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Programmed genome rearrangements in the ciliate *Oxytricha*

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Abstract

The ciliate *Oxytricha* is a microbial eukaryote with two genomes, one of which experiences extensive genome remodeling during development. Each round of conjugation initiates a cascade of events that construct a transcriptionally active somatic genome from a scrambled germline genome, with considerable help from both long and small noncoding RNAs. This process of genome remodeling entails massive DNA deletion and reshuffling of remaining DNA segments to form functional genes from their interrupted and scrambled germline precursors. The use of *Oxytricha* as a model system provides an opportunity to study an exaggerated form of programmed genome rearrangement. Furthermore, studying the mechanisms that maintain nuclear dimorphism and mediate genome rearrangement has demonstrated a surprising plasticity and diversity of non-coding RNA pathways, with new roles that go beyond conventional gene silencing. Another aspect of ciliate genetics is their unorthodox patterns of RNA-mediated, epigenetic inheritance, that rival Mendelian inheritance. This review takes the reader through the key experiments in a model eukaryote that led to fundamental discoveries in RNA biology and pushes the biological limits of DNA processing.

INTRODUCTION

Ciliates are microbial eukaryotes with separate germline and somatic nuclei. The DNA-rich somatic macronucleus forms by differentiation of a copy of the diploid, zygotic germline micronucleus, during sexual reproduction. The distinctive genome architectures of ciliates make them attractive model systems to study a wide range of key biological phenomena. These include complex genome rearrangements on a massive scale, a diverse range of non-coding RNA pathways, and several examples of non-Mendelian inheritance. In particular, ciliates belonging to the subclass *Stichotrichia*, such as *Oxytricha*, display the most exaggerated form of genome remodeling, stitching together somatic chromosomes from precursor gene segments, all under the epigenetic control of novel non-coding RNA pathways.

By contrast to these processes that actually reorder and recombine elements of germline DNA, many eukaryotes use RNA splicing to stitch together exons that are not linear in the genome. Such rearrangements at the RNA level can produce chimeric or permuted RNAs

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that reorder endogenous genetic material. Examples include the circularly permuted nuclear tRNA genes in the red alga *Cyanidioschyzon merolae* (1, 2, 3) and *trans*-splicing in both metazoa and protists, such as the *bursicon* gene in mosquitoes (4), at least two *Giardia lamblia* genes with pre-mRNAs transcribed from distinct chromosomal regions (5), the unusually spliced mitochondrial *cox3* of Dinoflagellates (6) and spliced leader *trans*-splicing in *Caenorhabditis elegans* and kinetoplastid protists (7, 8, 9), plus alternative splicing of 3'-UTRs in mammals (10) and countless other elaborate examples of alternative splicing (11). Numerous chimeric RNAs in humans have attracted attention due to their possible association with cancer (12, 13). One such example is the heterogeneously *trans*-spliced mRNA of the human transcription factor Sp1, which may alter the regulation of various genes (14, 15). Importantly, there is also evidence for the translation of such chimeric mRNAs in humans (16).

Although less common than rearrangement at the RNA level, programmed genomic remodeling at the DNA level is highly relevant to humans, since it underlies adaptive immunity. For example, V(D)J recombination (17) and class-switch recombination allow B and T cells to express a range of antigen receptors through the reshuffling of germline DNA sequences to produce genes for novel antigen receptors (18–20). Aberrant genomic rearrangements are also a hallmark of cancer (21–24). Similar to fusion proteins encoded by chimeric mRNAs, *trans*-spliced DNA segments may also encode malfunctioning proteins.

The process of genomic remodeling in *Oxytricha* also bears similarity to the developmentally regulated processes of chromosome elimination and diminution in metazoa, including nematodes, copepods, insects, jawless fish and marsupials (25). In the nematode *Parascaris univalens* and copepod *Mesocyclops edax*, the eliminated sequences account for ~90% of the germline genome as part of genome differentiation (26, 27). Similarly in Lamprey, an order of jawless fish, 20% of the germline sequences are eliminated during somatic differentiation. This may be an economical way to exclude genes from the soma that only function in the germline (28, 29). Sciarid flies go through a similar process of chromosome elimination during development, discarding entire chromosomes from their somatic genome (30).

The extensive genome remodeling in ciliates, while sharing some ostensible features with all these other types of genomic rearrangements, also illustrate the great power of non-coding RNA-mediated pathways. Small RNAs, together with long non-coding RNAs, in *Oxytricha* mediate the transgenerational transfer of information necessary to decrypt the germline genome during development of the soma. Surprisingly, ciliates have shown that RNAi-related pathways play a major part in genome remodeling (31, 32) in addition to their conventional role in gene silencing (33, 34). Moreover, these RNAi-related pathways have evolved to take on orthogonally different functions in the different branches of the ciliate clade (32).

Therefore, an emerging area of ciliate biology has been to study this intriguing form of non-Mendelian inheritance, which even permits mutations that accumulate in the soma to transmit to the sexual offspring. Epigenetic inheritance is ubiquitous among ciliates, due to the nature of their sexual life cycle [Fig. 1] (35). This allows maternal effects to transfer

epigenetic information for decrypting the germline after each round of sexual conjugation. This review will explore in detail the radical genome architecture of *Oxytricha* and the evidence for the RNA-guided mechanisms underlying its elaborate process of genome remodeling.

BODY

A) Background

i. Nuclear dualism and the life cycle of ciliates—In ciliates, the germline-soma differentiation of higher eukaryotes manifests in an unusual way. Each single celled organism contains a germline diploid micronucleus (MIC) and a somatic DNA-rich macronucleus (MAC) [Fig. 1]. During vegetative growth, cells divide asexually through binary fission [Fig. 1] as the MIC undergoes mitosis while the MAC replicates through amitosis. In the absence of spindle fibers, the MAC chromosomes may segregate randomly. During the asexual life cycle, the germline MIC is transcriptionally silent and all transcription for maintenance of the vegetative cell takes place in the somatic MAC (36).

Ciliates also undergo non-replicative conjugation under certain conditions, such as starvation in the laboratory setting [Fig. 1]. Conjugation leads to the formation of pairs between compatible mating types [Fig. 1]. This initiates meiosis and an ensuing cascade of events leading to the formation of a new zygotic nucleus that will become the new germline, while a copy of the germline will develop into the new somatic nucleus after major processing. This differentiation occurs during each round of sexual conjugation.

After completion of meiosis, one copy of a haploid MIC from each cell exchanges between the two mating partners to fuse with an endogenous haploid MIC forming a new, diploid, zygotic nucleus. Subsequently, the new zygotic nucleus undergoes mitosis, as the old maternal MAC degrades. One or more zygotic nuclei will be maintained as the germline, while others will differentiate into a new MAC [Fig. 1]. The developing MAC is called the “anlage” (plural anlagen). The numbers of each type of nuclei in a cell vary between different ciliate genera, up to hundreds in *Urostyla* (36).

The parental and newly developing macronuclei coexist in the same cell [Fig. 1], allowing for the direct transmission of cytoplasmic, surface, or other epigenetic factors from parent to offspring (35, 37). Compared to the nuclear differentiation process in Oligohymenophorea, the class to which the well-studied *Tetrahymena* and *Paramecium* belong, the genome remodeling process in stichotrichous ciliates, which include *Oxytricha trifallax*, is much more complex [Fig. 2].

ii. *Oxytricha trifallax* and its distinct genome architectures—The *Oxytricha trifallax* macronuclear genome contains over 16,000 unique gene-sized chromosomes. Averaging just 3.2 kb each with short telomeres, they are so small that they are sometimes called “nanochromosomes”. Each encodes just 1 to 8 genes, with 90% encoding only a single gene (38). In contrast to the MAC, the germline MIC harbors all the precursor macronuclear genomic sequences, plus a vast quantity of germline-limited DNA, on a set of ~120 long archival chromosomes (36).

Stichotrichous ciliates, such as *Oxytricha* and *Stylonychia*, differ from the oligohymenophorean ciliates *Tetrahymena* and *Paramecium* [Fig. 2] in important ways, such as discarding nearly all of their germline genome during formation of the somatic nucleus (compare over 90% in *Oxytricha* (39) and possibly 98% in *Stylonychia* (36) to ~33% in *Tetrahymena* (40, 41)) and the possession of thousands of scrambled genic segments in the germline that must be unscrambled to produce functional genes in the soma (42).

Somatic nanochromosomes in *Oxytricha* assemble from approximately 5% of the micronuclear genome after a sequence of events that includes chromosome polytenization, massive DNA elimination and chromosome diminution, DNA descrambling, chromosome fragmentation—likely coupled to telomere addition, and, finally, chromosome amplification. Genome differentiation begins with polytenization, where 15- to 32-fold amplification of the zygotic MIC genome produces giant polytene chromosomes (36, 43, 44). The first wave of amplification is followed by destruction of all germline-restricted DNA, including intragenic spacer sequences (**i**nternal **e**liminated **s**equences, or IESs) that interrupt macronuclear precursor loci [Fig. 3] (45), and intergenic DNA in the form of satellite repeats, transposons and even MIC-limited protein-coding genes (39). In stichotrichous ciliates and in a few other distant ciliates, such as *Chilodonella* (46), the ligation of DNA segments to reconstruct functional genes in the new MAC (**m**acronuclear-**d**estined **s**equences, or MDSs) may require DNA unscrambling (45, 47, 48) [Fig. 3]. In *Oxytricha trifallax*, approximately 20% of the 18,400 non-redundant genes have MDSs that are scrambled in the MIC and all require decryption by various combinations of DNA translocations or inversions during genome remodeling (39). Inverted MDSs map to the reverse orientation on the opposite strand in the germline precursor locus in the MIC [Fig. 3].

IESs are commonly flanked by 2–20 base-pair regions of microhomology called “pointers”, short, precise matches at the 3' end of the nth MDS and the 5' end of (n+1)st MDS [Fig. 3] (47). Several lines of evidence (49, 50) suggest a strong mechanistic preference for recombination between pointers, with one copy of the pointer retained in the MAC DNA. Because they can be as short as two base-pairs (or possibly a one base-pair perfect repeat, considering mismatches (50)) (see “Epigenetic regulation of genome rearrangement in *Oxytricha*” below), pointers are not sufficient to guide MDS unscrambling on their own, though pointers that map between scrambled MDSs do contain more information and are typically longer than the nonscrambled pointers that form the junctions between consecutive MDSs (eleven versus five base-pairs, respectively (39)). Chromosome fragmentation and telomere addition together yield nanochromosomes that are much smaller than the archival MIC chromosomes. Even though *Tetrahymena* fragments DNA at a well conserved 15-nucleotide chromosome breakage site (Cbs) (51, 52), and *Euplotes crassus* uses a different motif (*Euplotes* Cbs, E-Cbs) (53) to fragment chromosomes and to regulate IES excision (54, 55), no such motif has yet been identified in *Oxytricha*. Telomerase, originally discovered in ciliates (56), adds *de novo* telomeres to the ends of the first and last MDS. Alternative chromosome fragmentation, or alternative addition of telomeres to different sites, can produce nanochromosomes of variable length and with different 5' or 3' ends. This sometimes generates nanochromosome isoforms with different coding gene contents (38, 57,

58). Approximately 10% of *Oxytricha* nanochromosomes are alternatively fragmented, and most of these cases display a weaker pyrimidine to purine transition that may be important for chromosome breakage or telomere addition (38, 39). Amplification of telomere-capped nanochromosomes produces an average macronuclear chromosome copy number of 1900 (36).

iii. Epigenetically programmed, small RNA-mediated genome rearrangement in Oligohymenophorea—In the late 1980's and early 1990's, several pioneering experiments in *Paramecium*, as well as *Tetrahymena*, demonstrated a powerful, epigenetic influence of the maternal soma on the offspring in the next sexual generation (59–66). More recent experiments, summarized in detail elsewhere (52, 67, 68; and see *Tetrahymena* and *Paramecium* chapters in this volume), led to a widely accepted epigenetic model which accounts for some aspects of non-Mendelian inheritance in *Tetrahymena* and *Paramecium*, particularly the regulation of DNA deletions by small noncoding RNAs. Briefly, in this "scan RNA" model, MIC transcripts are processed into small RNAs that range in size among Oligohymenophorea (~28 and 25 nucleotides in *Tetrahymena* and *Paramecium*, respectively). After scanning the parental macronuclear genome to subtract MDS sequences, the remaining scnRNAs mark MIC-limited regions in the anlagen for elimination by RNAi-related pathways (31, 69–72). Recent experiments indicate involvement of multiple, parallel sRNA pathways, including "iesRNAs" that exclusively target IESs in *Paramecium* (73). Together, these collections of sRNAs are reminiscent of the extensively studied maternal mRNAs that drive the early steps of development in *Drosophila* embryos (74). But unlike the maternal effects of mRNAs in *Drosophila* and the scnRNAs in Oligohymenophorea that mark DNA sequences for deletion, the parental generation in *Oxytricha* supplies the daughter exconjugants with an enormous set of both small RNAs that mark DNA sequences in the anlagen for *retention*, and long noncoding RNAs that provide the information to rebuild the soma.

B) Epigenetic regulation of genome rearrangement in *Oxytricha*

i. Role of small RNAs in genome rearrangement—*Oxytricha*, like the oligohymenophorean ciliates, produces a huge quantity of conjugation-specific small RNAs (sRNAs), so abundant that they can be seen on an ethidium bromide stained acrylamide gel without radiolabeling (32, 75). Total RNA extracts from early conjugating pairs indicate an overwhelming enrichment of 27-nucleotide long 5'U sRNAs, which are absent in vegetative cells. Deep-sequencing of these sRNAs demonstrated that they map bidirectionally to the entire MAC genome, including pointer recombination junctions that are only formed in the MAC, and that these sRNAs are depleted in MIC-limited segments (32, 75) [Fig. 4A]. This is opposite to the scnRNAs and iesRNAs in *Tetrahymena* and *Paramecium* where the sRNAs are ultimately specific for primarily MIC-limited segments and IESs (73, 76), and not MAC-destined DNA. This suggests that a sign change occurred earlier in the evolutionary history of these distant ciliate classes, separated by over one billion years of evolution (77). Furthermore, immunoprecipitation with an antibody specific to Otiwi1, the *Oxytricha* ortholog of a PIWI protein (78, 79), demonstrates an association between Otiwi1 and *Oxytricha*'s conjugation specific 27-nucleotide sRNAs. Hence, they are labeled Piwi-interacting RNAs, or piRNAs (32). Immunofluorescence staining suggests that Otiwi1 may

participate in the transportation of piRNAs from the maternal MAC to the anlagen during early genome differentiation, a step that is essential for exconjugant viability and programmed macronuclear development, since knockdown of Otiwi1 results in nonviable offspring.

The contrast is striking between the model for piRNA guided-genome rearrangement in *Oxytricha* and the scan RNA model in Oligohymenophorea, in which scnRNAs mark IESs via an RNAi-related pathway for elimination. The piRNAs in *Oxytricha* that originate from the maternal MAC must mark and possibly tile the hundreds of thousands of MDSs that are retained in the anlagen, leaving most of the MIC genome, including all MIC-limited sequences (e.g. IESs and transposons), unmarked. The nature of this mark is unknown, but potential epigenetic marks are heterochromatin formation through the methylation of DNA or histones (see “Epigenetic marks for retained vs. deleted sequences” below); however, the small size of the tiniest MDSs suggests that it would be difficult for the marks to be on nucleosomes. Whether directly, by a physical block, or indirectly, via epigenetic marks, piRNAs may ultimately prevent DNA excision within MDSs by antagonizing transposases, part of the proposed catalytic machinery responsible for DNA elimination (80) (see “Genome remodeling mechanisms and machinery” below).

As experimental proof of this model in *Oxytricha*, microinjection into conjugating cells of synthetic piRNAs complementary to IESs that are normally deleted in wild type cells leads to retention of the IES in the new mature MAC (32) [Fig. 4B]. Furthermore, the retention of the IES in the new MAC is stable across many asexual, as well as sexual, generations. In a backcross between the F1 cells from such an experiment and a wild-type strain, Fang *et al*. (32) specifically demonstrated the biological production of new piRNAs that target the retained IES (but that differ in sequence from the injected piRNA), supporting the epigenetic model in Fig. 4. Hence, even the transient availability of piRNAs during early development can influence heritable changes in the soma that transfer across multiple sexual generations, similar to the transgenerational epigenetic programming effect of piRNAs in *C. elegans* (81, 82).

The orthogonality between the two sRNA pathways within ciliates is less surprising when one considers the deep-rootedness of the ciliate lineage among eukaryotes, and that the evolutionary distance between *Oxytricha* and *Tetrahymena* is as divergent as humans and fungi [Fig. 1] (83). The opposite piRNA pathways also make economic sense for the respective organisms, because in all cases the piRNAs mark the minority class of the germline, either for deletion (33% in *Tetrahymena* (40)) or retention (5–10% in *Oxytricha*). Furthermore, because some *Oxytricha* IESs are smaller than 27 nucleotides, these would be problematic if sRNAs had to exclusively mark eliminated sequences, like the iesRNAs of *Paramecium* (73). Along the same lines, it is also notable that both MDSs and IESs in *Oxytricha* can be smaller than the length of one nucleosome unit, suggesting that epigenetic marks are more likely to occur directly on the DNA. Accordingly, cytosine methylation has been noted as one developmental mark for deletion in *Oxytricha* (84). Additional distinguishing features in the respective sRNA pathways may explain *Tetrahymena*’s tolerance for imprecise IES excision. For example, *Tetrahymena* might lack the RNA-guided DNA proofreading available in *Oxytricha* (85) (see “RNA-guided DNA repair in

Oxytricha and broader implications of RNA templating" below). As a result of its imprecision, IESs in *Tetrahymena* usually reside within introns or intergenic regions and only very rarely disrupt open reading frames (86, 87). Like *Oxytricha*, IES excision in *Paramecium* is mostly precise; however unlike *Oxytricha*, the ends of *Paramecium* IESs possess a highly conserved TA pointer and uniform sequence motifs at both IES ends (52). This may constrain them from possessing enough information to support gene unscrambling and explain the lack of scrambled genes in *Paramecium*. (*Tetrahymena*, on the other hand, has no published cases of scrambled genes, because its IESs are mostly intergenic, with the exception of approximately ten cases that interrupt exons (87).) Curiously, *Stylonychia*, another stichotrichous ciliate with a complex, scrambled germline genome like *Oxytricha* [Fig. 2], may discard close to 98% of its MIC DNA during MAC development (36). Yet an earlier paper suggested that its sRNAs are more consistent with the scan RNA model (88); however that inference was based only on hybridization, with no sequence analysis, and numerous other factors could have affected these early results.

ii. Long non-coding maternal RNA templates

a. MDS unscrambling: The scan RNA model that extends to *Tetrahymena* and *Paramecium* genome rearrangement would be insufficient to program the precise DNA segment reordering and occasional inversion of MDSs in *Oxytricha* [Fig. 3]. Additionally, piRNAs are not likely candidates for guiding DNA rearrangements either, since microinjection of synthetic piRNAs has been unable to reprogram MDS order or to template RNA-guided DNA proofreading (32). Finally, the pointers, themselves, do not contain enough information to unambiguously reorder and invert MDSs, because they can be as small as 2 base-pairs, which would occur often by chance (39).

This conclusion, in addition to the observation of a surprising number of errors at MDS-MDS junctions during early rearrangement (49), suggested a need for error correction, possibly via a DNA or RNA template. Prescott *et al*. (89) and later Angeleska *et al*. (90) proposed a specific epigenetic model for RNA-guided DNA recombination acting via maternal templates.

Nowacki *et al*. (85) offered several lines of evidence that RNA templates derived from the maternal macronucleus do guide assembly of the new macronuclear chromosomes [Fig. 5A, B]. Either RNAi against specific, endogenous RNA templates [Fig. 5C] or microinjection of new, alternative DNA or RNA templates [Fig. 5D] resulted in aberrant rearrangements in the progeny. RNAi to reduce the concentration of available templates in the cell stalled DNA rearrangement [Fig. 5C], whereas injection of foreign templates programmed new MDS patterns in the targeted gene [Fig. 5D], respectively. Presumably, the injection of DNA templates (complete telomere-containing synthetic nanochromosomes) permitted their transcription to RNA *in vivo*, to mimic the effect of injecting RNA directly. These experiments historically provided the first opportunity to parse the specific steps in this complex process and helped develop *Oxytricha* as a powerful model system to study programmed genome rearrangement. Moreover, as for piRNAs, the reprogramming effect of the foreign templates propagated over multiple sexual generations. These data strongly supported the epigenetic models for sequence-dependent comparison between two genomes

(the parental and developing MAC) within a single cell. More broadly, these experiments revealed a new mechanism for epigenetic wiring and rewiring of cellular programs, and an RNA-mediated pathway for transmission of an acquired epigenetic state [Fig. 6].

b. Chromosome copy number regulation: In addition to programming the DNA rearrangement pattern during genome differentiation, the maternal RNA templates described in the previous section also establish DNA copy number after conjugation through an unknown epigenetic mechanism. Gene dosage and DNA copy number are critical for many aspects of cell biology, including the developmental regulation of gene expression. Plus, increases in DNA copy number can contribute to acquisition of new function by gene or segment duplication (91). Furthermore, defects in the regulation of gene dosage may contribute to diseases, including possibly cancer (92, 93), autism (94) and disorders associated with trisomy (95). Classic examples for regulating gene expression through DNA dosage include the *chorion* locus in *Drosophila* (96), the circular rRNA genes in *Xenopus* (97) and the *Tetrahymena* rDNA locus, which forms differentially amplified palindromic segments in the MAC that originate from a single *rDNA* chromosomal copy during rearrangement (98, 99). Many other ciliate species, including the stichotrichous ciliates *Oxytricha* and *Stylonychia*, also have an overamplified rDNA chromosome. However, the more general relationship in *Oxytricha* between nanochromosome copy number and gene expression levels is surprisingly modest, probably constrained by the limited amount of copy number variation relative to gene expression variation (38).

Two groups demonstrated that *Oxytricha* and *Stylonychia* can regulate whole nanochromosome copy number based on the abundance of the corresponding maternal RNA templates during each round of sexual conjugation (100, 101). This discovery offered a novel biological role for long, noncoding RNAs. Both Nowacki *et al*. (100) and Heyse *et al*. (101) showed that changes in the available quantity of maternal RNA templates affects DNA copy number in the exconjugants following conjugation. Microinjection of two-telomere-containing RNA copies corresponding to different nanochromosomes resulted in an increase in the chromosome copy number in the sexual progeny, whereas RNAi against the endogenous templates for these chromosomes led to a decrease in nanochromosome copy number. The F2 progeny in the first experiment also displayed increased copy number, demonstrating once again that somatically acquired traits triggered by exposure to a noncoding RNA may propagate across sexual generations, and established this phenomenon in two groups of stichotrichous ciliates. Point substitutions that marked the synthetic RNA templates did not transfer to the exconjugants, confirming amplification of the endogenous chromosomes, rather than reverse transcription or copying of the injected templates. Two possible models for RNA-template-mediated regulation of DNA copy number could entail an influence of the level of maternal RNA templates on the timing and/or efficiency of either DNA rearrangement (100) or endoreplication after rearrangement (101), leading to a testable prediction that there will be concordant levels of amplification among the maternal and exconjugant nanochromosomes, as well as the maternal RNA templates.

iii. RNA-guided DNA repair in *Oxytricha* and broader implications of RNA templating—The role of the maternal RNA templates is not even limited to programmed

genome remodeling and regulation of gene dosage, but also takes on a third level in DNA repair. Nowacki *et al*. (85) also demonstrated the transfer of point mutations close to the recombination junctions, or pointers, from the RNA template to the developing MAC. This occurred despite the absence of corresponding DNA substitutions in the germline, confirming the epigenetic nature of this transmission. For example, a C-to-T substitution four base-pairs away from the end of an MDS transferred faithfully from the template, implicating RNA-directed DNA repair, as had previously been seen in yeast (102). The epigenetic transfer of the somatic point substitution was inherited across at least three sexual generations in the MAC, even in the absence of the corresponding mutation in the germline (Wang and Landweber, in prep), similar to the RNA-mediated epigenetic inheritance of the three observed phenomena of IES retention, alternative MDS order, and chromosome copy number. *Oxytricha* was the first organism to offer experimental proof of the transient existence of an RNA templated cache of genetic information passed across generations (85); however, the epigenetic inheritance of genomic variation through transiently available, and hence elusive, RNA-templates may be more widespread than previously thought (102). On an evolutionary time scale, such events might influence the pace of adaptation to changing environments by permitting the propagation of fitter somatic variants, while preserving the opportunity to revert to wild-type.

RNA-templated DNA repair may also have broader implications for diseases, such as cancer. Chromosomal rearrangements, as well as *trans*-spliced RNAs, can produce fusion proteins that facilitate cell growth, as discussed in the introduction to this chapter (103–106). An additional consequence of *de novo* chimeric RNA formation in the absence of a corresponding DNA translocation may be to facilitate chromosomal rearrangements, with the *trans*-spliced or chimeric RNA acting as a template, similar to the action of RNA template-guided DNA recombination in *Oxytricha* (85, 90).

iv. Epigenetic marks for retained vs. deleted sequences—The physical detection of piRNAs (see “Role of small RNAs in genome rearrangement” above) precedes the onset of genome rearrangement in *Oxytricha* (32). This apparent absence of piRNAs during later development suggests that piRNAs may mark the precursor MIC DNA segments for retention through various epigenetic modifications. These could be alterations to the chromatin configuration, analogous to the heterochromatic regions discarded during rearrangement in *Stylonychia* and *Euplotes* (107, 108). Heterochromatin formation is a hallmark of RNAi silencing and studies in yeast indicate that heterochromatin may form as a result of histone H3 lysine-9 (H3K9) methylation directed by complementary small interfering RNAs (109, 110). Similarly, in *Tetrahymena*, H3K9 methylation occurs prior to DNA elimination and targets IESs for excision (111). Furthermore, scnRNAs are required for IESs to be marked by histone methylation and DNA elimination is significantly reduced in the absence of such methylation (112, 113). This provides direct evidence for the role of histone methylation and heterochromatin formation in genome remodeling in *Tetrahymena*. However, *Oxytricha*’s orthogonally different piRNA pathway presents challenges, because instead, 1) piRNAs mark sequences for retention rather than deletion, and 2) both MDSs and IESs may be shorter than one nucleosomal unit (whereas the smallest IES in *Tetrahymena* is 194 base-pairs long (87)). No stable epigenetic mark has yet been discovered for *Oxytricha*

IESs so far. However, recent evidence suggests development specific H3 variants may influence the fate of genome segments during *Stylonychia* genome rearrangement. The acetylated form of one variant specifically appears in association with *Stylonychia* MDSs, while another variant's expression and deposition depends on the piRNA pathway (114).

DNA methylation offers another epigenetic modification that can lead to heterochromatin formation (115, 116). Similar to H3K9 methylation in *Tetrahymena*, cytosine methylation may lead to heterochromatin formation and induce DNA excision in stichotrichous ciliates (117). In *Stylonychia, de novo* cytosine methylation occurs on transposon-like elements during differentiation of the anlagen (118). Therefore, DNA methylation may be required for the programmed removal of such MIC-limited segments from the anlagen. Similarly, very extensive cytosine methylation and hydroxymethylation has been observed in *Oxytricha* and may play a role in genome rearrangement by marking DNA for elimination and later degradation (84). This study identified cytosine methylation and hydroxymethylation in MIC-limited transposons and satellite repeats, as well as aberrant DNA rearrangement products and old MAC nanochromosomes that are destined for degradation during the later stages of nuclear development. Another candidate modification could be adenine methylation that was reported to increase during *Tetrahymena* genome rearrangement (119, 120).

v. Genome remodeling mechanisms and machinery

a. *cis*-acting sequence features: In many ciliates, except *Tetrahymena* (86, 87), IESs frequently interrupt coding regions and hence must be precisely excised (121). *Paramecium* and *Euplotes crassus* IESs have highly conserved 5'-TA-3' dinucleotide repeats demarcating their IESs, and this is part of a larger, conserved inverted repeat (41, 52, 122). Following excision, one copy of the TA pointer is retained in the new MDS junction (121), as for the more diverse sequence pointers in *Oxytricha*. While the presence of *cis*-acting sequences and retention of a TA pointer are conserved properties between the very distantly-related ciliates *Paramecium* and *Euplotes*, their excision by-products containing the released IESs have drastically different structures. Longer *Paramecium* IESs (> 200 base-pairs) circularize post excision (123), retaining a single TA precisely at the circle junction, whereas in *Euplotes* the observed circular by-products of both IES excision and deletion of transposon-like Tec elements retain both copies of the TA pointer, separated by a 10 base-pair heteroduplex region containing sequence flanking the IES (52, 124, 125). This supports the convergent evolutionary acquisition of two different mechanisms for deleting DNA sequences that map between a TA-pointer. In the absence of any clear, conserved motifs flanking IESs in the stichotrichs *Oxytricha* and *Stylonychia*, the recruitment of longer and highly diverse direct repeats at MDS-MDS junctions may support the ability of this lineage to reorder complex scrambled genes, a phenomenon that appears absent from *Paramecium* and *Euplotes crassus*. Accordingly, the circularized excision by-products of many IESs in *Oxytricha* typically display imprecisely joined sequences at complex circular junctions (50) (also see below).

Intriguingly, the set of all two base-pair pointers in *Oxytricha* display a strong preference for the TA dinucleotide (39) similar to *Paramecium* and *Euplotes*, consistent with a similar but

parallel evolutionary origin and common mechanistic constraints acting on all three of these independently acquired DNA splicing mechanisms (126). Furthermore, the set of all three base pair pointers in *Oxytricha* is enriched in 5'-ANT-3' (39), which is the characteristic target site duplication of the transposon-like telomere-bearing elements (TBEs) in *Oxytricha* (127). This suggests that IESs of this kind could be relics of ancient transposons from this family that mutated beyond sequence recognition, but retained simple features in the pointers that conform to the requirements or preferences of the machinery that catalyzes their excision (52, 128) (see “Transposases” below). Alternatively, pointers may have evolved to mimic the ends of transposons to facilitate their excision by transposases.

Both *Oxytricha* IESs and TBE transposons (127) appear often to be excised as DNA circles (129, 50). However, unlike the circular IES by-products in *Paramecium* and *Euplotes, the Oxytricha* IES circle junction sequences are more often highly imprecise, in contrast to the accurately spliced junctions between MDSs in mature macronuclear nanochromosomes (50). The circular junctions usually form near one of the endogenous pointers, but do not necessarily retain one complete copy of the pointer at the circle junction. Circularization often favors nearby cryptic pointers, which are direct repeats in the vicinity of at least one of the real pointers at the correct MDS borders (50). While aberrantly spliced MDS junctions at cryptic pointers (49) may be repaired by maternal RNA templates to yield precise MDS junctions in the mature macronuclear molecules, the by-products of IES excision, on the other hand, would lack an RNA template for DNA repair, and this absent step can explain their observed sequence heterogeneity. Accordingly, the circularized IESs appear to be methylated, presumably as a tag for their destruction (50).

b. Transposases: The sequence similarity between the TA or ANT repeats flanking IESs and the preferred target sequences of ciliate transposons is compelling. The TA dinucleotide flanking *Paramecium* IESs, as well as flanking both IESs and Tec elements in *Euplotes crassus*, is strikingly similar to the termini of transposons in the Tcl/*mariner* superfamily ((130, 131) and reviewed in (126) and (132)). This similarity suggested a possible recruitment of transposases for DNA elimination during genome rearrangement in ciliates (128). Indeed in *Tetrahymena* (*Tetrahymena piggyBac*-like transposase 2, TPB2) (133) and *Paramecium* (PiggyMac, Pgm) (134), domesticated *piggyBac*-like transposases participate in DNA elimination by inducing DNA double stranded breaks (*piggyBac* transposons reviewed in (135, 126)). These *piggyBac* transposases with a DDD catalytic domain may localize to heterochromatin, possibly with the additional help of the *cis*-acting signals discussed earlier, to cleave DNA, leaving behind a four nucleotide overhang at the 5' end (136).

In contrast to the domesticated *piggyBac* transposase genes in the macronuclear genomes of oligohymenophorean ciliates, *Oxytricha* bears thousands of active transposase genes within the Tc1/*mariner* superfamily TBE transposons in the MIC, and the encoded transposase proteins are implicated in the DNA elimination and rearrangement pathway during nuclear development (80). The *Oxytricha* macronuclear genome assembly does contain a modest number of somatic genes with transposase-like domains (38), including Phage_integrase, DDE_Tnp_IS1595 and MULE Pfam domains. Proteins containing the latter two domains offer additional candidates for a functional role in *Oxytricha*’s genome remodeling, because

they are highly expressed in a development-specific manner, contain the DDE catalytic motif, and also lack the characteristic terminal inverted repeats, indicating possible domestication, similar to the *piggyBac* transposases (38). However, their expression is predominantly later than the TBE transposases, and this may implicate them in a different DNA elimination pathway. The possible participation of multiple catalysts in the excision machinery may also explain the lack of conserved motifs marking IESs for excision. It is possible that different transposases are responsible for excising different types of IESs, as part of the intricate orchestration of the process that eliminates most of the germline genome and then reorganizes the hundreds of thousands of pieces that remain.

Herrick *et al.*'s initial description of the MIC-limited TBE transposons (127) identified three closely related types which contain 17 base-pair terminal telomeric sequences (C4A4) flanked by 5'-ANT-3' direct repeats. Full-length TBE transposons actually encode three proteins, including a 57 kDa zinc finger kinase and a 22 kDa protein (129, 137–139), both of unknown function, and notably a transposase with a DDE catalytic motif (137). The transposase proteins in the three transposon types, TBE1, 2, and 3, share >83% similarity at the amino acid level and appear to be under purifying selection (80), which further supported the hypothesis that they may play a biological role in genome differentiation (139). More recently, RNAi against all three groups of *Oxytricha* TBE transposase genes, exclusively expressed during rearrangement, leads to the aberrant or stalled elimination of not only the TBE transposon itself, but also of IESs, as well as incorrect or incomplete MDS reordering (80). The knock down experiments show accumulation of high-molecular-weight DNA, but significantly, the mutant phenotype is only observed after knockdown of all three TBE transposase types, indicating that their encoded transposase proteins may act redundantly in the DNA elimination process, and the thousands of transposase genes may collectively encode a massive quantity of transposase protein needed for such a tremendous number of DNA elimination steps during development.

One pressing question regarding the evolution of this mutualistic relationship between ciliates and active transposons is why *Oxytricha* recruited an army of active, germline-limited transposases for DNA elimination, whereas in the distant clade of *Tetrahymena*/*Paramecium*, the excision machinery was domesticated. This emphasizes the likelihood of independent origins, although domestication may have been a later evolutionary step, after recruitment of active transposases. In the latter scenario, *Oxytricha* could represent a more primitive state prior to transposase domestication in other lineages (126).

c. Germline-limited genes: The TBE transposase genes are truly germline limited, playing their roles early in development and then "expiring" in the soma, but retained in the archival germline. With the exception of these transposase genes, there have been no other extensive studies of germline-limited genes, including the two other genes encoded in the TBE transposons, though both appear to be under purifying selection, suggesting that they also play a role (139, Schwartz and Landweber, unpublished). Until the discovery of active germline transposases, *Oxytricha*'s micronucleus had generally been considered transcriptionally-silent. However, the recent sequencing of *Oxytricha*'s germline micronuclear genome revealed a surprising protein coding content of over one thousand MIC-limited protein-coding genes, including additional genes encoding DDE_Tnp_IS1595

protein domains (39). All are expressed only during conjugation, according to RNA-sequence data (38), and proteomic surveys confirmed translation of some of these genes, so they are not just predicted gene products. Complete gene elimination may seem an unconventional, but absolute, way to abrogate gene expression for proteins that are only required during early genome remodeling, analogous to genome diminution and the programmed loss of germline-specific genes from the soma of *Ascaris* and Lamprey (28, 29). Such extreme gene expression regulation would certainly prevent the ectopic expression of genes with germline-specific roles that could otherwise contribute to uncontrolled cell growth (140), and hence may be important for prevention of disease states or the maintenance of genome integrity. The MIC-limited gene products in *Oxytricha* may provide important proteins required for nuclear differentiation, bridging the period of growth between destruction of the old MAC and nascent gene expression from the developing MAC. Future studies of *Oxytricha*'s rich set of germline-limited genes have the potential to reveal fundamental aspects of *Oxytricha*'s life-cycle and its division of labor between the two genomes, in addition to revealing more missing components of the excision apparatus that are no longer needed in vegetative cells and clandestinely, if not permanently, silenced by elimination, only to be resuscitated again by another round of sexual division.

CONCLUSION

Investigating genome remodeling in *Oxytricha* has uncovered many new and fundamental discoveries in RNA biology and explored the biological limits of DNA processing in a model eukaryote. Its extremely exaggerated genome plasticity and diversity of non-coding RNA pathways, plus unorthodox inheritance patterns owing to its sexual life cycle, together make *Oxytricha* a compelling model for studying all these phenomena that relate to genome integrity. Though many aspects of its biology seem, at first glance, to defy conventional genetics, it can also be viewed as an extended examination into the limits of genomic architecture and nucleic acid biodiversity on our planet.

Many unanswered questions remain, including the biogenesis of *Oxytricha* piRNAs and how they mark MDSs for retention. Furthermore, many of the components of the remodeling machinery have not yet been identified, including the source of proofreading from an RNA template. Similarly, the mechanism of gene expression regulation in *Oxytricha*, with such a tiny quantity of noncoding DNA in the macronucleus, is unknown. Future work should also examine the possible roles of other transposase-related genes in the macronucleus, as well as the two additional protein coding genes (zinc finger kinase and 22 kDa unknown protein) present on the TBE transposons, themselves. And finally, what are the roles of the thousand MIC-limited protein coding genes? The answers to these questions over the next several years will greatly improve our understanding of the genome dynamics of *Oxytricha* and relatives, near and far, including many eukaryotes that harbor more conventional twists on this creative RNA-regulated genome biology.

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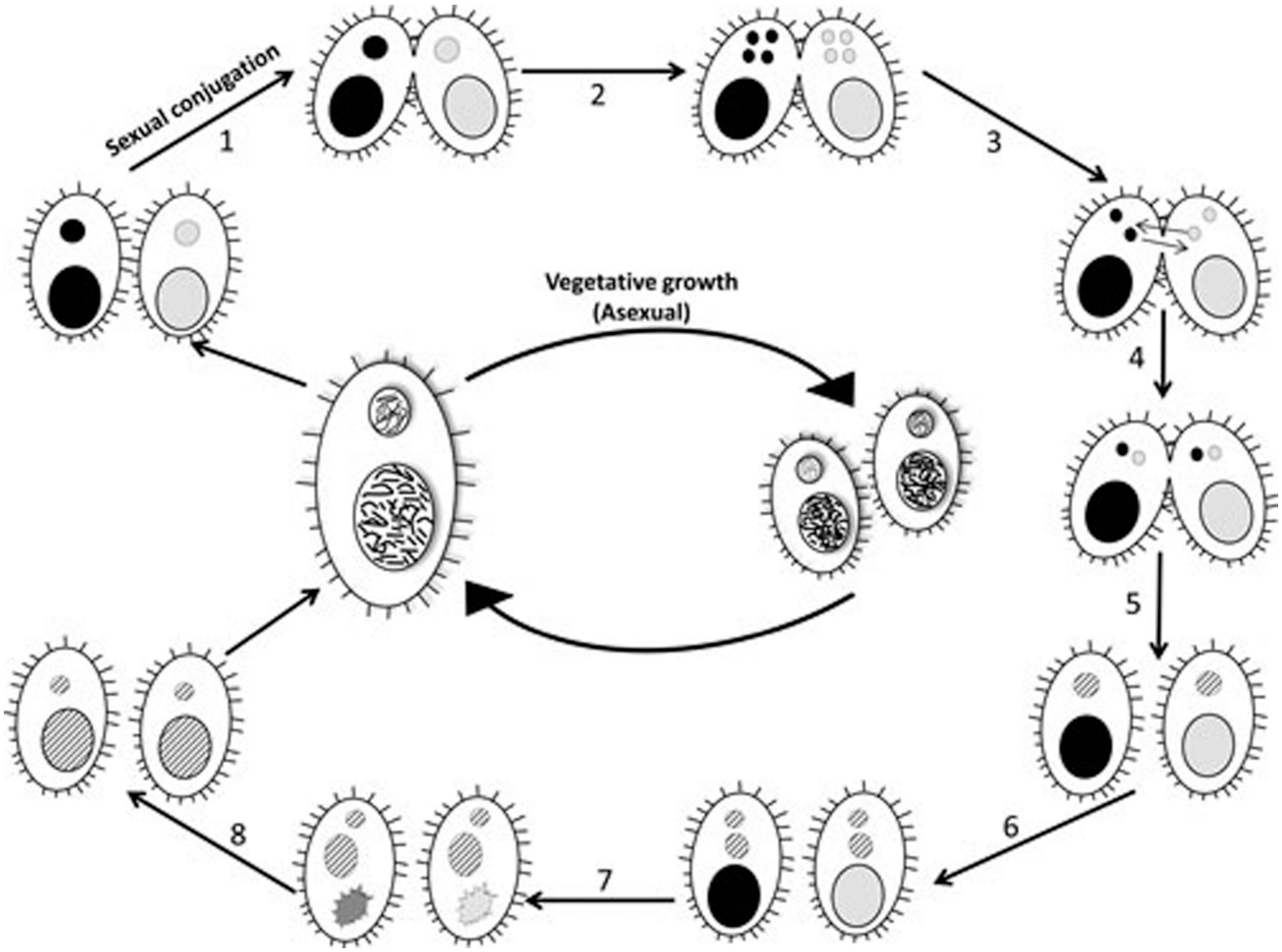


Figure 1.

Oxytricha vegetative and sexual life cycles. *Oxytricha* cells contain one somatic macronucleus and two identical, germline, micronuclei. For simplicity only one MIC is shown. *Oxytricha* divides asexually through binary fission producing clonal offspring. (1) Under starvation conditions, two cells of different mating type form pairs. (2) The diploid MIC undergoes meiosis and produces four haploid gametes. (3) Three of the haploid gametes degrade while the one remaining divides mitotically. (4) The pair exchange a copy each of these haploid micronuclei. (5) The haploid micronuclei fuse, producing a new, diploid, zygotic nucleus shown with cross hatching. This produces exconjugants with identical zygotic genomes. (6) The zygotic nucleus divides mitotically, producing two identical zygotic genomes. (7) One copy of the zygotic genome differentiates into a new soma, while the old maternal soma degrades. The developing MAC at this stage is called the “anlage” (plural anlagen). The other zygotic nucleus will maintain the new germline.

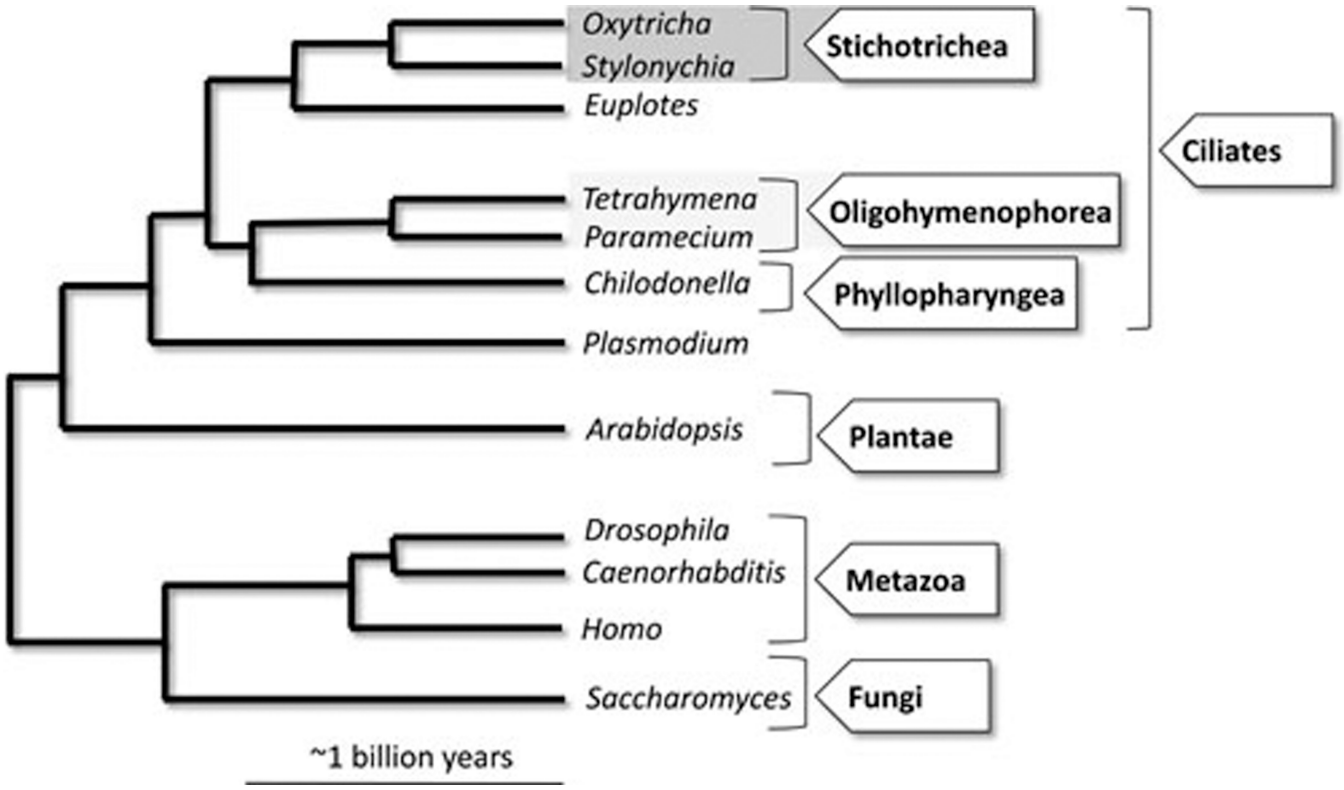


Figure 2.

The inferred evolutionary relationship of *Oxytricha* to other well-studied ciliate lineages and representative eukaryotic genera. Figure modified from Bracht *et al*. (77). Branch order, branch lengths and scale bar are based on estimates in Parfrey *et al*. (83) and Chang *et al*. (141).

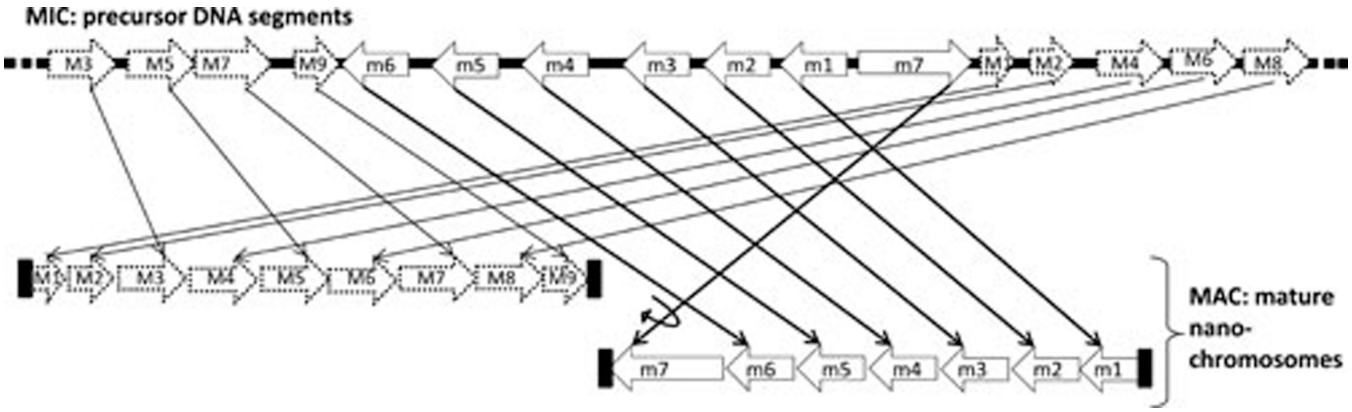


Figure 3.

Schematic illustration of gene unscrambling in stichotrichous ciliates. Black bars represent IESs to be eliminated. These IESs separate the MDSs (shown as white arrows) in the micronuclear precursor region. MDS labeled M1-9 and drawn with dotted lines are the precursors for one somatic nanochromosome, whereas MDSs drawn with solid lines (m1-7) assemble into a separate nanochromosome. The numbers represent the order in which the MDSs appear in the mature MAC nanochromosomes. Mature nanochromosomes in the MAC form following IES excision, MDSs descrambling and chromosome fragmentation. Addition of *de novo* telomeres (black tall rectangles) at the boundaries of the first and last MDS produces mature nanochromosomes.

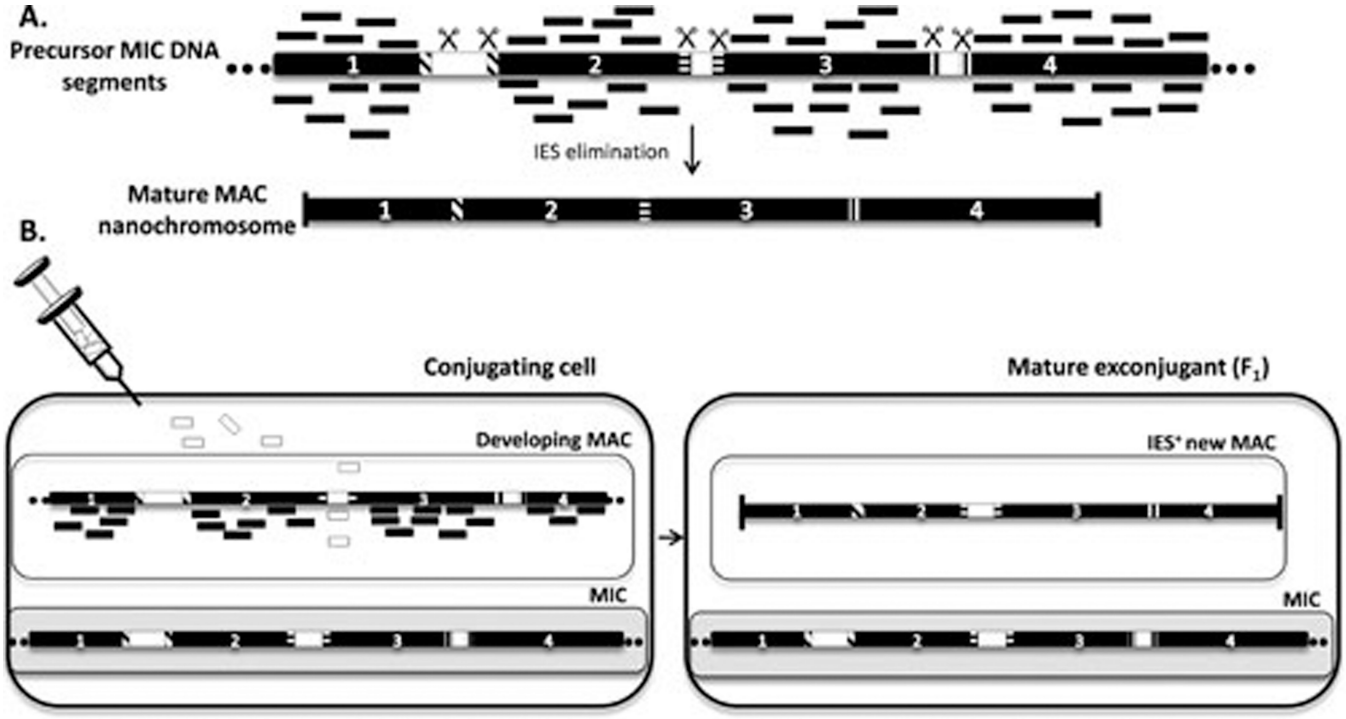
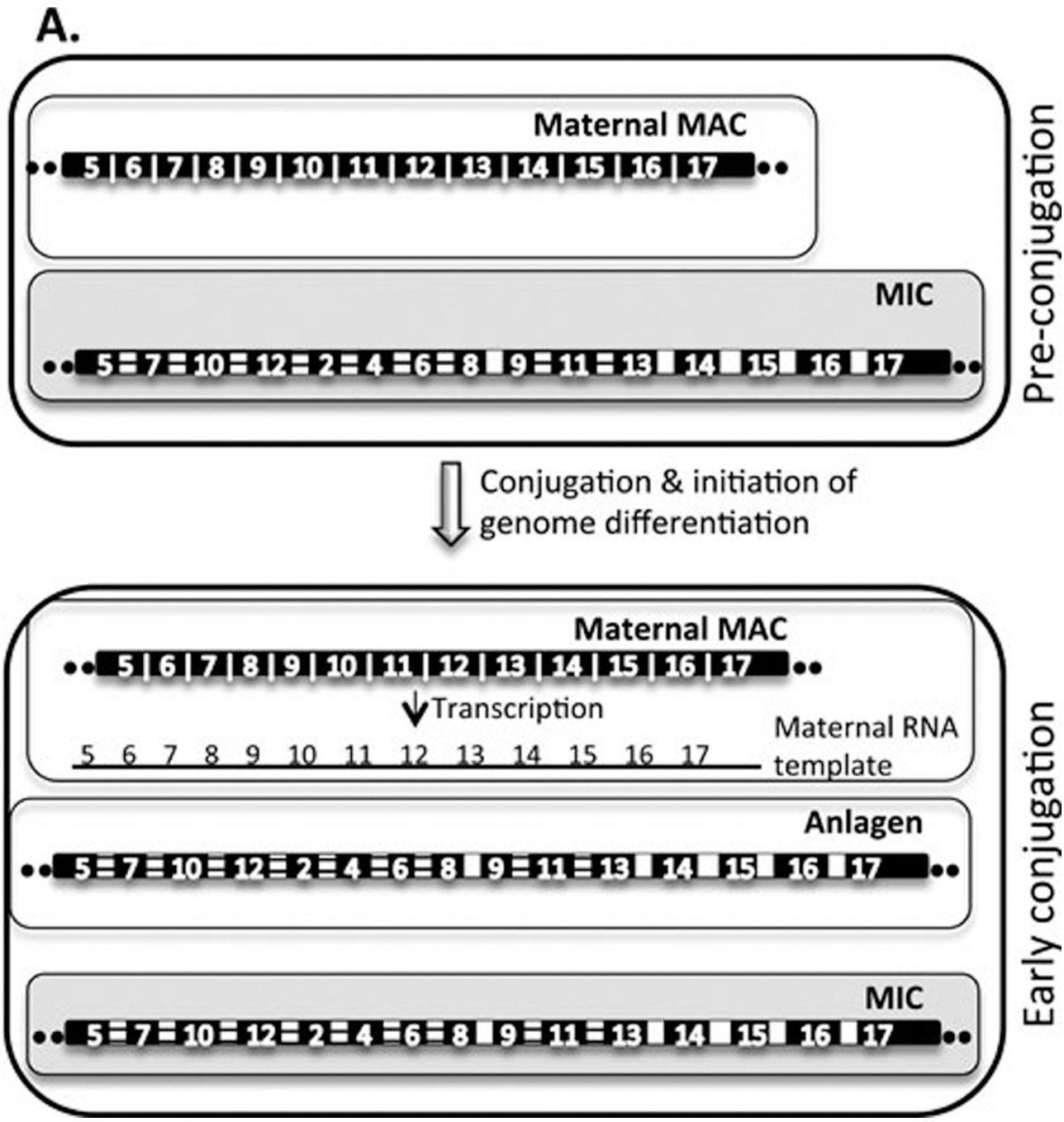
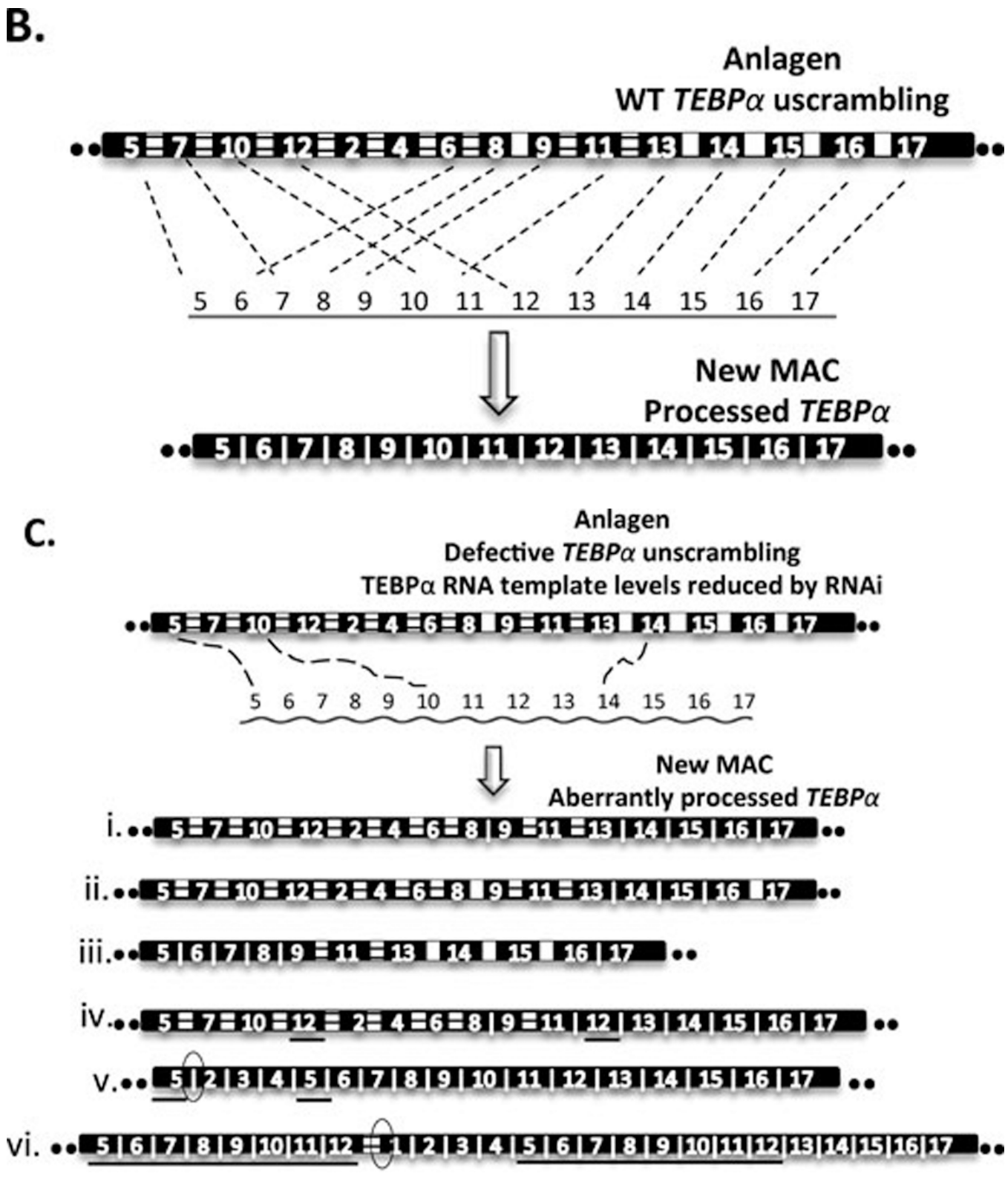


Figure 4.

Oxytricha piRNAs mark MDSs for retention during genome rearrangement. **A**. 27-nucleotide piRNAs (short black bars) derive from RNA copies of the maternal somatic genome. These piRNAs mark and protect MDSs for retention. DNA segments to be eliminated, such as IESs (shown as white rectangles between MDSs in the precursor DNA segment), lack corresponding piRNAs. Direct repeats (pointers) flanking the IESs are shown as small, patterned vertical rectangles. Identical pointers that join two consecutive MDSs are marked with the same pattern. The excision machinery (represented as scissors) can cleave the regions not protected by piRNAs and permit recombination between pointers. This leads to IES elimination, with retention of one copy of the pointer between MDSs in the mature nanochromosome. Dots at the ends of precursor loci in the micronucleus and developing macronucleus represent sequences that continue beyond the regions shown. Mature, macronuclear nanochromosomes terminate in telomeres (thin, vertical, black rectangles). **B**. Microinjection into conjugating cells of synthetic 27-nucleotide piRNAs (short white bars) complementary to IESs that are normally deleted in wild type cells, leads to retention of the IES in the mature MAC of the exconjugants and subsequent sexual offspring (32). This creates IES+ strains.





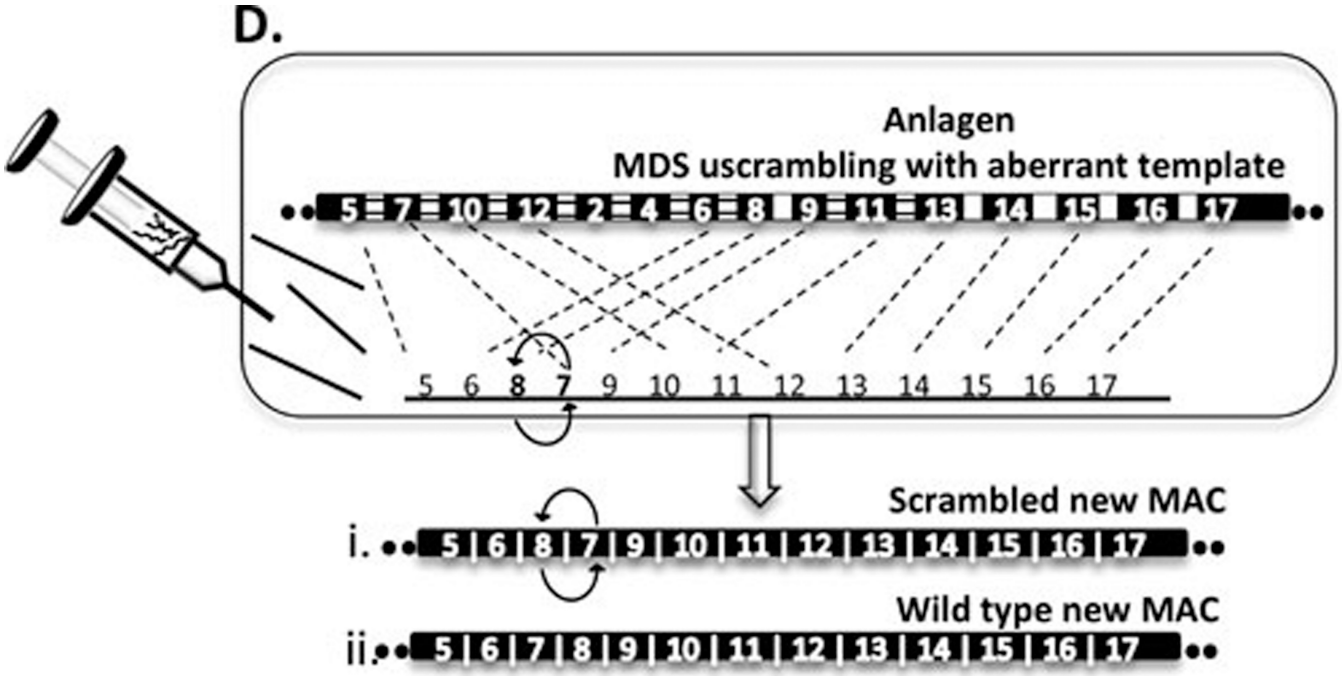


Figure 5.

Genome differentiation in *Oxytricha* is mediated by long non-coding maternal RNA templates. **A**. Nowacki *et al*. (85) demonstrated the transient presence of two-telomere-containing, bidirectional RNA transcripts of maternal nanochromosomes in conjugating cells. Panel A shows part of the *O. trifallax* TEBPα (Telomere End Binding Protein α) locus between segments 5 and 17 and the corresponding region of the RNA template (long thin lines). **B**. These templates may guide rearrangement in the anlagen by interacting with the MIC precursor DNA. Numbered black rectangles represent MDSs in the linear MAC order. White rectangles indicate nonscrambled IESs (interrupting consecutive MDSs in the MAC) and striped rectangles are scrambled IESs that map between nonconsecutive MDSs. **C**. RNAi targeting the maternal *TEBP*α RNA templates (wavy line) leads to gross rearrangement defects in the corresponding gene (85). A PCR assay of the region between MDS 5 to 17 revealed the presence of molecules that are longer than wild type MAC sequences because they often retain unspliced IESs, with a strong bias towards retaining IESs between scrambled segments (examples i, ii, iv). Consistent with studies of earlier timepoints in the rearrangement cascade (49), example (ii) only eliminated a subset of the nonscrambled IESs in this region, and examples (i), (iv), (v) and (vi) eliminated *all* nonscrambled IESs. In addition, cases (i) and (ii) stalled before any reordering. The presumed decrease in template abundance also leads to accumulation of partially unscrambled molecules, such as (iii), (iv) and (v). Case (iv) correctly repaired MDS 12 between MDS 11 and 13, but with an unexpected duplication of MDS 12, retaining a copy in its scrambled location as well. The duplications in (iv) and (vi) indicate that intermolecular DNA rearrangement may be tolerated. Aberrantly spliced junctions, marked by thin vertical ovals, occur at cryptic repeats, instead of endogenous pointers. **D**. Microinjection of synthetic *TEBP*α templates with the order of segments 7 and 8 transposed (indicated with curly arrows) produces new, scrambled MAC nanochromosomes with the

reprogrammed order. Wild type, unscrambled nanochromosomes coexist in the cell with the permuted chromosomes, because the endogenous wild type templates were not destroyed in this experiment (85).

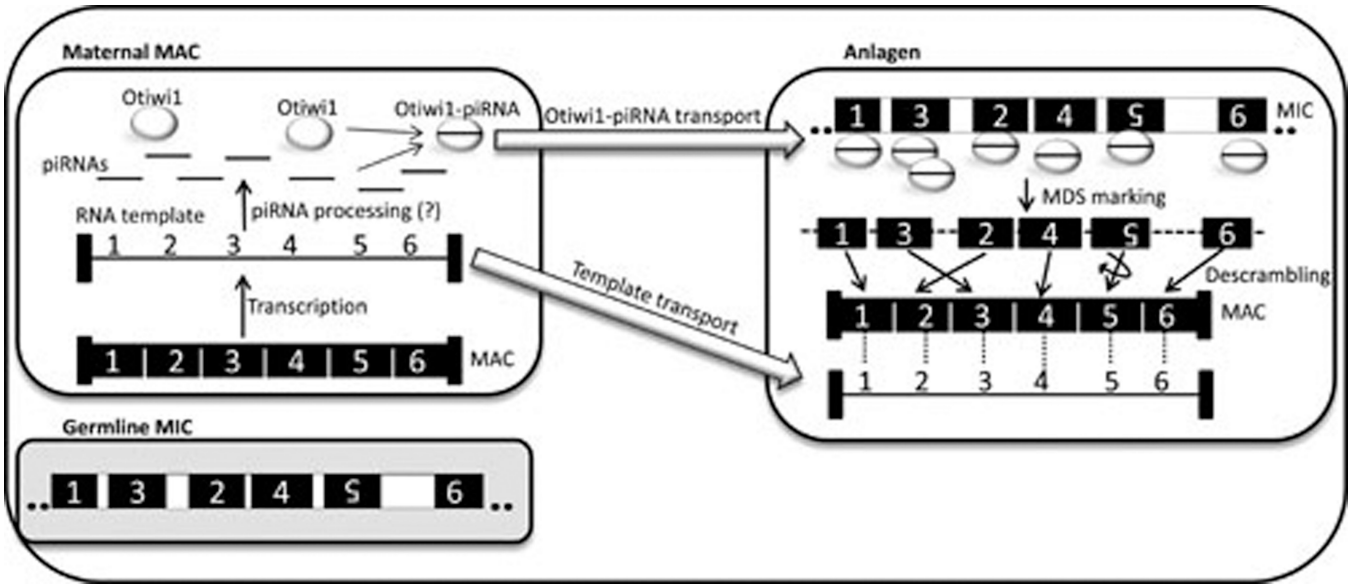


Figure 6.

A model for non-coding RNA mediated genome rearrangement in *Oxytricha*. Figure modified from Bracht *et al*. (77). The bidirectional transcription of the maternal MAC genome produces whole-chromosome transcripts which includes telomeres (long thin line flanked by black vertical rectangles). Either this maternal RNA template or other MAC transcripts are processed into 27-nucleotide piRNAs (short line segments) that interact with the *Oxytricha* Piwi protein, Otiwi1 (drawn as white ovals). Otiwi1 transports the piRNAs to the anlagen, in conjunction with the template RNAs. Here, piRNAs interact with the MIC precursor DNA to differentiate MDSs (black boxes) from IESs (white intervening rectangles). The template RNA then guides MDS descrambling, which, together with chromosome fragmentation and *de novo* telomere addition, forms mature MAC nanochromosomes.