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PRIMER

Small-RNA-guided histone modifications and somatic genome elimination in ciliates

Thomas Balan¹ | Leticia Koch Lerner¹ | Daniel Holoch^{1,2} | Sandra Duharcourt¹ ¹Université Paris Cité, CNRS, Institut Jacques Monod, Paris, France²Institut Curie, INSERM U934/CNRS UMR 3215, Paris Sciences et Lettres Research University, Sorbonne University, Paris, France**Correspondence**

Sandra Duharcourt, Université Paris Cité, CNRS, Institut Jacques Monod, F-75013 Paris, France.

Email: sandra.duharcourt@ijm.fr**Funding information**

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Edited by: Astrid Haase, Associate Editor and Jeff Wilusz, Editor-in-Chief**Abstract**

Transposable elements and other repeats are repressed by small-RNA-guided histone modifications in fungi, plants and animals. The specificity of silencing is achieved through base-pairing of small RNAs corresponding to these genomic loci to nascent noncoding RNAs, which allows the recruitment of histone methyltransferases that methylate histone H3 on lysine 9. Self-reinforcing feedback loops enhance small RNA production and ensure robust and heritable repression. In the unicellular ciliate *Paramecium tetraurelia*, small-RNA-guided histone modifications lead to the elimination of transposable elements and their remnants, a definitive form of repression. In this organism, germline and somatic functions are separated within two types of nuclei with different genomes. At each sexual cycle, development of the somatic genome is accompanied by the reproducible removal of approximately a third of the germline genome. Instead of recruiting a H3K9 methyltransferase, small RNAs corresponding to eliminated sequences tether Polycomb Repressive Complex 2, which in ciliates has the unique property of catalyzing both lysine 9 and lysine 27 trimethylation of histone H3. These histone modifications that are crucial for the elimination of transposable elements are thought to guide the endonuclease complex, which triggers double-strand breaks at these specific genomic loci. The comparison between ciliates and other eukaryotes underscores the importance of investigating small-RNAs-directed chromatin silencing in a diverse range of organisms.

This article is categorized under:

Regulatory RNAs/RNAi/Riboswitches > RNAi: Mechanisms of Action

KEYWORDSDNA elimination, histone modifications, *Paramecium*, small RNA, transposable elements

Thomas Balan, Leticia Koch Lerner, and Daniel Holoch are equally contributed in this study.

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1 | INTRODUCTION

Eukaryotic organisms must contend with mobile genetic elements and repetitive sequences which, left unchecked, can undermine the stability of their genomes. An ancient solution for dealing with this challenge is powered by small RNAs, which provide the specificity for recruiting specialized machineries to these loci in order to ensure their efficient repression (Malone & Hannon, 2009). Small RNAs are loaded onto Piwi/Argonaute-family proteins (Bobadilla Ugarte et al., 2023), which they then guide to target RNAs in the cytoplasm or in the nucleus through sequence complementarity. In a diverse range of species and biological contexts, the binding of these small-RNA complexes to nascent transcripts triggers the local recruitment of histone methyltransferases to the corresponding genomic sequences, resulting in chromatin-level silencing (Castel & Martienssen, 2013; Holoch & Moazed, 2015).

Studies of nuclear small-RNA pathways in an array of model organisms have revealed a variety of distinctive features as well as recurring themes. One of the most thoroughly explored examples is the fission yeast *Schizosaccharomyces pombe*, whose pericentromeric DNA repeats are assembled into constitutive heterochromatin through the action of a small-RNA-guided Argonaute complex, RITS (Verdel et al., 2004; Volpe et al., 2002). Pericentromeric heterochromatin is vital for genome stability because of its essential contribution to correct chromosome segregation during cell division (Bernard et al., 2001; Nonaka et al., 2002). Localization of RITS to pericentromeric regions is mediated by base-pairing with nascent noncoding transcripts (Shimada et al., 2016), and promotes heterochromatin formation by recruiting Clr4, a conserved histone methyltransferase that methylates histone H3 on lysine 9, through a direct physical interaction (K. Zhang et al., 2008). RITS also recruits the small RNA biogenesis machinery to its binding sites, thus amplifying the corresponding small RNA populations and enhancing RITS targeting (Motamedi et al., 2004). Finally, RITS also binds with high affinity to Histone H3 dimethylated on K9 (H3K9me2) (Partridge et al., 2002), which, in conjunction with small-RNA-dependent recruitment, further stabilizes its association with target regions. Altogether, these positive feedbacks between small-RNA-mediated targeting, small-RNA production and histone methylation vigorously reinforce the silencing signal and ensure robust maintenance of heterochromatin and chromosome stability (Figure 1a).

The self-reinforcing nature of pericentromeric repeat silencing in *S. pombe* finds echoes in many distantly related small-RNA-mediated phenomena. Among the best studied of these is RNA-directed DNA methylation (RdDM) in flowering plants. Silencing of transposable elements (TEs), as in mammals, is achieved through cytosine-5 DNA methylation in plants, but its proper genomic targeting requires the action of two plant-specific RNA polymerases. RNA polymerase IV (Pol IV) generates individual precursors which are then converted to mature small RNAs by homologs of classical small-RNA pathway members in a highly coordinated manner (Chan et al., 2004; Herr et al., 2005; Onodera et al., 2005; Zhai et al., 2015). Meanwhile, RNA polymerase V (Pol V) produces nascent noncoding transcripts that serve as the targets for small-RNA-guided Argonaute proteins and fairly precisely define the regions to be methylated (Wierzbicki et al., 2008, 2009). The synergy between these polymerases is highlighted by artificial recruitment assays in which tethering both Pol IV and Pol V to a reporter locus enables dramatically stronger silencing than either one alone (Gallego-Bartolomé et al., 2019). The DNA methylation step itself is carried out by DRM2, recruited through an interaction with the Argonaute protein AGO4 (X. Cao et al., 2003; Zhong et al., 2014). Thus, as in small-RNA-directed heterochromatin assembly in *S. pombe*, RdDM works according to a nascent transcript model and relies on a physical connection between a small-RNA complex and the writer of the transcriptional silencing mark. RdDM is also replete with additional physical interactions that combine to form a strong positive feedback loop. For instance, the H3K9 methyltransferase KYP binds directly to 5-methylated cytosines (5mC), via its SRA domain (Johnson et al., 2007), while at the same time further promoting local DNA methylation through recruitment of Pol IV, whose SHH domain recognizes H3K9me2 (Law et al., 2013). KYP thus facilitates self-propagation of the 5mC silencing mark. Meanwhile, the Pol V binding partners SUVH2 and SUVH9 also contain SRA domains, which help tether Pol V to RdDM target sites and reinforce existing DNA methylation (Johnson et al., 2014). Pol V also possesses a conserved Argonaute-binding domain (El-Shami et al., 2007), further strengthening the small-RNA-mediated targeting of AGO4 to the nascent transcripts it produces. Altogether, these various interactions work in concert to perpetuate the repression of target TEs (Figure 1b).

In animal gonads, TEs are also silenced by small RNAs through the action of Piwi proteins programmed with a class of small RNAs called piRNAs (Ozata et al., 2019; X. Wang, Ramat, et al., 2023). Part of this silencing occurs post-transcriptionally, through a process known as the “ping-pong cycle” that couples the destruction of TE transcripts to the biogenesis of new piRNAs (Aravin et al., 2007; Brennecke et al., 2007; Gunawardane et al., 2007). Nevertheless, piRNAs also act in the nucleus to repress TEs at the transcriptional level. Indeed, Piwi proteins mediate cytosine-5 DNA methylation of TEs in the mouse testis (Aravin et al., 2007, 2008; Carmell et al., 2007) as well as transcriptional

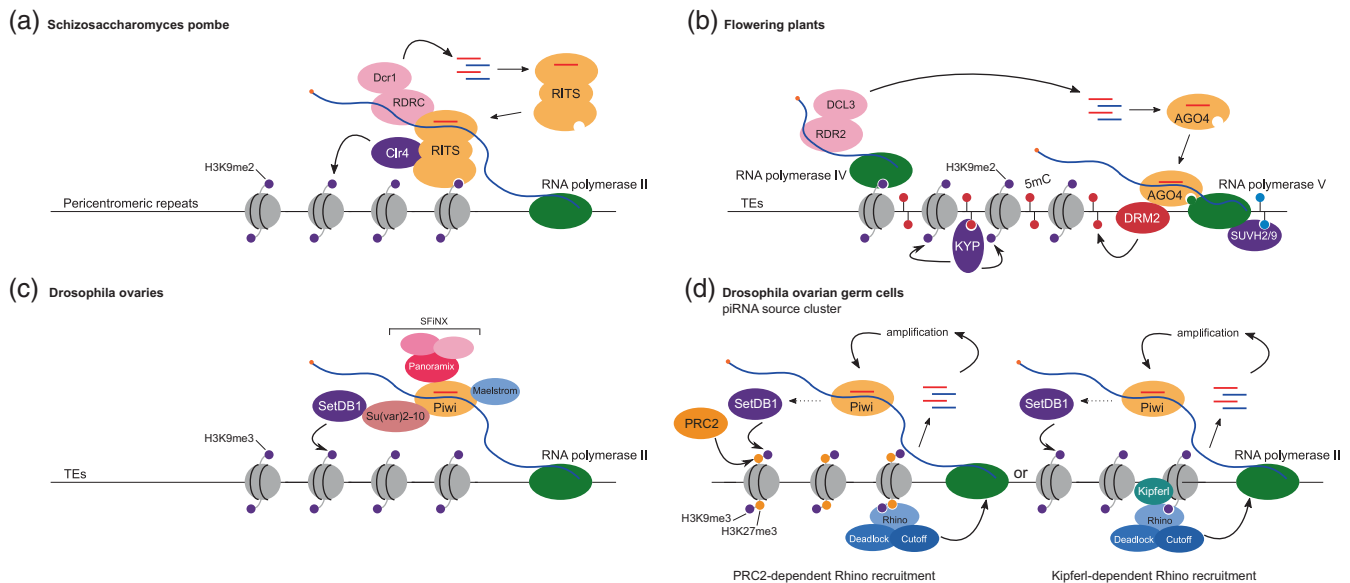


FIGURE 1 Conserved role of small RNAs in targeting chromatin silencing by pairing with nascent transcripts. (a) In *Schizosaccharomyces pombe*, assembly of pericentromeric repeats into heterochromatin marked with the conserved repressive modification H3K9me requires the action of the RITS complex loaded with small RNAs complementary to noncoding nascent transcripts. RITS binds with high affinity to H3K9me2, and physically interacts with the H3K9 methyltransferase Clr4 and the small RNA biogenesis machinery (RDR2 and Dcr1, not discussed in the text). Together, these physical interactions form the basis of a powerful positive feedback. (b) Transposable elements (TEs) in flowering plants are repressed through RNA-directed DNA methylation. The RNA molecules involved in this process are produced by the plant-specific polymerases Pol IV and Pol V, which respectively generate precursor transcripts converted to small RNAs by RDR2 and DCL3 (not discussed in the text) and nascent transcripts to be targeted by these small RNAs once they are loaded into AGO4. DNA methylation by DRM2 ensues, assisted by a direct interaction with AGO4. The process is self-reinforcing, as Pol V associates with partners SUVH2/9 that bind to methylated DNA and contains an Argonaute-interacting domain that binds tightly to AGO4. Furthermore, the H3K9 methyltransferase KYP also binds directly to sites of DNA methylation, where its H3K9me2 deposition activity in turn helps recruit Pol IV. (c) At TEs in *Drosophila* ovaries, the Piwi protein loaded with piRNAs is also targeted through base-pairing with nascent transcripts, and mediates repression in two ways: recruitment of the SetDB1 H3K9 methyltransferase through the E3 SUMO ligase Su(var)2-10, as well as the silencing factors Maelstrom and Panoramix, the latter within the SFInX complex (not discussed in the text). (d) In ovarian germ cells, H3K9me3 at dual-strand piRNA clusters is also critical for further piRNA biogenesis because it recruits the Rhino-Deadlock-Cutoff complex, which in turn promotes transcription of piRNA precursors. Rhino binding relies on the zinc-finger protein Kipferl at most sites (right); meanwhile, binding at Kipferl-independent sites requires dual recognition of H3K9me3 and H3K27me3, the latter catalyzed by PRC2 (left). Although the piRNAs thus produced are destined for ping-pong amplification in the cytoplasm mediated by paralogs of Piwi, Piwi itself is required for their accumulation, suggesting a positive feedback loop wherein the piRNAs guide Piwi to piRNA clusters to maintain H3K9me3 deposition and Rhino-Deadlock-Cutoff recruitment.

silencing and H3K9 trimethylation (H3K9me3) of TEs in the *Drosophila* ovary (Huang et al., 2013; Le Thomas et al., 2013; Rozhkov et al., 2013; Sienski et al., 2012). Mechanistic investigations of piRNA-mediated chromatin silencing in *Drosophila* have uncovered features reminiscent of the fungal and plant systems described above. Like the *S. pombe* and plant Argonaute proteins, Piwi complexes appear to be targeted through interactions with nascent transcripts, which in turn allows Piwi to recruit a series of silencing factors, including the H3K9 methyltransferase SetDB1, to repress the corresponding loci (Figure 1c) (Batki et al., 2019; Ninova et al., 2020; Sienski et al., 2012, 2015; Yu et al., 2015). Meanwhile, in germline cells, H3K9me3 also accumulates at the clusters that serve as the source of piRNAs, leading to the binding of a dedicated reader of this histone mark called Rhino (Klattenhoff et al., 2009). At most sites, Rhino is recruited by the recently described Rhino-associated zinc finger protein, Kipferl (Baumgartner et al., 2022), while at the Kipferl-independent sites, it requires dual recognition of H3K27me3 and H3K9me3 for binding (Akkouche et al., 2024). Rhino acts together with its partners Deadlock and Cutoff to promote the synthesis of new piRNA precursors (Le Thomas et al., 2014; Mohn et al., 2014; Z. Zhang et al., 2014). Thus, through a positive feedback loop, piRNA-mediated targeting of histone methylation begets more piRNA production, in so doing ensuring repression of corresponding TEs elsewhere in the genome (Figure 1d). It is worth noting that asexually reproducing rotifers also rely on piRNAs to restrict TE activity. A remarkable recent study of rotifers of the bdelloid class found evidence that a

cytosine-4 DNA methyltransferase, recently acquired from a prokaryote through horizontal transfer, is co-targeted with a SetDB1 H3K9 methyltransferase ortholog, each recognizing the modification catalyzed by the other and acting preferentially at repressed TEs (Rodriguez et al., 2022). This proposed mutual communication between DNA methylation and histone methylation resembles what is observed in RdDM in plants, but the involvement in this case of a DNA methyltransferase acquired just tens of millions of years ago speaks to the inherent adaptiveness of this type of self-reinforcement.

These genome defense pathways from fungi, plants and animals differ widely in their specific functions and molecular players, but collectively they illustrate the staying power of chromatin silencing mechanisms based on targeting by complementarity of small RNAs to nascent transcripts. Positive feedback loops that promote further small-RNA production ensure persistence of repression. In yet another branch of the eukaryotic lineage, the ciliates, this problem of persistence is solved in an entirely different and audacious manner when the somatic genome is faced with TEs and their remnants: the unwanted sequences are simply removed once and for all.

2 | DNA ELIMINATION DURING SOMATIC DEVELOPMENT IN CILIATES

In ciliates, the process of DNA elimination occurs at each sexual cycle. While multicellular organisms present distinct somatic and germ cells, ciliates contain both somatic and germline nuclei within the same cell. Ciliates belong to the Alveolata phylum in the SAR (Stramenopiles, Alveolata and Rhizaria) clade, with more than 8000 species described so far (Burki et al., 2020; Grattepanche et al., 2018). Ciliate cells contain one or more germline micronuclei (MICs) and one or more somatic macronuclei (MACs), with different genomes, epigenomes and modes of division. The model ciliate *Paramecium*, which will be the focus of this review, belongs to the *Paramecium aurelia* complex, a group of 15 cryptic species that share at least three past whole-genome duplications (WGDs), the most recent one shortly predating the speciation events in the formation of the group (Aury et al., 2006; Gout et al., 2023; McGrath, Gout, Doak, et al., 2014; McGrath, Gout, Johri, et al., 2014). *Paramecium aurelia* species are well suited for genetic analyses because of their two alternative modes of sexual reproduction, as well as extremely low mutation rates such as observed in *P. tetraurelia* (Sung et al., 2012).

In *Paramecium*, the two micronuclei are diploid and their chromosomes appear similar to those of metazoans: they are large, contain both telomeres and centromeres, as well as transposable elements (TEs) and other repetitive sequences. MICs divide mitotically and chromosome segregation is achieved by spindle fibers formed by microtubules (Brygoo et al., 1980). MICs are transcriptionally silent, and appear to be dispensable during vegetative growth (Betermier & Duharcourt, 2014; Lhuillier-Akakpo et al., 2014). The MAC, on the other hand, is actively transcribed and supports gene expression and metabolism. During vegetative growth, the MAC divides amitotically, splitting in two halves without chromosome condensation or the formation of a mitotic spindle (Tucker et al., 1980).

Mild starvation triggers meiosis of the MICs, leading to sexual reproduction that occurs either by autogamy (self-fertilization) or by conjugation of two cells of compatible mating types (Sonneborn, 1937). The fusion of two gametic nuclei produces the zygotic nucleus, which divides twice and gives rise to four diploid nuclei that differentiate into two new MICs and two new MACs. After MIC meiosis, the maternal MAC is broken into fragments, which remain in the cells during the first divisions before being diluted out and are actively transcribed until the new MACs are functional (Figure 2). During formation of the new MAC, *P. tetraurelia* eliminates a defined portion of its germline genome (approximately one-third, or 25–30 Mbp) that encompasses a wide variety of sequences of different sizes and copy numbers (Arnaiz et al., 2012; Guérin et al., 2017; Sellis et al., 2021). The majority of eliminated sequences are large regions comprising repetitive sequences, such as transposable elements (TEs) and minisatellites. These are imprecisely eliminated during MAC development, leading either to chromosome fragmentation with de novo telomere addition to new MAC chromosome ends or to intrachromosomal deletions with variable boundaries (Betermier et al., 2023).

In contrast, the remaining eliminated sequences, which correspond to approximately 45,000 unique elements, known as internal eliminated sequences (IESs), are excised precisely (Arnaiz et al., 2012). The vast majority (93%) are shorter than 150 bp. While IESs are scattered throughout the MIC genome, most of them are localized inside coding exons. Their precise elimination is therefore crucial for the assembly of functional ORFs. IESs are flanked by one TA dinucleotide at each end, one of which remains after excision, and they possess a loosely conserved 8-bp consensus sequence, reminiscent of the inverted repeats found at the ends of Tc/mariner transposons (Klobutcher & Herrick, 1995). Consistently, most IESs in present-day genomes are relics of transposable elements (Arnaiz et al., 2012; Sellis et al., 2021). Excision of all IESs and most TE-containing regions depends on the activity of a domesticated

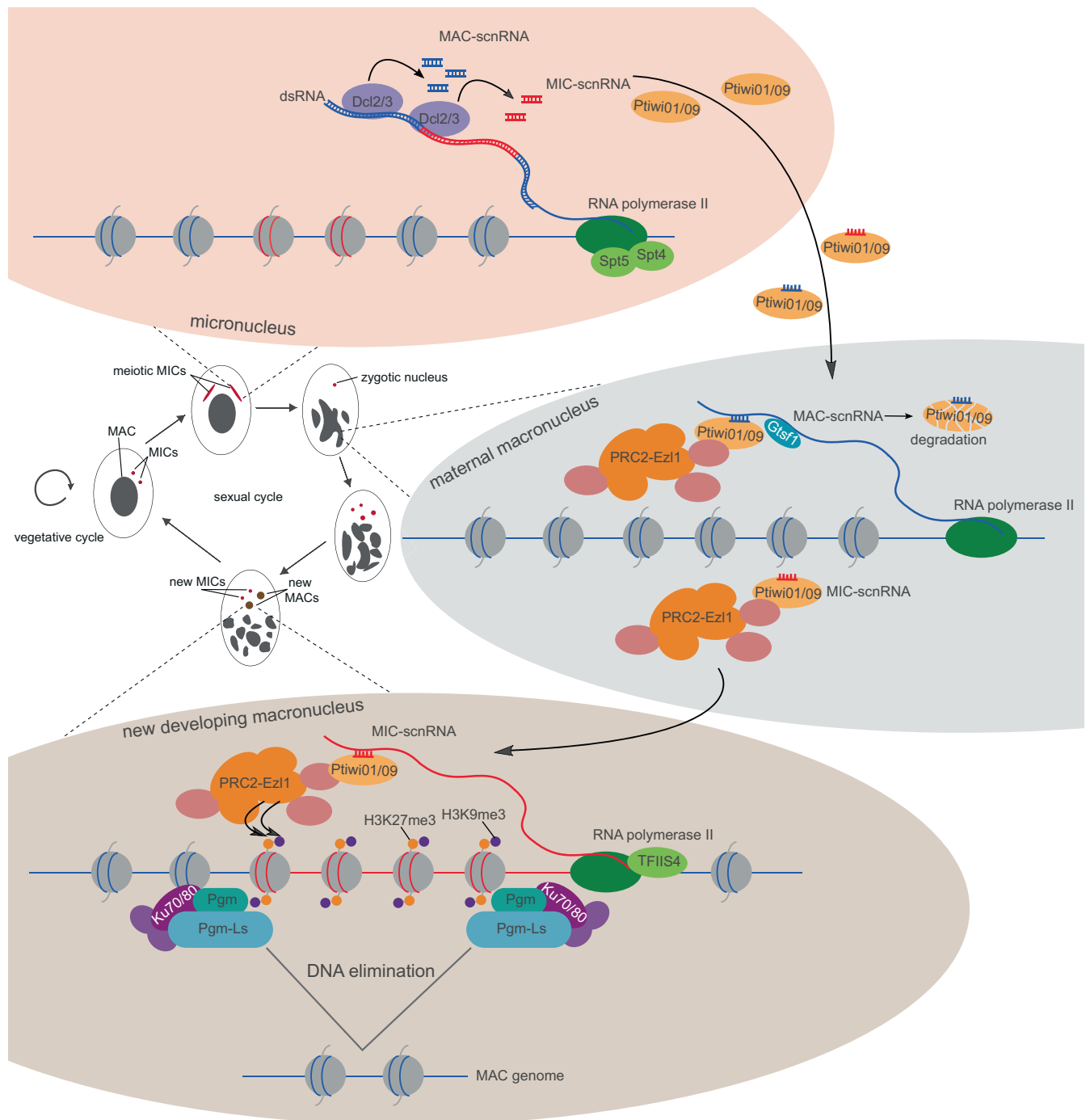


FIGURE 2 Model of DNA elimination during *Paramecium* sexual cycle. During vegetative growth, the MICs undergo mitosis while the MAC divides amitotically at each cell division. Starvation triggers entry into the sexual cycle. The MICs undergo meiosis, and two haploid nuclei fuse to give rise to a diploid zygotic nucleus. In the meiotic MIC, the whole genome is transcribed and gives rise to double-stranded RNA that is processed by Dicer-like proteins 2 and 3 into 25 nucleotide-long RNAs called scan-RNAs (scnRNAs). These scnRNAs are loaded onto Ptiwi01/09 proteins and transported to the maternal macronucleus. scnRNAs then hybridize by sequence complementarity to nascent transcripts. Paired scnRNAs corresponding to MAC-specific sequences are degraded through a mechanism involving the PRC2 complex and Gtsf1. Thus, only the MIC-specific scnRNAs, which by definition do not pair with nascent transcripts in the maternal MAC, remain. These selected scnRNAs are then exported to the new developing MACs where, in contrast to the maternal MAC, they are able to pair with non-coding nascent transcripts, since the sequences to be removed from the genome are still present. This pairing enables the recruitment of the PRC2 complex through the Rf4 protein and the subsequent deposition of both H3K9me3 and H3K27me3 onto the MIC-limited sequences. The presence of the two histone modifications allows the recruitment of the Pgm excision complex which introduces double-strand breaks followed by DNA repair through the NHEJ pathway.

transposase, PiggyMac (Pgm) (Arnaiz et al., 2012; Baudry et al., 2009), which introduces double-strand breaks (DSBs) at IES ends together with its Pgm-like partners, PmgL1 to 5 (Baudry et al., 2009; Bischerour et al., 2018). This is coupled to canonical non-homologous end-joining (NHEJ) repair and gap filling of the cleavage sites (Abello et al., 2020; Betermier & Duhaucourt, 2014; Kapusta et al., 2011; Marmignon et al., 2014). A list of proteins involved in DNA elimination in 4 different genera of ciliates (*Paramecium*, *Tetrahymena*, *Oxytricha* and the less studied *Blepharisma*) can be found in Table S1.

3 | Small-RNA-GUIDED HISTONE MODIFICATIONS TRIGGER THE ELIMINATION OF TRANSPOSABLE ELEMENTS

No strictly conserved sequence motif has been identified at the boundaries of either IESs or TE-containing regions, which raises the question of how the Pgm excision machinery is consistently recruited. Part of the answer comes from the epigenetic control exerted by the maternal MAC. For a subset of IESs, the presence of the IES in the maternal MAC inhibits the excision of the corresponding sequence during the formation of the new MAC (Duhaucourt et al., 1995). To explain this maternal homology-dependent epigenetic control, it was proposed that the sequences to be eliminated are defined through a comparison between the MAC and MIC genomes. The underlying mechanisms involve a process termed genome scanning (Mochizuki et al., 2002) that involves different classes of non coding RNAs. Low levels of non-coding transcripts (ncRNAs) are continuously generated from both strands of the MAC genome (Lepère et al., 2008). Then, during meiosis, 25-nt-long small RNAs called scnRNAs, are produced by two Dicer-like proteins Dcl2/3 (Lepère et al., 2009; Sandoval et al., 2014). Despite this Dicer-dependent mode of biogenesis, which is not a feature of piRNAs, scnRNAs exhibit strong piRNA hallmarks including a similar size, interaction with PIWI proteins, and germline origin. Their likely precursors are long dsRNA molecules transcribed from the MIC chromosomes by RNA polymerase II in association with the MIC-specific transcription factor Spt4-Spt5 (Gruchota et al., 2017; Owsian et al., 2022). A recent study confirmed earlier findings that the entire MIC genome is transcribed and produces scnRNAs (Miró-Pina et al., 2023), that are subsequently loaded onto Ptiwi01 and 09, two ohnologs of the PIWI subclade of Piwi/Ago proteins (Bouhouche et al., 2011; Furrer et al., 2017).

Ptiwi01/09, loaded with scnRNAs, shuttle to the maternal MAC where the genome scanning process is thought to occur. ncRNA transcripts present in the maternal MAC are believed to be probed by the Piwi-bound scnRNAs, with scnRNAs complementary to a ncRNA target being degraded, and the remaining scnRNAs being transported to the new MAC. This would result in the selection of a scnRNA population exclusively corresponding to sequences not found in the maternal MAC, and therefore to be eliminated from the new MAC. The details of the selection mechanism remain unclear. Some proteins have been implicated in the selective degradation of scnRNAs, such as the RNA-binding proteins Nowa1 and 2 (Nowacki et al., 2005), Pdsg1 (Arambasic et al., 2014), or the *Tetrahymena* RNA helicase Ema1 that appears to stimulate the interaction between scnRNAs and noncoding transcripts (Aronica et al., 2008). Interestingly, recent findings have identified roles for proteins traditionally associated with different functions in other organisms in scnRNA selection in ciliates, such as PRC2 and its cofactors (Miró-Pina et al., 2023; C. Wang et al., 2022) and Gtsf1 (Charmant et al., 2023; C. Wang, Lv, et al., 2023).

In the new MAC, scnRNA-Ptiwi complexes guide the PRC2 methyltransferase Ezl1 to catalyze both H3K9 and H3K27 trimethylation over MIC-specific sequences to be eliminated (Frapporti et al., 2019). This dual substrate activity is strikingly different from the human PRC2 methyltransferase EZH2, which has a strong preference for H3K27 (Margueron et al., 2008), even though they share structural similarities (Frapporti et al., 2019). By analogy to the small-RNA-guided heterochromatin formation mechanism described in *S. pombe*, scnRNA-Ptiwi complexes are likely recruited to their targets in the new MAC by nascent transcripts synthesized by TFIIIS4 (Maliszewska-Olejniczak et al., 2015). Similarly to the recruitment of H3K9 methyltransferases by small-RNA-Argonaute complexes in other organisms, PRC2 is likely recruited to TEs via its known physical interaction with Ptiwi09 (Miró-Pina et al., 2022; C. Wang et al., 2022). The resulting H3K9me3 and H3K27me3 marks are required for the elimination of all TEs and around 70% of IESs (Frapporti et al., 2019; Lhuillier-Akakpo et al., 2014). Small RNAs of another type, called iesRNAs, are produced by Dcl5, are bound to Ptiwi10/11 and contribute to the elimination of IESs, apparently acting synergistically with scnRNAs (Allen et al., 2017; Furrer et al., 2017; Sandoval et al., 2014). Once H3K9me3 and H3K27me3 are deposited, however, it is not entirely understood how they are interpreted by the cell to trigger DNA elimination.

4 | HISTONE MODIFICATIONS AND THE RECRUITMENT OF THE EXCISION COMPLEX

The simplest model proposed so far is that the endonuclease itself is tethered to chromatin by the histone marks (Baudry et al., 2009; Betermier & Duhaucourt, 2014; Coyne et al., 2012). Both the Pgm (*Paramecium*) and Tpb2 (*Tetrahymena*) endonucleases possess a cysteine-rich domain (CRD). While the CRD domain of Tpb2 has been reported to show a modest preference for H3K9me3 or H3K27me3 peptides (Vogt & Mochizuki, 2013), it remains to be assessed whether the CRD domain preferentially binds modified nucleosomes. Meanwhile, the CRD domain of Pgm has been shown to have a weak affinity for the electrostatic charge of histone H3 N-terminal tail independently of its methylation status (Guérineau et al., 2021).

Alternatively, other factors might link the excision machinery to histone modifications. The Pgm excision complex is believed to include 5 Pgm-like proteins which are catalytically inactive but necessary to introduce double strand breaks (Bischerour et al., 2018). These or other Pgm-interacting proteins could serve as intermediaries between the excision machinery and histone marks. In other organisms, H3K9me3 and H3K27me3 are each recognized by distinct families of chromodomain-containing proteins (Bannister et al., 2001; R. Cao et al., 2002; Kuzmichev et al., 2002; Lachner et al., 2001). The presence of regions marked by both H3K9me3 and H3K27me3 in *Paramecium* raises the possibility that the same chromodomain-containing protein reads the two marks, as has been shown for Rhino in *Drosophila* (Akkouche et al., 2024). Despite the large number of chromodomain proteins encoded in the *Paramecium* genome, or perhaps because they are so numerous, none has so far been linked to Pgm complex recruitment to eliminated regions. Further work is needed to uncover what bridges histone modifications and the Pgm excision complex.

It is not known whether Pgm cuts throughout the TE-containing regions that are covered with H3K9me3 and H3K27me3, or only at their boundaries. In the first scenario, Pgm would only need the repressive marks to guide its recruitment, whereas the second model would imply that Pgm can sense the junction between Ezl1-modified and unmodified chromatin.

As for the IESs, it is not formally known if they are marked with H3K9me3 and H3K27me3, although the excision of 2/3 of IESs is dependent on PRC2 (Lhuillier-Akakpo et al., 2014; Miró-Pina et al., 2022; C. Wang et al., 2022). It seems difficult to explain how they could be excised with nucleotide-level precision through histone modifications alone. Indeed, the position of the modified nucleosomes relative to the IES length (93% shorter than 150 bp) does not appear to provide the necessary resolution. Nevertheless, positioning of modified nucleosomes with respect to IES likely plays a direct role in the recruitment of the Pgm complex at their vicinity (Coyne et al., 2012; Lhuillier-Akakpo et al., 2014). Access to DNA cleavage sites may then be facilitated by chromatin remodelers (de Vanssay et al., 2020; Singh et al., 2022).

IESs are excised in a time-dependent manner according to their size (Zangarelli et al., 2022). The small IESs, which are also the oldest ones, are excised early compared to the longer, and more recent IESs (Sellis et al., 2021). These longer, late-excised IESs require PRC2 for excision, while the shorter early-excised ones most often do not (Lhuillier-Akakpo et al., 2014; Sellis et al., 2021). Interestingly, disruption of IES excision has been found to have cascading effects, with dysregulation of IES-containing genes impairing the subsequent excision of other IESs (Bazin-Gélis et al., 2023). This raises the possibility that the early events of DNA elimination might re-establish the reading frames of genes whose transcription is required for the later PRC2-dependent steps of DNA elimination. Proper timing of IES excision is thus crucial for completing the DNA elimination process. Depletion of PRC2, like that of the excision machinery, leads to dysregulation of developmental genes, although this is likely a consequence of IES retention rather than a direct repressive effect of PRC2 on gene expression (Bazin-Gélis et al., 2023; Frapporti et al., 2019).

5 | DYNAMIC 3D ORGANIZATION OF THE NUCLEUS AND DNA ELIMINATION

At the cytological level, H3K9me3 and H3K27me3 display a dynamic pattern of localization in the new MAC. The marks are initially diffuse and then form foci that coalesce into a single spot, before finally disappearing (Lhuillier-Akakpo et al., 2014). Interestingly, the formation of these foci is abolished when DNA elimination is impaired (Lhuillier-Akakpo et al., 2014), suggesting that nuclear 3D organization is important for the DNA elimination process. However, it remains unknown whether the foci are a prerequisite for or a consequence of DNA elimination in *Paramecium* (Lhuillier-Akakpo et al., 2014). In *Tetrahymena*, DNA elimination has been proposed to occur downstream the formation of these foci (Kataoka & Mochizuki, 2017).

One could imagine that chromodomain-containing proteins that recognize H3K9me3 and H3K27me3 are at play. Studies have shown that the metazoan chromodomain protein HP1 is a driver of liquid–liquid phase separation, allowing the split of heterochromatin and euchromatin into different nuclear compartments (Strom et al., 2017; Larson 2017). By analogy, MIC-limited and MAC-destined sequences could be physically separated by a similar mechanism in *Paramecium* and *Tetrahymena*. This would enable genome compartmentalization and the creation of a suitable chromatin environment for the excision machinery.

Alternatively, if the foci are a consequence of DNA elimination, their formation may represent modified histones that aggregate together with DNA after excision from the chromosome. Their disappearance might result from the subsequent active degradation of these DNA/protein aggregates by mechanisms yet to be determined.

6 | CONCLUSION

Analogous small-RNA-guided strategies are used in fungi, plants, animals and ciliates to recruit histone methyltransferases to repeated sequences and transcriptionally silence them. Instead of H3K9 methyltransferases, ciliates such as *Paramecium* have apparently rewired the RNAi machinery toward a Polycomb protein, which has the ability to trimethylate both K9 and K27 of histone H3.

While PRC2 is generally associated with maintenance of gene repression in animals and plants (Blackledge & Klose, 2021; Holoch & Margueron, 2017; Schuettengruber et al., 2017), in *Paramecium* and *Tetrahymena* it has been shown to control the elimination of TEs and their transcriptional repression (Frapporti et al., 2019; Miró-Pina et al., 2022; C. Wang et al., 2022; Zhao et al., 2019). Interestingly, an association between H3K27me3 and transposable elements has also been reported in several eukaryotic lineages beyond ciliates (Délérís et al., 2021). Enhancer-of-zeste proteins, which are the catalytic subunits of PRC2, have recently been implicated in the transcriptional control of TEs in these organisms (Hisanaga et al., 2023), supporting the idea that this may constitute the ancestral function of PRC2 (Délérís et al., 2021). Beyond participating in transcriptional repression of TEs directly, PRC2 was recently found to contribute to small-RNA biogenesis (Akkouche et al., 2024), suggesting that H3K27me3 can help control TEs at multiple levels.

To ensure the persistence of repression, other organisms have developed self-reinforcing regulatory loops that lead to lasting accumulation of small-RNAs and histone modifications. At first glance, it appears that such a mechanism might not be necessary in a system in which the repressed sequences are removed. Yet several rounds of genome endoreplication occur before transposable elements are eliminated (Zangarelli et al., 2022). This leaves open the possibility that ciliates may also possess positive feedback loops to enhance scnRNA production and accumulation of histone modifications during this time window preceding TE elimination.

The similarities and differences between ciliates and other eukaryotes highlight the value of studying small-RNA-directed chromatin silencing in a wide variety of model systems. Continuing to explore these questions through the lens of evolutionary comparison will be critical to reach a more thorough understanding of the principles governing genome stability.

AUTHOR CONTRIBUTIONS

Thomas Balan: Conceptualization (equal); investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Leticia Koch Lerner:** Conceptualization (equal); investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Daniel Holoch:** Conceptualization (equal); investigation (equal); writing – original draft (equal); writing – review and editing (equal). **Sandra Duharcourt:** Conceptualization (equal); funding acquisition (equal); supervision (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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ORCID

Sandra Duhaucourt  <https://orcid.org/0000-0002-8913-8799>

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