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**SPECIAL ISSUE: Non-coding RNAs**
**• REVIEW •**

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# Non-coding RNAs mediate the rearrangements of genomic DNA in ciliates

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Most eukaryotes employ a variety of mechanisms to defend the integrity of their genome by recognizing and silencing parasitic mobile nucleic acids. However, recent studies have shown that genomic DNA undergoes extensive rearrangements, including DNA elimination, fragmentation, and unscrambling, during the sexual reproduction of ciliated protozoa. Non-coding RNAs have been identified to program and regulate genome rearrangement events. In *Paramecium* and *Tetrahymena*, scan RNAs (scnRNAs) are produced from micronuclei and transported to vegetative macronuclei, in which scnRNA elicits the elimination of cognate genomic DNA. In contrast, Piwi-interacting RNAs (piRNAs) in *Oxytricha* enable the retention of genomic DNA that exhibits sequence complementarity in macronuclei. An RNA interference (RNAi)-like mechanism has been found to direct these genomic rearrangements. Furthermore, in *Oxytricha*, maternal RNA templates can guide the unscrambling process of genomic DNA. The non-coding RNA-directed genome rearrangements may have profound evolutionary implications, for example, eliciting the multigenerational inheritance of acquired adaptive traits.

**RNAi, gene silencing, scnRNA, piRNA, rearrangement, elimination**

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Ciliates are a group of protozoa characterized by large and transparent body. Research in ciliates has led to many groundbreaking discoveries including ribozyme [1], telomere and telomerase [2,3], and RNA editing [4]. Recent studies have revealed a new role for ciliates as a model organism in understanding the mechanism of the regulation of genome integrity [5–11].

Most ciliates have two structurally and functionally different nuclei, the diploid germline micronucleus and the polyploidy somatic macronucleus, in a single cell. The genomes of ciliates are maintained in the two distinct compartments. During vegetative growth, the germline genome is transcriptionally inactive; the somatic genome is highly active in gene expression. Ciliates can undergo both asexual and sexual reproduction.

Ciliates proliferate asexually by binary fission in the presence of abundant nutrients. The micronucleus undergoes mitosis; the macronucleus elongates and splits in half amitotically; the parental cell then divides into two daughter cells; each of the two daughter cells receives a copy of the micronuclear and macronuclear genomic DNA. In contrast, when food is scarce, two cells of compatible mating types conjugate and elicit the sexual reproduction pathway. The micronucleus undergoes meiosis, fertilization, and forming of zygotic nucleus which gives rise to the new micronucleus and macronucleus. The parental macronucleus is destroyed at the end of sexual reproduction. Most ciliates undergo extensive genome rearrangements in the development of macronucleus during conjugation, a process that requires the elimination of repetitive DNA and the excision of the non-coding elements that are known as internally eliminated sequences (IESs). The excision of IESs leads to the loss of

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15%–95% of micronuclear genomic sequences.

The critical issue is how ciliates select specific sequences for elimination and unscrambling, whereas other sequences are retained at the same time. Studies in *Tetrahymena thermophila*, *Paramecium tetraurelia*, and *Oxytricha trifallax* have pinpointed the important roles for non-coding RNAs in these genome rearrangement events.

## 1 Small RNA-mediated DNA elimination

In *Tetrahymena thermophila* and *Paramecium tetraurelia*, small RNAs have been shown to guide DNA eliminations [10,12–15]. We will focus on *Tetrahymena* in this review. *Tetrahymena* has two nuclei, a transcriptionally active somatic macronucleus and a germline silent micronucleus that is transcribed transiently during early conjugation. The micronucleus is diploid and contains 10 chromosomes (five per haploid genome). The macronucleus has over 20000 chromosomes. During sexual reproduction, the new macronucleus is differentiated from the parental micronucleus. This process includes the elimination of repetitive DNA and the excision of non-coding elements IESs. Thirty percent of the micronuclear genome is eliminated by the removal of approximately ~6000 internal DNA segments [16,17]. The remaining macronuclear genome is then endoreplicated to about 45 copies and followed by the addition of telomere at each end of the chromosomes.

IESs are 0.5–20 kb in size and a total of around 20 Mbp (~15%) of DNA in the micronuclear genome [18]. Most IESs exist in the intergenic region, whereas others are present in introns [19]. Many IESs are transposon-like repeats or other repeated sequences [18,20,21]. IESs are located to specific genomic region with relatively precise boundaries [22]; yet, no sequence motif has been identified either in the IESs or in the flanking regions.

A scan RNA model has been proposed to explain the elimination of IESs (Figure 1) [8,10,12,23]. In this model, the sexual conjugation of two ciliate cells first triggers a bi-directional transcription and generates double-stranded RNAs (dsRNAs) from the entire micronuclear genome. These dsRNAs are cleaved to 28–30 nt small RNAs called

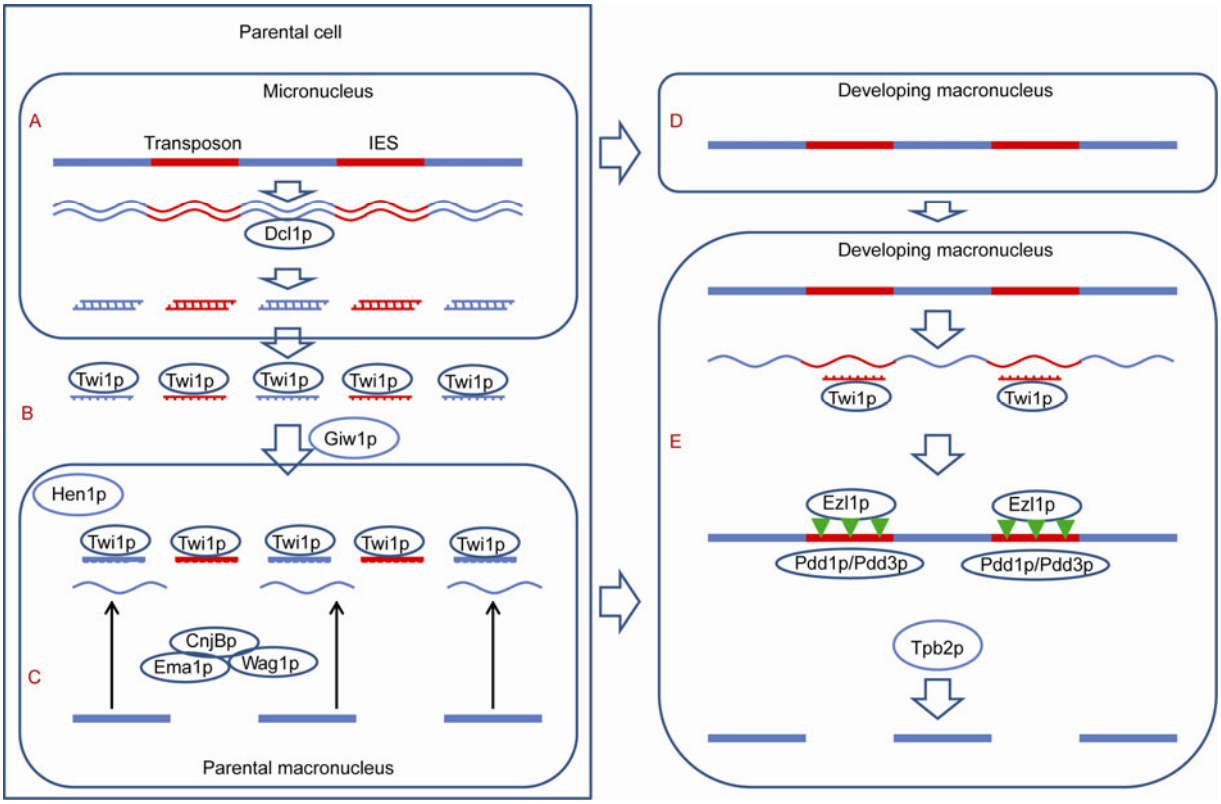


**Figure 1** scnRNA-mediated DNA elimination in *Tetrahymena*. A, During sexual reproduction, the micronuclear genome is transcribed bi-directionally to generate dsRNAs, which are processed by Dcl1p to produce scnRNAs. B, scnRNAs are transported to the cytoplasm where they associate with Twi1p. One of the two strands of scnRNA is degraded by Twi1p. The scnRNA-Twi1p complex is transported into the old macronucleus with the aid of Giw1p. C, The parental macronuclear genome expresses transcripts that recognize scnRNAs, which are 2′-O-methylated by Hen1p in the parental macronucleus. Those scnRNAs that are complementary to macronuclear sequences are degraded, a process depending on Ema1p, CnjBp, and Wag1p. D, The developing (new) macronucleus is derived from the parental micronucleus. E, The remaining scnRNAs that contain micronuclear specific sequences are transported from the parental (old) macronucleus into the developing macronucleus and associate with nascent transcripts. The Twi1p-scnRNA complex elicits the methylation of histone H3K9 and H3K27 (green triangles) and the formation of heterochromatin, a process depending on the enhancer-of-zeste homolog Ezl1p and the two chromodomain proteins Pdd1p and Pdd3p. The transposase Tpb2p excises the transposons and IES elements.

scnRNAs by the RNAi-related machinery. ScnRNAs then bind to the Argonaute protein Twi1p and transport to the parental macronucleus [24], where scnRNAs are depleted of those sequences that are complementary to sequences present in the parental macronuclear genome. The remaining scnRNAs, with micronucleus-specific sequences, are retained. In later stages of the sexual reproduction, these scnRNAs translocate to the developing macronucleus, target IES sequences, and elicit the excision of these IESs in the macronuclear genomic DNA.

The components of RNAi machinery have been shown to act in the DNA elimination process in *Tetrahymena*. In the canonical RNAi pathway, dsRNA is recognized and cleaved by the RNase III-like enzyme Dicer to generate small RNAs (approximately 20–30 nt), which associate with the conserved Argonaute proteins [25,26]. The *Tetrahymena* genome encodes three Dicer [27–29] and 12 Argonaute proteins [27,30]. Each of the Dicer and Argonaute protein has distinct roles in generating and binding different classes of small RNAs.

During the prophase of meiosis, the entire micronuclear genome becomes transcriptionally active. RNA polymerase II translocates to the micronucleus and transcribes dsRNAs bi-directionally [31–33]. DsRNAs are cleaved by the Dicer protein Dcl1p in the micronucleus to produce scnRNAs [12,28,29]. ScnRNAs are exported to the cytoplasm where they interact with the Argonaute protein Twi1p [34]. Both Dcl1p and Twi1p are required for DNA elimination in *Tetrahymena*. Dcl1p localizes to the micronucleus during meiosis [28]. The mutation of Dcl1p leads to the loss of scnRNA production and the over-accumulation of long micronuclear RNA, indicating that Dcl1p processes micronuclear transcripts into scnRNAs in the micronucleus during meiosis.

The Twi1p-scnRNA complex translocates to the parental macronucleus at the mid-conjugation stages, a process depending on Dcl1p, the Twi1p-associated protein Giw1p, and the endoribonuclease activity of Twi1p itself [24]. Furthermore, the endoribonuclease activity of Twi1p is also required for its association with Giw1p. Since the endoribonuclease activity is necessary for Twi1p to remove one of the two strands of scnRNA, Giw1p most likely monitors the state of Twi1p-associated scnRNAs and selectively transports mature Twi1p-scnRNA complexes into the parental macronucleus.

Like some classes of small RNA in other organisms, scnRNAs in *Tetrahymena* are 2′-O-methylated at their 3′-termini by the conserved RNA methyltransferase Hen1p [35], which is necessary for DNA elimination. Loss of Hen1p leads to the complete abolishment of scnRNA methylation, a gradual reduction in the accumulation of scnRNAs, and the defect in DNA elimination [35]. Hen1p localizes to the parental macronucleus and only methylates single-stranded RNAs, indicating that scnRNAs are 2′-O-methylated after the Twi1p-scnRNA complex releases one of the two scnRNA strands and translocates into the parental macronucleus. The 2′-O-methylation stabilizes scnRNAs to facilitate DNA elimination.

Three Twi1p-binding proteins, including Ema1p (RNA helicase), CnjBp (GW repeat and Zinc-finger protein), and Wag1p (GW repeat protein), have been shown to specifically degrade the scnRNAs that are complementary to the genomic sequences (or their transcripts) in the parental macronucleus. Ema1p mediates the interaction between the nascent non-coding transcript and the scnRNA-Twi1p complex [32]. CnjBp and Wag1p are two proteins containing the GW (glycine and tryptophan) repeat sequence that interacts with Argonaute proteins [36]. Wag1p and CnjBp colocalize with Twi1p in the parental macronucleus in the early conjugation and in the new developing macronucleus. Wag1p and CnjBp likely have redundant roles in the selective degradation of specific scnRNAs, the formation of DNA elimination structures, and DNA excision.

When the new macronucleus is formed, the selected Twi1p-scnRNA complex translocates from the parental macronucleus to the new macronucleus [12]. The Twi1p-scnRNA complex induces methylation of histone H3 at lysines 9 (H3K9me) and 27 (H3K27me) and the formation of heterochromatin, a process depending on the enhancer-of-zeste homolog Ezl1p [37,38] and the two chromodomain proteins Pdd1p and Pdd3p [38,39]. During the course of DNA elimination, IESs are found in dense heterochromatic regions at the nuclear periphery [39,40]. Twi1p, Ezl1p, and Pdd1p are all required for the formation of heterochromatic structure and DNA elimination, suggesting that the nuclear peripheral heterochromatic structure plays important roles in DNA elimination. Pdd1p has been shown to specifically accumulate on IESs in a scnRNA-dependent manner. Interestingly, the tethering of Pdd1p to a locus using a LexA-Pdd1p fusion protein and the DNA sequence for LexA binding can elicit the ectopic DNA elimination [41].

The mechanism underlying the excision of IESs remains unclear. Recently, a PiggyBac transposase-like protein, Tpb2p, has been identified as essential for DNA elimination in *Tetrahymena* [42,43]. Tpb2b is encoded in the macronuclear genome. In the developing macronucleus, Tpb2p localizes to the nuclear peripheral heterochromatic structure. *In vitro* studies have shown that Tpb2p is an endonuclease that produces double-strand breaks with four-base 5′ protruding ends, similar to the ends that are generated during DNA excision.

However, a recent research pointed to a new model in which the biased transcription and selective degradation can shape the pattern of DNA elimination in *Tetrahymena* [44]. Using deep sequencing, scnRNAs were found to be produced exclusively from the micronucleus, with templates preferentially derived from the sequences destined to elimination. This preference is determined at the level of transcription. In addition, scnRNAs are also derived from the macronuclear-destined sequences; and these scnRNAs are degraded during the course of sexual reproduction. Therefore, the pattern of DNA elimination in the new macronucleus is directed by the biased transcription in the micronu-

cleus and the selective degradation of scnRNAs in the parental macronucleus.

## 2 Small RNA-mediated DNA retention

In spirotrichous ciliates, such as *Euplotes*, *Stylonychia*, and *Oxytricha*, more than 95% of the germline genome is eliminated in the macronucleus during sexual reproduction [11]. Moreover, in the macronucleus, the remaining genome is severely fragmented; and these fragments are sorted and reordered under the guidance of transcripts from the parental macronucleus to produce protein-coding genes [5,45,46]. In *Oxytricha*, the tiny macronuclear "nanochromosomes" typically encode single, protein-coding gene, contain minimal noncoding regions, vary from 469 bp to 66 kb long, differentially amplify to an average of ~2000 copies, and encode approximately ~18500 genes [11]. Interestingly, the nanochrosomes also undergo extensive alternative fragmentation, which complicates the macronuclear genome.

A recent study by Fang et al. [47] has uncovered the mechanism of DNA elimination in *Oxytricha*. In contrast to *Tetrahymena*, small RNAs in *Oxytricha* elicit the retention of homologous genomic DNA, and eliminate the sequences that are not complementary to the small RNAs (Figure 2). Soon after mating, a class of abundant 27 nt small RNAs (piRNAs) are produced from the parental macronucleus [48]. *Oxytricha* expresses a Piwi-like protein, Otiwi1, that associates with piRNAs [47]. During sexual conjugation, Otiwi1 first appears in the parental macronucleus, and then translocates to the cytoplasm and the developing zygotic macronucleus. Knockdown of Otiwi1 expression leads to the loss of piRNAs and the failure of complete conjugation.

Unlike scnRNAs in *Tetrahymena*, piRNAs in *Oxytricha* map to the retained somatic genome, but not to germline-restricted sequences [47]. Consistent with their macronuclear origin, some piRNAs even map across the junctions that are left behind by IES elimination. Interestingly, the injection of synthetic piRNAs complementary to an IES that is normally eliminated induces the retention of the targeted IES sequence. The newly retained IES is inherited to progeny and serves as a template for new piRNA generation. This result suggests that the self sequences are marked by piRNAs and are transmitted from the germline to the somatic genome, whereas the nonself sequences are discarded. On the contrary, the injection of small RNA duplexes with 2 nt 3′ overhang to *Paramecium* elicits the deletion of IESs [13], suggesting an opposite mechanism between the two species of ciliates.

Transposases likely conduct the DNA excision in *Oxytricha* [49]. The eliminated sequences in *Oxytricha* contain telomere-bearing elements (TBEs), which encodes for a 42 kD protein of a superfamily of transposases, characterized by a DDE catalytic motif. *Oxytricha* genome encodes for three transposases, TBE-1, -2, and -3. The gene expression of transposases occurs during the germline-soma differentiation; the silencing of transposase by RNA interference leads to abnormal DNA rearrangement in the progeny.

The mechanism underlying the piRNA-mediated genome retention is also applied in *Stylonychia*. It has been shown that RNAi-mediated knock-down of *Stylonychia* PIWI protein leads to the loss of histone H3 lysine 9 methylation and an arrest in the development of new macronuclei [50].

However, it is unclear why the functions of *Tetrahymena* scnRNAs and *Oxytricha* piRNAs are different. There are four major differences between these two classes of small RNAs. First, scnRNAs are processed from dsRNA precur-

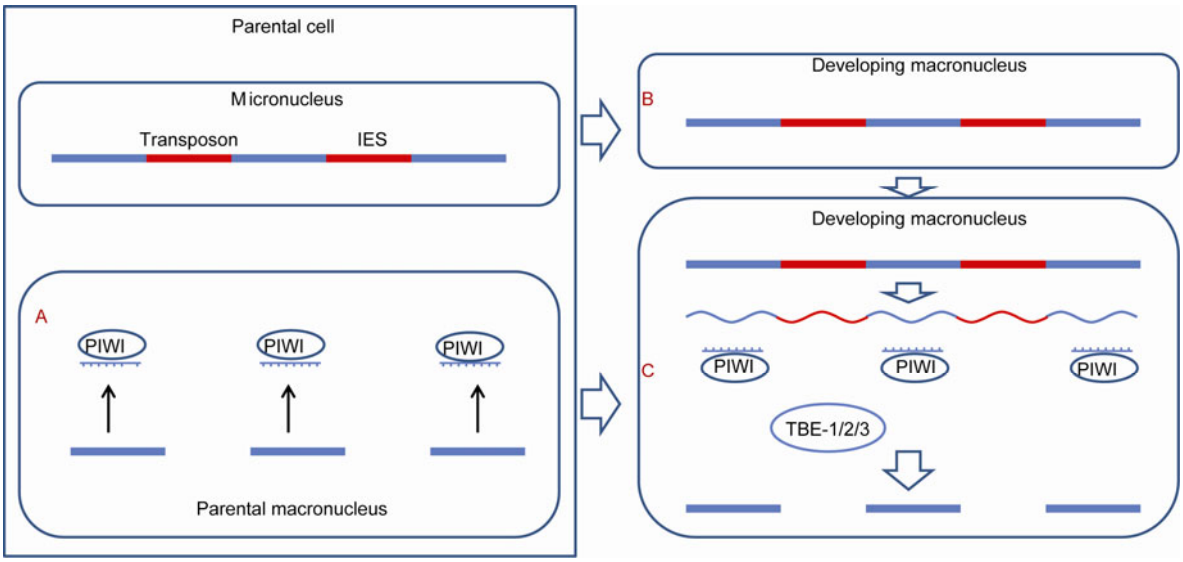


**Figure 2** piRNA-mediated DNA retention in *Oxytricha*. A, During sexual conjugation, the macronucleus expresses piRNAs. B, The developing (new) macronucleus is derived from the parental micronucleus. C, piRNAs are transported from the parental (old) macronucleus into the developing macronucleus and associate with nascent transcripts. The genomic DNA that is complementary to piRNA sequences are protected from elimination. TBE-1, TBE-2, and TBE-3 are transposases that are required for DNA elimination.

sors by Dicer proteins that likely form duplexes with 2 nt 3′ overhangs [51]. A prominent 5′-UNG signature has been observed in scnRNAs. On the other hand, piRNAs from *Oxytricha* lack this kind of signature and it is even unclear whether they derive from single-stranded RNAs or dsRNAs. Second, *Tetrahymena* scnRNAs are 3′-O-methylated, whereas *Oxytricha* piRNAs lack 3′-end modifications [35,47]. Third, *Tetrahymena* scnRNAs are generated in the micronucleus, whereas *Oxytricha* piRNAs are produced in the old macronucleus. Forth, *Tetrahymena* scnRNAs bind to Twi1p in the cytoplasm, while *Oxytricha* piRNAs associates with PIWI protein in the old macronucleus.

## 3 RNA-mediated genome unscrambling

In *Oxytricha*, the macronuclear genome can be further unscrambled by permutation and inversion after the elimination of IESs (Figure 3). During macronuclear development, the ‘scrambled’ protein-coding sequences in the micronucleus are sorted and reordered (unscrambled) into the proper order in the macronucleus. Approximately 20%–30% of genes in spirotrichs are scrambled in the micronucleus. DNA unscrambling is guided by the RNA templates which are inherited from the parental macronucleus. Nevertheless, unlike piRNAs, these RNA templates are larger in size comparable to protein-coding genes, suggesting a distinct mechanism other than RNAi may be involved. The disruption of these RNA molecules disabled the rearrangement of corresponding genes. The injection of an artificial RNA template can trigger the rearrangement of DNA sequences that are complementary to the injected RNA in the macronucleus [52]. Interestingly, the RNA molecules inherited from parental cells during sexual reproduction also regulate chromosomal copy numbers in the macronucleus [53].

The genome sequencing has identified three types of *Oxytricha*-specific macronuclear-encoded transposase-like domains: Phage_integrase, DDE_Tnp_IS1595, and MULE [11]. All of these domains belong to proteins encoded on complete nanochromosomes. Interestingly, the nanochromosomes encoding these DDE transposase domain lack terminal inverted repeats, the characteristic of transposons in *Tetrahymena*. Therefore, it is likely that these transposases might be involved in *Oxytricha*-limited functions, e.g., DNA unscrambling.

Although genome rearrangements in *Oxytricha* are exquisitely programmed by non-coding template RNAs, other epigenetics modifications are important as well. *De novo* cytosine methylation and hydroxymethylation have been found in three classes of eliminated DNA: germline-limited transposons and satellite repeats, aberrant DNA rearrangements, and DNA from the parental genome undergoing degradation [54]. Treating *Oxytricha* with the inhibitor of DNA methyltransferase induces demethylation of both somatic and germline sequences during genome rearrangements, which leads to elevated levels of germline-limited repetitive sequences in progeny cells.

## 4 Perspectives

A critical mission of germline cells in eukaryotes is to limit the establishment or spread of mobile or selfish nucleic acids in the genome. At the same time, germline cells need to specifically protect germline-expressed genes from repression. piRNAs have been shown to be involved in silencing mobile elements in germline cells in many species. For ex-

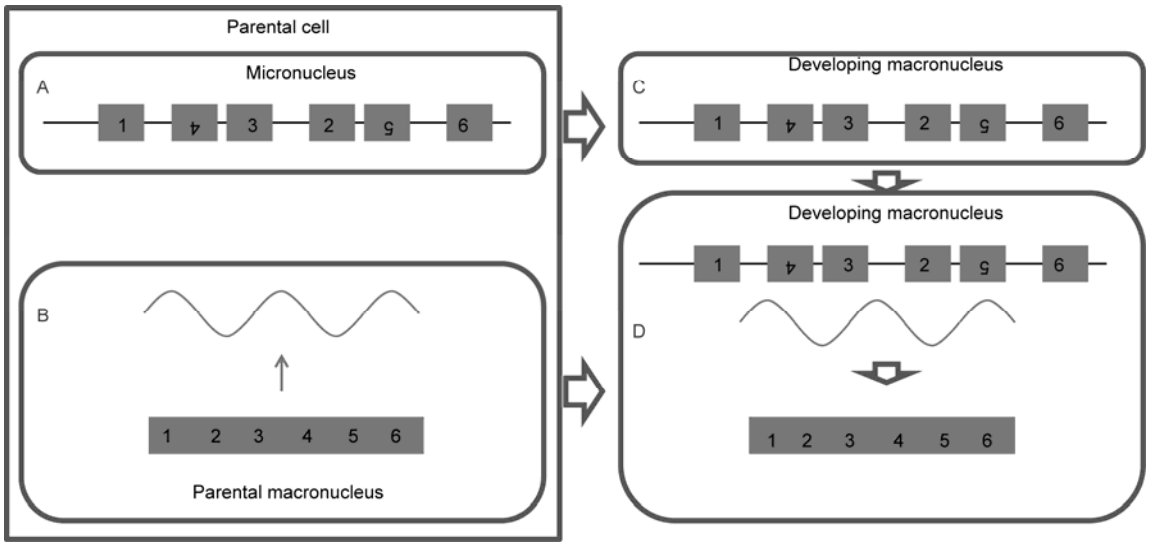


**Figure 3** The model of RNA-guided genome unscrambling in *Oxytricha*. A, In parental cells, the micronucleus contains scrambled DNA sequences. B, Transcripts are produced from the macronuclear genome in an ordered manner. C, The developing (new) macronucleus is derived from the parental micronucleus. D, The transcripts with sequences corresponding to the old macronucleus are transported into the developing macronucleus. Then, these transcripts direct the unscrambling of the ‘scrambled’ sequences in the micronuclear genome into their proper order, which generates a new macronuclear genome corresponding to that of the old macronucleus.

ample, in *C. elegans*, piRNAs act in a genome surveillance system to recognize and silence nonself sequences and promote a multigenerational epigenetic memory [55–57]. However, the ultimate way of silencing is physical removal of these mobile or selfish elements from the somatic genome as is done in ciliates.

DNA elimination occurs broadly in a variety of eukaryotes. For example, approximately ~80% of the genome is eliminated in somatic cells in the parasitic nematodes *Ascaris* and *Parascaris* [58,59]. In the jawless vertebrate, sea lamprey, hundreds of millions of base pairs (and at least one transcribed locus) from many somatic cell lineages are eliminated during embryonic development [60]. The most outstanding example of chromosome elimination is found in sciarid flies, where the whole chromosomes of exclusive parental origin are selectively eliminated at different developmental stages [61]. Strikingly, nearly all vertebrate species undergo a few programmed genome rearrangements during development, e.g., the remodeling of adaptive immune receptor locus [62].

It is unclear why DNA elimination and chromosome fragmentation have evolved in ciliates. It was suggested that IES elimination can serve as a defense system against foreign DNAs. In accordance with this model, many IES sequences in ciliates are sequence-related to transposable elements. When a heterokaryon was created with the bacterial transposon Tn5 neomycin resistance gene that was introduced into the micronucleus, but not the (old) macronucleus, this gene was eliminated from the new macronucleus that was formed after conjugation, just like IESs [63].

In ciliates, the RNAi-related mechanism is required for the formation of heterochromatin structures and the elimination of IESs. Small RNA-mediated comparison of the complete micronuclear and macronuclear genomes is proposed to search for the DNA sequences to be eliminated or retained. In other eukaryotes, transposons are silenced by the RNAi machinery both transcriptionally and posttranscriptionally [26,64]. However, in ciliates, transposable elements are silenced by the complete elimination from the genomic DNA in the transcriptionally active macronucleus. The macronucleus-encoded PiggyBac-like transposase is most likely involved in the DNA excision, whereas the mechanism is still unclear.

Many questions remain unknown. For example, how the production and amplification of dsRNAs are started and regulated; how scnRNAs and piRNAs are generated and selected; how enzymes are recruited to the heterochromationized IESs to conduct the elimination; and why the functions of scnRNAs in *Tetrahymena* and piRNAs in *Oxytricha* are different. It is also unclear whether the non-coding RNA-mediated DNA rearrangement is conserved in other organisms. Nevertheless, a recent study in *Ascaris* has found that the elimination of repetitive sequences may not be targeted by small RNAs [59,65].

In summary, ciliates have adopted an elegant way to maintain their genomic DNA, which likely functions to defend the genome from parasitic mobile nucleic acids and regulate gene expression. Further investigations into the mechanism underlying small RNA-mediated genome rearrangement will shed light on the control of genome integrity in eukaryotes.

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