heritable piRNA-induced silencing through the use of many proteins involved in the nuclear RNAi pathway, including NRDE-2 and HRDE-1 [3,26]. These maintenance 22Gs spread further upstream than the initial piRNA on the targeted mRNA and are able to act *in trans* to silence genes that were not directly targeted by the initial piRNA signal [10]. These molecules have been described as 'tertiary 22Gs' as they are thought to require secondary 22Gs as the template for their biogenesis [10]. These tertiary 22Gs may function in a manner analogous to the piRNA ping-pong amplification pathway observed in other animals [48], generating a feed-forward amplification loop to maintain high levels of 22Gs in successive generations independent of the initial trigger [10].

Interestingly, piRNA initiated silencing (RNAe) has many similarities with RNAi-induced heritable silencing. The maintenance of both these pathways relies on many WAGO-22G pathway proteins [8,9], including the core nuclear RNAi pathway protein HRDE-1 [3] as well as other components of the nuclear RISC. Unlike RNAi-initiated TEI, it is not yet clear whether RNAe is able to be inherited in the complete absence of the target locus. RNAe is able to act *in trans* [8–10] and so it is possible that it also relies on locus-independent factors. While both the RNAi and piRNA pathways seem to converge on the amplification of WAGO-22Gs, somehow their downstream factors still diverge into very different mechanisms of TEI, with vastly different penetrance and duration of silencing. How exactly these molecules are differentiated for their participation in the RNAi or piRNA pathways is still unknown. Whether these pathways are truly separate or just branches of an overarching mechanism is also undetermined.

4. Regulatory small RNAs—CSR-1-22Gs

CSR-1 is a WAGO protein that binds to a specific subset of 22G RNAs. CSR-1-22G RNAs are critically different from other

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WAGO-22Gs as they do not require any known primary siRNAs for their biogenesis by the RdRP, EGO-1. This biogenesis may be triggered by CSR-1 catalytic activity, which cleaves inefficiently translating mRNAs in the cytosol [49]. These cleavage products can then become the template for EGO-1-mediated 22G synthesis. CSR-1-22Gs can direct both silencing and licensing of transcripts, unlike WAGO-22Gs, which only play a role in silencing. These molecules are far less characterized than their WAGO counterparts and so their mechanisms of action are still largely unknown, though they have been implicated in a variety of essential roles including controlling germline and histone gene expression and regulating chromatin organization in the early embryo [50–53]. These roles have meant that CSR-1-22Gs are recognized mainly for their silencing actions (outside the scope of this paper, but discussed extensively in references [49–53]) but recently their licensing role has given them a potential link to epigenetic inheritance.

CSR-1-22G RNAs do not directly have a role in TEI but they are involved in the heritable licensing of self-transcripts and are thought to be a safeguard that allows piRNA silencing to target unknown foreign sequences without harming endogenous transcripts [12,13,35,54]. CSR-1 tethering has been shown to protect transcripts from RNAe [55,56]. CSR-1 gene licensing, termed RNA activation (RNAa), has been shown to be a non-permanent process similar to heritable paramutation. When *C. elegans* strains containing licensed (active) transgenes are crossed with a strain containing a silenced transgene the licensing can be passed to the silenced counterparts, protecting the transgenes from silencing for multiple generations [57]. The CSR-1 pathway also promotes euchromatin formation, with its loss causing aberrant repressive histone modifications and decreased transcription at the target loci [58,59].

There is evidence to suggest that CSR-1-22Gs may also play a role in regulating the RNAi pathway. WAGO-4 is a protein required for heritable RNAi that is necessary for the silencing of germline expressed genes and may have a role in transmitting small RNAs from parents to zygotes [60,61]. Sequencing of WAGO-4-bound 22Gs has found that they share many mRNA targets with the CSR-1 pathway and that mutants that show a decrease in WAGO-4-bound 22Gs show a simultaneous increase in CSR-1-bound 22Gs targeting the same transcripts [60]. Because of this, it has been suggested that these two pathways compete for binding of the same cohort of 22Gs to control the expression of germline genes [13]. Interestingly, these proteins comprise two of the only three components currently shown to localize to the newly identified Z-granules [61,62] (see below). This overlap could provide an explanation for the silencing observed against some of the CSR-1-22G-targeted genes; possibly the CSR-1-class 22Gs that cause silencing are bound not by CSR-1, as their name suggests, but instead by WAGO-4.

Several other factors have also been discovered that further complicate the TEI pathways: GLH-1, a conserved RNA helicase; CDE-1, a nucleotidyltransferase; HRDE-2 and HRDE-4, two novel worm-specific factors, have all been identified from heritable RNAi-defective screens [5]. CDE-1, in particular, is of interest as it is thought to destabilize CSR-1-22Gs by catalysing their uridylation [5] and this has been hypothesized to promote the transition of CSR-1-22Gs to WAGO-4 [60], adding yet another layer to this regulatory pathway. The activities of CSR-1-22Gs and their proximity to known TEI effectors suggest that these molecules likely play a much greater role in TEI than is currently understood. Many other molecules are involved in this pathway in ways that have not yet been discovered, but it is obvious that these systems are far more complex than can be currently modelled.

5. RNA localization—perinuclear granules

Eukaryotic cells contain many ribonucleoprotein (RNP) droplets that are RNA–protein assemblies held together by a combination of RNA–RNA, protein–RNA and protein–protein interactions. These RNP droplets are non-membrane-bound organelles that self-assemble via liquid–liquid phase separations [63–65] and rely on RNA molecules to seed their nucleation [66,67]. Some RNP droplets, also termed granules, are specific to germ cells. These include germ granules (termed P granules in *C. elegans*) and Mutator foci, both of which have been implicated in heritable TEI [5,68,69]. These granules localize to the outer nuclear membrane adjacent to nuclear pores. Evidence suggests that these granules play a role in the post-translational processing of mRNAs as they pass through the nuclear pores into the cytoplasm. P granules are crucial for proper germline function as functional P granules prevent the improper expression of sperm and somatic genes within germline tissues [53,70,71].

P granules are intimately involved in the production of heritable RNAs. A single generation of dysfunctional P granules leads to the creation of aberrant siRNA molecules that can be inherited and silence genes incorrectly for multiple generations after P granule formation is restored [69,72]. How does this happen? Newly synthesized mRNAs congregate in P granules after export from the nuclei [73], suggesting that sorting of mRNAs for silencing and/or licensing by the different small RNA pathways occurs in P granules. Many small RNA pathway proteins localize to P granules, including Dicer, PRG-1 and CSR-1 [38,50,74,75]. The functions of these proteins are heavily intertwined with correct P granule formation: these proteins depend on P granule compartmentation to carry out their functions and P granules depend on these proteins for their proper structure and localization [62,74,75].

The fact that these granules contain the key proteins required for both the CSR-1 and piRNA pathways further suggests that these pathways compete for nascent transcript binding. P granules localize to nuclear pores, allowing these competing pathways direct access to newly synthesized mRNAs and P granule localization protects transcripts from piRNA-induced silencing [50,68], likely owing to the proximity of protective CSR-1-bound 22Gs. However, the mechanism appears to involve more than just simple competition. Studies have found compelling evidence that piRNAs somehow influence the loading of 22G RNAs onto the appropriate argonaute protein (CSR-1 or other WAGOs), as a lack of piRNAs during *de novo* 22G establishment results in sterile animals with CSR-1-22Gs improperly loaded onto other WAGO proteins [41,42].

Though it is still not fully clear how nascent transcripts are flagged for silencing or activation in the P granules, the emerging model in the field suggests that Mutator foci interact with these P granules at the nuclear periphery to coordinate small RNA silencing of specific nascent transcripts [13,76,77]. These condensates are a key site of secondary siRNA amplification within the cell and get their name from the Mutator complex, which localizes to these foci [13,16,17,76]. This complex is

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comprised of many RdRPs that use mRNA transcripts as templates for the biogenesis of secondary 22G RNAs (WAGO-22Gs). WAGO-22Gs can then initiate RISC silencing of their respective genes. As WAGO-22Gs are the downstream effectors of all the primary silencing RNA pathways, including those required for TEI, the loss of the Mutator foci obviously disrupts these pathways [42,44,76].

Recently, other condensates such as Z granules have been identified. These droplets are not yet well characterized but they have been observed to associate with P granules and Mutator foci [78]. Proteins with established involvement in TEI, including ZNFX-1 and WAGO-4, have been shown to localize to P granules early in development, but split off and form Z granules in later developmental stages [61,74].

The different granules congregate to form multi-condensate structures within *C. elegans* germ cells [16,69], creating a complex liquid droplet network on the nuclear periphery. The structural organization of these granule assemblies facilitates complex interactions between their components. It has been suggested that these granules may act as organizational hubs that connect regulatory siRNAs to mRNAs, allowing them to form the complexes necessary for gene regulation. It is clear that the full spectrum of perinuclear granules and their roles is not completely characterized. New granules are still being identified, and it is likely that this droplet network also varies throughout the organism's development. But altogether this suggests a mechanism by which newly synthesized mRNAs are passed through a system of biomolecular condensates, allowing these mRNAs to be sorted, silenced and processed before their export into the cytoplasm.

6. Histones and chromatin

A key silencing action of RISC is to recruit histone modifiers to the targeted genomic loci. These modifiers are known to deposit repressive marks, creating regions of heterochromatin around the targeted gene and so contributing to silencing. These histone modifications, established in the parental germline, appear to initiate a multigenerational repressive chromatin footprint that persists for a few generations after the initial trigger has disappeared [4,60,61,79–81]. There are two well-known histone modifications induced by nuclear RNAi: these are the tri-methylation of histone 3 lysine 9 (H3K9me3) and histone 3 lysine 27 (H3K27me3). Although their true function within the nuclear RNAi pathway is still unclear, their deposition onto the targeted genomic loci is induced by the HRDE-1-bound sub-class of WAGO-22G RNAs [2].

Of these modifications, H3K9me3 has been more widely studied. H3K9me3 relies on nuclear RNAi machinery, and HRDE-1, NRDE-2 and NRDE-4 mutants all display a progressive loss of H3K9me3 over generations [2,82]. Most H3K9me3 in *C. elegans* is driven by the histone methyltransferases SET-25 and MET-2 [83]. Although SET-25 is able to generate low levels of all three methylation states alone [84], in embryos these proteins optimally function sequentially to bring about H3K9me3; MET-2 mediates the mono- and di-methylation of the lysine, while SET-25 deposits tri-methylation [27,83–86] (figure 1). In adult germ cells, this may not be the case, as H3K9me3 is still observed at wild-type levels in the absence of MET-2 [47]. SET-32 has also been implicated in H3K9me3 regulation [5,83], although the evidence for its exact role is not clear [27] and a recent report suggests that SET-32 may actually be responsible for the deposition of another newly described histone mark, H3K23me3 [87] (discussed further below). Interestingly these proteins seem to have vastly different roles to play in TEI. MET-2 mutants display either no significant difference [27] or enhanced transgenerational silencing [88], while SET-32 and SET-25 mutants have greatly impaired transgenerational silencing [3,5,27,85,88]. SET-32, and sometimes SET-25, is crucial for the establishment of heritable RNAi, but dispensable for its long-term maintenance [27,83,85]. Interestingly, there also seem to be different pathways for the silencing of endogenous genes and foreign transgenes. Both SET-32 and SET-25 are required for the silencing of a green fluorescent protein (GFP) transgene, but SET-25 is dispensable for the silencing of the endogenous genes, *oma-1* [85,89] and *sid-1* [90], though the mechanisms behind this difference are yet to be discovered. This calls into question the exact role of H3K9me3 in TEI: is it simply required to signal the start of heritable RNAi and then dispensable for maintenance over generations? Is it an artefact of other roles of these crucial proteins? Or are other, as-yet unidentified, methyltransferases required for maintenance of the heritable signal?

The other well-known histone modification, H3K27me3, is deposited by MES-2, a subunit of polycomb-like chromatin repressive complex (PRC2) in *C. elegans* [91]. This deposition can be triggered by both the piRNA and RNAi pathways and requires nuclear RNAi machinery such as HRDE-1, NRDE-2 and NRDE-3 [83,92]. MES-2 mutants are defective for RNAi and RNAi-induced H3K27me3 is inherited for multiple generations after removal of the dsRNA trigger [92], and so it has been suggested that this mark may play a role in TEI but this had not been confirmed. H3K27me3 and H3K9me3 colocalize on endogenous RNAi targets [83] but their exact role in TEI is unclear.

An additional nuclear RNAi-triggered chromatin modification, H3K23me3, has recently been reported. This modification also lasts for multiple generations after the RNAi trigger and requires both HRDE-1 and SET-32 for its establishment [87] (figure 1). This mark is enriched in heterochromatic regions in *C. elegans*, specifically around endogenous RNAi targets. This modification displays some linkage to H3K9me3, as these marks display very similar signals at endogenous RNAi targets [83,87]. This may just be from a common requirement of SET-32 but it may also indicate the cooperation or redundancy of these marks [87].

Another histone mark, H3K4 methylation, plays crucial roles in the regulation of lifespan and germline mortality, though it is not directly connected to RNAi or RNAe. The loss of the H3K4me1/me2 demethylase SPR-5 causes both an extended lifespan and a mortal germline phenotype [93,94]. This system also has an interaction with H3K9 methylation, as SPR-5 mutants display an accumulation of H3K4me2 and a decline in H3K9me3 over the generations of progressive sterility. The loss of H3K9 methyltransferase MET-2 and putative methyltransferase SET-26 also accelerate the transgenerational sterility of SPR-5 mutants, while the loss of the H3K9me3 demethylase JMJD-2 suppresses this sterility [93]. This illustrates that histone marks have wide-reaching consequences for the organism, beyond that linked to RNAi- or RNAe-triggered silencing.

Many known TEI-related proteins also play crucial roles in heterochromatin formation and maintenance. Components of the nuclear RNAi machinery including HRDE-1, NRDE-2, NRDE-3 and MORC-1 have been directly linked to small

RNA-induced chromatin compaction [25]. This compaction is thought to be driven by the HPL-1-like protein, HPL-2 [25]. HPL-2 is crucial for RNAe silencing [3] but has not yet been investigated in RNAi-induced TEI. In yeast, HPL-1-like proteins are known to be recruited by H3K9me3 [95]. But in *C. elegans*, HPL-2 *in vivo* binding correlates more closely with H3K9me1/2 than H3K9me3 when mapped by CHIP-seq, and HPL-2 chromatin binding is still observed in animals that lack H3K9me3 [96,97]. It is still unknown whether chromatin compaction is actually required for TEI in either RNAe- or RNAi-triggered pathways, although it seems plausible that chromatin compaction may only be necessary for RNAe, since this pathway requires the silenced locus for the transmission of heritable silencing. Further research is required to investigate this hypothesis.

The heritable RNAi system seems to have its own built-in regulations that limit the duration of the inherited silencing. Heritable enhancer of RNAi (HERI-1) is a protein that associates with chromatin around RNAi-targeted loci. The loss of HERI-1 causes an enhanced transgenerational silencing phenotype that allows RNAi-induced silencing to persist for over 20 generations. This extended silencing is accompanied by an extension in both the H3K9me3 footprint and targeted siRNAs [4]. Interestingly, the recruitment of HERI-1 to RNAi-targeted genes requires both HRDE-1 and SET-32 [4] (figure 1), somehow implicating these pro-silencing factors in this negative regulatory pathway. Could the recently discovered H3K23me3 modification be the additional mark necessary to recruit regulatory proteins such as HERI-1 to the targeted loci?

The exact role of histone modifications in RNAi-triggered TEI remains unclear. They have been shown to mediate the penetrance and duration of transgenerational silencing, but cannot be the main effector of TEI transmission as nuclear RNAi-induced gene silencing can occur in the absence of the target DNA locus [29]—and therefore in the absence of histone modifications attached to this locus. So, despite the requirement of histone methyltransferases in establishing TEI and the presence of a multigenerational chromatin footprint, histone modifications cannot be the epigenetic signal inherited between generations. This suggests a locus-independent mechanism that likely relies on the transmission of RNA molecules where these histone marks may act primarily to maintain and amplify the signal throughout the organism's lifetime. RNAe silencing on the other hand could require the target locus and so these histone modifications and/or chromatin compaction may play a greater role in heritability mediated by this pathway.

7. Environmental triggers of transgenerational epigenetic inheritance

True TEI has been clearly demonstrated in numerous animal models, from *C. elegans* to fish to mice [3,9,90,98,99], but most of this evidence is from artificial, laboratory-based conditions. How does this translate into the natural environmental context? And how do these epimutations relate to environmental fitness and evolution?

Recent studies have found that the progeny of animals exposed to a variety of environmental stressors including pathogen exposure, heat and starvation can display long-lasting adaptations to their environment. These effects can sometimes be passed on to future generations before waning. These phenotypes are not permanent, suggesting that the changes cannot be genetic, implicating an environmentally triggered epigenetic mechanism.

(a) Environmental stressors

Environmental temperature is a major factor that can induce transgenerational changes in gene expression, though this response is not necessarily adaptive. One generation of growth at an elevated temperature triggers a decrease in piRNA biogenesis and causes decreased fitness in offspring for 1–3 generations post-exposure [100]. Heat exposure also causes the multigenerational derepression of 'junk DNA'. Both multicopy transgene arrays and a subset of endogenous repetitive elements were erroneously expressed for up to 7 generations after a single generation of growth at an elevated temperature [101]. This derepression response correlated with the level of H3K9me3 on the affected loci and may be controlled by SET-25. Heat exposure also leads to multigenerational changes in siRNA levels and temperature-sensitive siRNAs are enriched for genes that are targeted by the piRNA and WAGO-22G pathways [102]. No adaptive physiological response has yet been linked to either these siRNA or histone modification changes.

Many other environmental stressors have been shown to cause an intergenerational benefit to offspring. Parental exposure to osmotic stress or heavy metals both provide increased resistance to these same stressors in future generations [103]. Parental dietary restriction causes a reduced brood size with larger, increasingly starvation-resistant offspring [104], though this effect lasts for only one generation. Ancestral food availability has also been suggested to trigger transgenerational responses [104–106], but no clear mechanisms have been presented to link this observed resistance to any known epigenetic pathways. Lack of food in the early life of *C. elegans* can result in two distinct responses: L1 arrest or dauer diapause. When *C. elegans* hatch in the absence of food they arrest in the L1 life stage, whereas when they hatch in the presence of limited food and a high population density worms become dauers [107]. Extended starvation in both stages has been suggested to trigger transgenerational starvation resistance. Worms can remain as dauers in starvation culture for up to 45 days with a 90–100% survival rate. Great-grand progeny of these extended dauers then exhibit resistance to starvation, with mildly increased growth rates, brood size and lifespan upon recovery compared to non-postdauer progeny [105]. The utility of a transgenerational response to starvation is clear: extended starvation is likely to be common in a species that relies heavily on the acute proximity of food. But this response has a trade-off as primed parents have offspring that are more prone to larval arrest and more sensitive to osmotic stress [108,109], so having this response hard-wired into the genetic code is not likely to be advantageous.

Extended parental starvation in the L1 larval stage has also been suggested to increase offspring starvation resistance [106]. This starvation caused 20% lethality in exposed animals and sterility and developmental defects in others. The majority of surviving animals had a reduced brood size with lowered embryo quality. Interestingly, only the grand-progeny of the worms that displayed developmental defects directly after starvation showed an increase in starvation resistance or resistance to heat stress [106]. It is hard to say whether starvation triggers this epigenetic response or instead selects for pre-existing epigenetic variation within the population. *C. elegans* have a wide range of spontaneous

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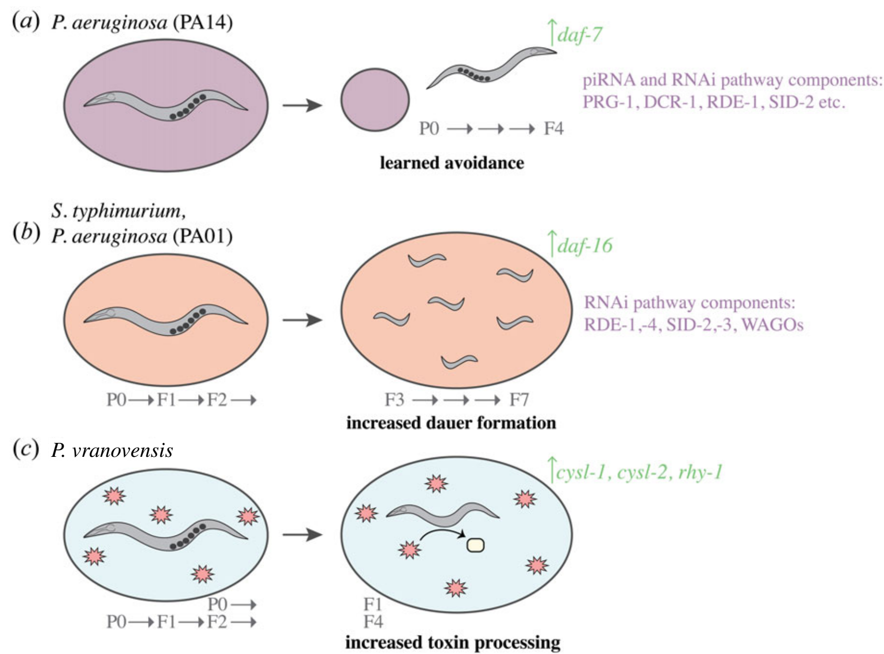

Figure 2. *C. elegans* multigenerational immune priming responses are highly specific to each bacterial pathogen. (*a*) The *P. aeruginosa* PA14 strain is able to induce transgenerational pathogen avoidance behaviour. This occurs directly in the exposed generation and persists for four generations after exposure [45,46]. (*b*) *S. typhimurium* and the *P. aeruginosa* PA01 strain trigger increased dauer formation in progeny. This persists for five generations after removal from the pathogen [111]. (*c*) Larval pathogen resistance after brief parental exposure is observed against *P. vranovensis*. Increased larval survival is observed in the direct offspring of exposed parents after a single parental exposure and in the grand-offspring after three generations of pathogen priming [108]. Purple signifies proteins required for the multigenerational response, green signifies upregulated genes. CYSL-1 and CYSL-2, cysteine synthases; RHY-1, hypoxia inducible factor.

epimutations that occur 25 times more frequently than DNA sequence changes and last anywhere from 2 to 10 generations [110]. It seems plausible that L1 starvation, with its high lethality rate and the level of selection required to see a transgenerational response, may in fact be selecting for one of these transient epimutations.

(b) Pathogen exposure

Multigenerational immune priming is a type of phenotypic plasticity by which parental exposure to a pathogen alters their offspring's immune defences. This process encompasses what we would define as both transgenerational and intergenerational effects and has been observed across a wide range of taxa.

In *C. elegans*, a transgenerational avoidance response lasting for up to four generations [45] has been observed following exposure to the pathogen *Pseudomonas aeruginosa* (PA14) (figure 2*a*). This exposure induces neuronal gene expression changes, including upregulation of the TGF-β receptor *daf-7* in some chemosensory neurons of progeny, which subsequently avoid consuming PA14. The transmission of this behaviour, and the upregulation of *daf-7*, require the piRNA argonaute PRG-1 and its downstream molecular components, providing a link between this well-studied TEI pathway and a natural environmental trigger. This avoidance response also seems to require HRDE-1 and SET-32, as mutant animals were defective in aversive pathogenic learning even in the P0 generation [45]. This brief exposure has quite a low fatality rate in the P0 generation, indicating that this is a newly established response, and not environmental selection for worms predisposed to avoidance of the pathogen. In fact, a recent study showed that a single PA14 non-coding RNA (ncRNA) is responsible for the transgenerational avoidance behaviour in a process that requires both functional P granules and components of the piRNA and RNAi pathways [46]. Interestingly, this PA14 ncRNA has homology to a native *C. elegans* gene, *maco-1*, and RNAi against this gene is sufficient to induce this transgenerational learning behaviour [46]. Could this indicate an immune pathway by which *C. elegans* incorporates the bacterial genome? Or do the bacteria incorporate *C. elegans* genes to disguise themselves from the host? Or is this homology just purely coincidental? Either way, it is clear to see how advantageous it would be to have offspring that naturally avoid a virulent pathogen in a highly targeted manner, given that *Pseudomonas* species are a major *C. elegans* food source [45]. This epigenetic response is able to occur far faster than DNA sequence changes and so it is beneficial to use this fast, plastic response even if avoidance occurs for only four generations before returning to the naïve state.

Another interesting pathogen exposure-related response in *C. elegans* is the increase in dauer formation after exposure to *P. aeruginosa* (PA01) or *Salmonella typhimurium* [111] (figure 2*b*). In these experiments, worms were exposed to the pathogenic bacteria for three generations, then placed on a non-pathogenic food source for up to five generations before being exposed to the pathogenic bacteria again. These exposed animals displayed a significant increase in dauer formation, with up to 10% of their offspring forming dauer larvae. Dauer formation is an extremely effective avoidance strategy against bacterial pathogens, as *C. elegans* are primarily colonized through their consumption of bacteria and the blocked oral cavity of dauers makes them unable to consume any bacteria [111]. Dauer arrest has the effect of delaying reproduction, which may allow these worms to avoid the worst effects of the pathogen. This response makes sense from an evolutionary perspective, especially in a clonal species, as keeping a small amount of the population free from pathogenic bacterial

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contamination may allow the population to survive a particularly virulent strain without the huge trade-off of delayed reproduction for all members of the generation. Many components of the RNAi pathway, including RDE-1, RDE-4, SID-2, SID-3 and the WAGO proteins [111], are required for this pathogen-induced diapause formation, showing another possible link between these well-studied pathways and an adaptive environmental response.

Intergenerational pathogen responses have also been demonstrated in *C. elegans*. *Pseudomonas vranovensis* is a natural pathogen of *C. elegans* that causes 95% lethality in newly hatched larvae. Brief parental exposure to this pathogen promotes significant resistance to this infection, with a 10–50-fold increase in the survival of exposed progeny larvae [108] (figure 2*c*). Three consecutive generations of this pathogen priming can trigger resistance for the two subsequent generations. This heritable resistance correlates with upregulation of the genes for the cysteine synthases, CYSL-1 and CYSL-2 and the hypoxia inducible factor RHY-1 previously reported to break down hydrogen cyanide and promote resistance to hydrogen sulfide [112,113]. It has been suggested that this may be a mechanism that increases the ability of progeny to break down the lethal toxins produced by *P. vranovensis* [108].

Interestingly, these particular responses are highly specific to each bacterial strain. Only *P. vranovensis* was able to induce larval pathogen resistance [108]. Learned avoidance behaviour is observed against *Serratia marcescens* and *P. aeruginosa* (PA14), but not against any non-virulent forms of *P. aeruginosa* [45,114,115] and is only inherited against *P. aeruginosa* (PA14) [45]. *S. typhimurium* and *P. aeruginosa* (PA01) trigger the increased dauer formation but PA14 cannot [111]. The genetic requirements for this response also seem to differ between bacterial strains as NRDE-3, a somatic argonaute in nuclear RNAi, is required for F2 dauer formation against *P. aeruginosa* (PA01), but not against *S. typhimurium* [111].

The antiviral response of *C. elegans* has been less studied. It is commonly thought that RNAi may have evolved as an antiviral mechanism, though this cannot be proven. Certainly, the RNAi pathway is required for an effective antiviral response [116–119]. RNAi-based TEI would be a perfect antiviral pathway, as 'vaccinating' offspring against a virus present in the parental lifetime would be incredibly beneficial to offspring survival. *C. elegans* has as yet only one known native virus, the Orsay virus [120], and there is conflicting data on whether a heritable protective response can be seen in the offspring of infected animals [121,122]. On the other hand, parental antiviral small RNAs generated by non-native viral infections have been suggested to transmit a protective antiviral response to their progeny [123,124]. More naturally occurring *C. elegans* viruses will need to be discovered to conclusively determine whether TEI can commonly protect against native viral infection in *C. elegans*.

8. Conclusion

Small RNA molecules play a large role in the transmission of epigenetic information and environmental phenomena can trigger these transgenerational responses. Although this review has focused on *C. elegans*, these systems translate into a wide range of taxa. Many additional factors not specifically mentioned in this review—such as sperm tsRNAs—have also been implicated in the epigenetic landscape. These molecules have been shown to be responsive to diet and transmitted into the F2 generation in mice [125–127] and likely play a role in TEI in mammalian systems, though they have not been well-studied in *C. elegans*. Multigenerational immune priming has been observed in a range of invertebrates [128] eliciting a variety of effects in offspring. These include priming offspring for correctly timed immune gene expression, constitutively elevating baseline immune effectors or even creating a metabolic shift in offspring [129–131]. In vertebrates, intergenerational immune priming has clearly been shown in fish, mice and humans through the direct transmission or epigenetic maintenance of active immune components [128,132]. Studies in fish have also shown changes in immune gene expression in the offspring and grand-offspring of exposed individuals [133], indicating the possibility of a transgenerational pathway for immune priming in vertebrates. Parental diets and trauma have also been linked to heritable responses in mammals [98,126,127], indicating the possibility of a wider role for transgenerational responses in preparing offspring for harsh environmental conditions.

These epigenetic responses likely function as a gentler, less permanent mechanism of variation that serves to allow populations to survive temporarily harsh conditions, without permanently altering their genetic code in ways that may otherwise harm their viability. *C. elegans* exist in the wild as clonal, isolated populations so the ability to adapt quickly to harsh conditions is crucial. But these adaptive epigenetic responses all have trade-offs, from the avoidance of potentially viable food sources [45,46] to increasingly stress-sensitive offspring [108,109]. These are acceptable in the short-term but are detrimental for long-term population survival, indicating the utility of these plastic, transitory responses.

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