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Programmed DNA elimination in *Tetrahymena*: a small RNA-mediated genome surveillance mechanism

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Abstract

RNA interference (RNAi) was initially discovered as a post-transcriptional gene silencing mechanism in which short RNAs are used to target complementary RNAs for degradation. During the past several years, it has been demonstrated that RNAi-related processes are also involved in transcriptional gene silencing by directing formation of heterochromatin. The dynamic DNA rearrangement during sexual reproduction of the ciliated protozoan *Tetrahymena* provides an extreme example of RNAi-directed heterochromatin formation. In this process, small RNAs of ~28-29 nt, which are processed by the Dicer-like protein Dcl1p and are associated with the Argonaute family protein Twi1p, induce heterochromatin formation at complementary genomic sequences by recruiting the histone H3 lysine 9/27 methyltransferase Ez11p and chromodomain proteins. Eventually these heterochromatinized regions are targeted for DNA elimination. In many eukaryotes, one of the major roles for RNAi-related mechanisms is silencing transposons, and DNA elimination in *Tetrahymena* is also believed to have evolved as a transposon defense by removing transposon-related sequences from the somatic genome. Because DNA elimination is achieved by the coordinated actions of non-coding RNA transcription, RNA processing, RNA transport, RNA-RNA and RNA-protein interactions, RNA degradation and RNA-directed chromatin modifications, DNA elimination in *Tetrahymena* is a useful model to study 'RNA infrastructure'.

Keywords

DNA elimination; *Tetrahymena*; ciliate; RNAi; small RNA; siRNA; piRNA; CRISPR; non-coding RNA; epigenetics; heterochromatin; transcriptional silencing; transposon silencing; Argonaute; Piwi; Dicer

Introduction

Transposable elements and viruses are genomic 'parasites' that are capable of moving between cells and from one genomic position to another. It is imperative that they should be inactivated because they are otherwise potentially detrimental to genome stability. Therefore host cells need to distinguish these molecular parasites from their own genomes to selectively silence the former.

Prokaryotic bacteria and archaea employ a restriction-modification (R/M) system, which is composed of restriction enzymes and modification methylases, for selective digestion of non-modified invading DNAs. This system serves as a defense against invading viruses (aka bacteriophages).¹ Recently, the CRISPR (Clustered Regularly Interspaced Short

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Palindromic Repeat) system, another bacteriophage defense mechanism, has been discovered (Fig. 1A).²⁻⁴ CRISPR loci consist of multiple short nucleotide repeats (typically 25-40 nt) separated by similarly-sized unique spacer sequences that are homologous to bacteriophage genomes and plasmid sequences. The entire CRISPR loci are transcribed as primary transcripts containing a full set of repeats and spacer sequences. The transcripts are processed into small RNAs (~25-40 nt) by a set of Cas (CRISPR-associated) proteins encoded adjacent to the CRISPR clusters. Each small RNA includes a unique spacer sequence. The small RNA associates with an effector Cas protein complex and directly promotes degradation of bacteriophage DNA as well as possibly the RNA product that is complementary to the small RNA.⁵⁻⁷ In this system, the unique spacer sequences serve as a catalog of bacteriophages to be silenced. Moreover, new spacer sequences are acquired from novel phage genomes.⁸ Therefore, the RNA-based CRISPR system provides a heritable acquired immunity against bacteriophages.

Eukaryotes also utilize small RNAs for gene silencing. However, the mechanism of small RNA production is most likely evolutionarily unrelated to that employed by the CRISPR system.⁴ In eukaryotes, RNAi-related pathways are responsible for generating and employing small RNAs. RNAi was first discovered as a post-transcriptional gene silencing mechanism in which double-stranded RNAs trigger degradation of complementary mRNAs.⁹ In this pathway, double-stranded RNA is processed by a Dicer enzyme into small (20-30 nt) RNAs that subsequently form effector complexes with Argonaute proteins, which induce small RNA-directed RNA degradation.¹⁰ An RNAi-related mechanism can also induce transcriptional gene silencing at the chromatin level in organisms ranging from unicellular eukaryotes to plants and humans.¹¹ Although RNAi has intrigued biologists as a useful genetic tool, it may have evolved as a guardian of cellular genomic DNA from molecular parasites.¹²

The RNAi-related mechanism plays important roles in the defense against viruses.¹³ Some viruses possess a double-stranded (ds) RNA genome, and single-stranded RNA viruses also have a dsRNA phase of their replication cycles. These dsRNAs are recognized by the RNAi machinery and processed into small RNAs, which direct degradation of viral genomes and their transcripts. RNAi-related pathways also act in silencing repeated elements (Fig. 1B). Because of the ability to amplify their own genomes, successfully integrated retrotransposons such as LINEs and SINEs are repeated in their host genome. Read-through transcripts from adjacent genes result in an accumulation of dsRNAs for these elements.¹⁴ These dsRNAs are processed to small RNAs by RNAi-related pathways, which then induce degradation of transcripts from molecular parasites as well as transcriptional silencing through alteration of chromatin structure. By these mechanisms, any dsRNA is recognized as a signature of invaders.

Recent studies in mammals and flies have revealed that these metazoans also silence transposons with small RNAs derived from loci concentrated in degenerated transposon sequences (Fig. 1C).^{15, 16} These loci, called piRNA clusters, produce single-stranded RNA that is processed to small Piwi-associated (pi) RNAs, probably by sequential actions of endoribonucleolytic activities of two Piwi proteins.^{16, 17} Argonaute proteins of the Piwi subfamily associate with piRNAs and induce silencing of transposons complementary to piRNAs at the post-transcriptional and transcriptional levels. Although this is reminiscent of the CRISPR system in prokaryotes, the machinery employed in piRNA-mediated transposon silencing in metazoans differs from that in the prokaryotic system.

The ciliated protozoan *Tetrahymena thermophila* employs a unique strategy to silence transposable elements, utilizing an evolutionarily conserved RNAi-related mechanism (Fig. 1D). Its nuclear dimorphism allows *Tetrahymena* to identify transposable elements by

comparing the whole genome of vegetative and germline nuclei and to completely eliminate the transposons from the transcriptionally active vegetative nucleus. Small RNAs produced in the germline nucleus are selected for transposable elements specific to the vegetative nucleus; these RNAs then induce heterochromatin formation followed by DNA elimination in the newly developing vegetative nucleus. This small RNA-directed programmed DNA elimination is orchestrated by amazingly complex RNA infrastructure¹⁸, which is composed of spatiotemporally regulated processes including non-coding RNA transcription, RNA processing, RNA transport, RNA-RNA and RNA-protein interactions, RNA degradation and RNA-directed heterochromatin formation. This chapter reviews roles of these processes in DNA elimination in *Tetrahymena* and discusses evolutionary relationship between RNA-directed DNA elimination and transposon-silencing mechanisms in other eukaryotes.

Nuclear dimorphism in *Tetrahymena*

Tetrahymena thermophila is a ciliated protozoan, a free-living unicellular eukaryote. *Tetrahymena* and most other ciliates have two structurally and functionally different nuclei within a single cell: a diploid micronucleus and a polyploid macronucleus (Fig. 2). The micronucleus is mostly inert transcriptionally (an exception to this generalization is described below), but it has the ability to make both the macro- and micronuclei during sexual reproduction. In contrast, the macronucleus is responsible for all gene expression but is degraded after sexual reproduction. Elimination of 'junk' DNA (see below) and an ~50-fold endoreplication of its chromosomes make the macronucleus a highly specialized machine for gene expression. This division of labor between micro- and macronuclei is reminiscent of the germline-soma separation in metazoans.¹⁹

When enough nutrients are available, *Tetrahymena* vegetatively (asexually) proliferates by binary fission, and the diploid micronucleus divides mitotically while the polyploid macronucleus divides by amitosis. In the absence of plentiful nutrients, *Tetrahymena* undergoes the sexually reproductive process of conjugation (Fig. 3). During conjugation, two cells of complementary mating types partially fuse to make a pair and their micronuclei undergo meiosis (Fig. 3B). One of the four meiotic products in each cell divides once mitotically to produce two haploid pronuclei while the other three meiotic products are degraded (Fig. 3C, D). The paired cells exchange one of their pronuclei, then the stationary and exchanged pronuclei fuse to make a zygotic nucleus (Fig. 3E, F). The zygotic nucleus divides twice more. In the second post-zygotic nuclear division, the spindles are parallel to the anteroposterior axis of the cells, and the two daughter nuclei in the anterior cytoplasm differentiate into macronuclei while the other two in the posterior cytoplasm become micronuclei (Fig. 3G, H). In parallel, the parental macronucleus is eliminated by an apoptosis-like process (Fig. 3H).

The micronucleus possesses five chromosomes per haploid set. During differentiation of the new macronucleus, these germline chromosomes are fragmented into approximately 250 pieces by chromosome breakage, which is coupled to the addition of new telomeres.²⁰⁻²² Moreover, 15-20 Mbp (~15%) of DNA in the micronuclear genome is removed from the new macronuclear chromosomes (Fig. 4).²³ DNA destined to be eliminated is called an Internal Eliminated Sequence (IES). IESs are ~0.5-20 kb in size and it has been estimated that there are approximately 6000 IES loci in the micronuclear genome. IESs have not been found in gene coding sequences in *Tetrahymena*, although some are located in introns. The majority of known IESs are transposon-like repetitive DNAs and other repeated sequences, which are often categorized as 'junk' DNA in eukaryotic genomes.²⁴⁻²⁶ Elimination of IESs from the somatic macronucleus is thought to be essential for cell viability, because all known mutant strains with defective DNA elimination produce non-viable progeny.²⁷⁻³⁶

This may be because transposons in IESs are activated in the macronucleus and compromise essential cellular functions.

Epigenetic regulation of DNA elimination by small RNAs

IES elimination in *Tetrahymena* occurs very precisely such that all given IESs are eliminated in each sexual reproductive event, and their boundaries are conserved to within several base pairs (Fig. 5A). However, no common sequence signature that might support precise IES elimination has been identified in or around IESs. Chalker and Yao³⁷ demonstrated that IES elimination in *Tetrahymena* is epigenetically controlled by a mechanism that prevents sequences within the parental macronucleus from being eliminated in the newly developing macronucleus. They first introduced an ectopic IES element, which is normally absent from the mature macronucleus, into the macronuclear genome of the parental strains. Conjugation was then induced using these IES-introduced parental strains, and DNA elimination in their progeny was observed. They found that endogenous IES elements complementary to the artificially introduced IESs failed to be eliminated from the new macronuclear genome (Fig. 5B). Conversely, it has also been reported that a transgene introduced only into the micronucleus of the parental strain is deleted from the developing new macronucleus irrespective of where the transgene is inserted (Fig. 5C).^{38, 39}

These observations indicate that the new macronucleus epigenetically inherits the pattern of DNA elimination from the parental macronucleus. In other words, *Tetrahymena* can compare micronuclear and parental macronuclear DNA sequences to remove micronuclear-specific DNAs (i.e., IESs) from the newly developing macronucleus. Because there are ~6000 different IESs, it had been expected that DNA or RNA molecules, but not sequence-specific DNA binding proteins, would be used to identify IESs for epigenetic inheritance.²⁶

The requirement for the Argonaute protein Twi1p in DNA elimination and accumulation of conjugation-specific small (~28-29 nt) RNAs immediately suggested that these small RNAs, produced by an RNAi-related mechanism, direct DNA elimination.³² Indeed, later studies have revealed that these small RNAs (named scan RNAs or scnRNAs) are produced by the Dicer-like protein Dcl1p, which is also essential for DNA elimination,^{31, 33} and form a complex with the Argonaute protein Twi1p.⁴⁰ Moreover, it has been demonstrated that injection of dsRNA into conjugating cells can induce ectopic elimination of DNA that is complementary to the injected RNA.³⁹ Injected dsRNAs are probably processed to small RNAs which then induce IESs elimination. These results strongly argue that scnRNAs are the primary factors that determine which sequences will be eliminated. In the following sections, we describe our current understandings about how scnRNAs epigenetically direct DNA elimination event.

Biogenesis of scnRNA

It has been known for several decades that transcription in the micronucleus can be detected only at early stages of conjugation during prophase meiosis.^{41, 42} The micronuclear transcripts are ~0.2-1.0 kb in length and are produced from both strands of the genome (Early Stage in Fig. 6).⁴³ Although the exact mechanism that produces these transcripts is not clear, temporal micronuclear localization of RNA polymerase II (RNAPII) during prophase meiosis⁴⁴ indicates that they are transcribed by RNAPII. Knockout strains for *DCL1* do not produce scnRNA and result in over-accumulation of micronuclear RNA,^{31, 33} indicating that micronuclear transcripts are precursors of scnRNAs. Since all IESs and macronuclear-destined sequences examined so far are transcribed during meiotic prophase, it was proposed^{27, 43} that the entire micronuclear genome is transcribed in this stage. However, a large-scale genome-wide analysis of micronuclear transcripts will be necessary to confirm this assumption.

The scnRNAs produced by Dcl1p in the micronucleus are thought to be transported to the cytoplasm where they form a complex with the Argonaute protein Twi1p (Early Stage in Fig. 6)⁴⁰, since Dcl1p exclusively localizes to the micronucleus while Twi1p first appears in the cytoplasm.³¹⁻³³ In the absence of Twi1p, scnRNAs disappear rapidly.⁴⁰ Therefore, complex formation between Twi1p and scnRNAs must stabilize scnRNAs. The mechanisms by which scnRNAs are exported to the cytoplasm and loaded into Twi1p are not yet well understood.

Although the scnRNAs processed by Dcl1p are double-stranded, one of the strands (the passenger strand) is removed from the scnRNA-Twi1p complex.³⁵ Like other Argonaute proteins,⁴⁵⁻⁵⁰ Twi1p possesses endoribonuclease (Slicer) activity that cleaves the passenger strand of scnRNA. This endoribonucleolytic cleavage is necessary to remove the passenger strand from the complex and to eliminate DNA.³⁵

It has been shown that scnRNAs are modified by 2'-*O*-methylation at their 3' ends (Mid Stage in Fig. 6).⁵¹ The RNA methyltransferase Hen1p interacts with Twi1p and is responsible for modification and stable accumulation of scnRNAs.⁵¹ Therefore, 2'-*O*-methylation at their 3' ends probably protects scnRNAs from exonuclease attack. Since *HEN1* knockout strains show a partial defect in DNA elimination, stabilization of scnRNAs is important to induce proper DNA elimination.⁵¹ Hen1p modifies only single-stranded scnRNAs, both *in vitro* and *in vivo*,⁵¹ indicating that Hen1p modifies scnRNA after the passenger strand of scnRNA has been removed from the scnRNA-Twi1p complex.

scnRNA selection

Twi1p first localizes to the cytoplasm in early conjugation and is then exclusively detected in the parental macronucleus at mid-stages of conjugation.³² In *DCL1* KO strains, which lack scnRNAs, and in Slicer-defective *TWI1* mutant strains, where Twi1p cannot remove the passenger strand of scnRNA, Twi1p does not localize to the parental macronucleus and remains in the cytoplasm.³⁵ According to these observations, it has been suggested that formation of the scnRNA-Twi1p complex and removal of the passenger strand of scnRNA occur in the cytoplasm (Early Stage in Fig. 6). In the late stages of conjugation, the scnRNA-Twi1p complex translocates from the parental to the new macronucleus where it induces DNA elimination (Late Stage in Fig. 6).

DNA elimination occurs in the new macronucleus. Why then does the scnRNA-Twi1p complex localize first to the parental macronucleus? A key experiment was conducted by Mochizuki and Gorovsky.⁴⁰ scnRNAs extracted at different stages of conjugation were radiolabeled and hybridized to genomic DNAs extracted from isolated macro- and micronuclei on a Southern blot. scnRNAs extracted from cells at early stages of conjugation, when Twi1p was predominantly in the cytoplasm, hybridized to both macro- and micronuclear DNA. This is consistent with the prediction that the entire micronuclear genome is transcribed to produce scnRNAs. In contrast, scnRNAs complementary to micronuclear-restricted DNA were gradually enriched during mid-stages of conjugation when the scnRNA-Twi1p was localized to the parental macronucleus, and those extracted from late stages of conjugation, when Twi1p localizes to the new macronucleus, preferentially hybridized to micronuclear DNAs. These observations indicated that, although scnRNAs complementary to both macronuclear-destined sequences and IESs were produced, those complementary to IESs are specifically selected in the parental macronucleus. Thus, the scnRNA-Twi1p complex must localize first to the parental macronucleus in order to interact with the macronuclear chromosome to induce scnRNA selection (Mid Stage in Fig. 6).

The exact molecular mechanism for this scnRNA selection process remains unknown; however, two possible mechanisms have been proposed. The first is the selective degradation of scnRNAs complementary to macronuclear sequences. Since scnRNA is highly unstable in the absence of Twi1p,^{32, 40} selective degradation of scnRNAs can be achieved if interaction of scnRNA to the macronuclear genome induces dissociation of the scnRNA-Twi1p complex. An alternative possibility is the selective amplification of scnRNAs complementary to the micronuclear-limited sequence. In the yeast *Schizosaccharomyces pombe* and the plant *Arabidopsis*, siRNAs are amplified by an RNA-dependent RNA polymerase complex, and in the fly *Drosophila* and mouse germlines, piRNAs are amplified by 'ping-pong' cycles mediated by Argonaute proteins.⁵² Therefore, small RNA amplification mechanisms are widespread among eukaryotes, and a system to selectively amplify scnRNAs that are complementary to IESs may exist in *Tetrahymena*.

Whatever the molecular mechanism for scnRNA selection, scnRNAs complementary to the macronuclear genome must interact by base pairing either directly to macronuclear DNA, or indirectly to RNA transcribed from the parental macronucleus. Our recent study supports the latter possibility (Mid Stage in Fig. 6). ncRNAs transcribed from the parental macronucleus can be co-immunoprecipitated with Twi1p²⁷ and the interaction between Twi1p and chromatin is RNA-dependent (KM, unpublished results). Moreover, the RNA helicase Ema1p has been identified as an essential factor for the Twi1p-ncRNA interaction, the Twi1p-chromatin interaction, scnRNA selection and DNA elimination.²⁷ All of these results suggest that the scnRNA-Twi1p complex interacts with macronuclear chromatin via base-pairing between scnRNA and nascent macronuclear non-coding transcripts, and that this interaction either induces degradation of scnRNA or inhibits scnRNA amplification. Ema1p possibly modulates the structures of nascent macronuclear non-coding transcripts to facilitate interactions of the scnRNA-Twi1p complex with chromatin.

scnRNA-induced heterochromatin formation and DNA elimination

When the new macronucleus forms, the scnRNA-Twi1p complex together with Ema1p translocates from the parental macronucleus to the new macronucleus (Late Stage in Fig. 6).^{27, 32} Like scnRNA selection in the parental macronucleus, it has been demonstrated that ncRNAs transcribed from the new macronucleus can be co-immunoprecipitated with Twi1p²⁷ and Twi1p interacts with new macronuclear chromatin in an RNA-dependent manner (KM, unpublished results). Disruption of Ema1p abolishes this interaction and hinders DNA elimination.²⁷ These observations strongly suggest that the interaction between scnRNA and chromatin via nascent non-coding transcripts is required for targeting DNA for elimination in the newly developing macronucleus.

Methylation of histone H3 at lysines 9 (H3K9me) and 27 (H3K27me) occurs in the newly developing macronucleus.^{30, 53} These modifications are the hallmarks of heterochromatin in diverse eukaryotes from plants, yeasts to humans. Chromatin immunoprecipitation analyses using anti-H3K9me and H3K27me antibodies reveal that these methylated histones accumulate on IESs but not on macronuclear-destined regions.^{30, 53} Disruption of the histone methyltransferase Ez1p, which catalyzes methylation of H3K9 and H3K27 in *Tetrahymena*, as well as amino acid substitutions at H3K9 or H3K27, inhibit DNA elimination.^{30, 54} Therefore, these histone H3 modifications are required for DNA elimination. H3K9me and H3K27me bind to the chromodomain protein Pdd1p.^{30, 53} Pdd1p is also localized to heterochromatin of the newly developing macronucleus and is essential for DNA elimination.²⁹ Elimination of parental *PDD1* expression greatly reduces H3K9me level,⁵³ indicating that there may be a positive amplification loop through which association of Pdd1p with H3K9/27me recruits Ez1p to induce greater accumulation of H3K9/27me and Pdd1p. All of these observations strongly suggest that IES elimination is mediated by

heterochromatin formation, which includes histone H3 modification (H3K9/27me) and accumulation of the chromodomain protein Pdd1p (Late Stage in Fig. 6). Since disruption of the Dicer gene *DCL1* and the Argonaute gene *TW1* greatly inhibit H3K9me accumulation or its IES targeting^{31, 33, 54} the RNAi-related pathway is upstream of heterochromatin formation. On the other hand, Ezl1p and Pdd1p are not required for scnRNA accumulation.³⁰ There may be no feedback mechanism that heterochromatin regulates the RNAi-related pathway in *Tetrahymena* although such mechanism has been reported in the fission yeast.⁵⁵

How heterochromatin structure induces precise DNA elimination is largely unknown. Since artificial tethering of the heterochromatin component Pdd1p to a locus is sufficient to induce its ectopic DNA elimination,⁵³ Pdd1p can recruit all downstream proteins required for DNA elimination. Cytological observations have shown that heterochromatinized IESs accumulate into nuclear peripheral foci within the developing new macronucleus when DNA elimination occurs.^{56, 57} It has been suggested that the foci include a putative protein complex called 'Excisase', which catalyzes double-stranded DNA breaking and ligation for each fragmented DNA (Late Stage in Fig 6). Recently it was reported that a PiggyBac transposase-like protein is required for DNA elimination in both *Tetrahymena* and the other ciliate *Paramecium*.^{58, 59} Therefore, PiggyBac transposase-like proteins are a likely component of Excisase and may directly recognize heterochromatin structures to excise IESs.

Evolutionary considerations

The process of DNA elimination in *Tetrahymena* bears a striking similarity to piRNA-directed transposon silencing in metazoans. Although several different RNAi-related pathways collaborate with siRNA, miRNA and piRNA in eukaryotes, they all associate with core effector Argonaute family proteins.¹² The Argonaute family proteins can be divided into two subfamilies: AGO and Piwi. siRNAs and miRNAs are associated with AGO proteins while piRNAs bind Piwi proteins. piRNAs (~24-30 nt) are slightly, but significantly, longer than si- and miRNAs (~21-24 nt). Most identified piRNAs are 2'-O-methylated at their 3' ends for all metazoans studied.⁶⁰⁻⁶⁵ In *Tetrahymena*, scnRNAs specifically interact with the Piwi protein Twi1p.⁴⁰ scnRNAs are about 28 to 29 nt in length³² and are modified by 2'-O-methylation at their 3' ends.⁵¹ Therefore, the biochemical features of scnRNAs in *Tetrahymena* are similar to those of piRNAs in metazoans.

In the germlines of flies, fish and mice, Piwi proteins play pivotal roles in transposon silencing at post-transcriptional as well as at transcriptional levels.^{52, 66, 67} In *Tetrahymena*, the scnRNA-Twi1p complex is essential for IES elimination. Since many IESs are similar to transposons, this process might have evolved as a transposon silencing mechanism for discarding transposons from the transcriptionally active macronucleus. Therefore, transposon silencing might be a task common to the scnRNA-Twi1p complex in *Tetrahymena* and piRNA-Piwi protein complexes in metazoans.

Despite biochemical and functional similarities between scnRNAs and piRNAs, their biogenesis pathways are notably different. scnRNAs are generated in *Tetrahymena* from long double-stranded precursor RNAs by the Dicer protein Dcl1p. Therefore, in terms of biogenesis, scnRNAs must be classified as endogenous siRNAs. In contrast, piRNAs are produced from single-stranded RNAs by a Dicer-independent mechanism.¹⁴ It is believed that piRNAs are produced by the sequential action of two Slicer activities of Piwi proteins described as a 'ping-pong' mechanism.^{16, 17} It is not yet clear how Dicer-dependent production of Piwi-associated small RNAs (scnRNAs) in *Tetrahymena* and Slicer-dependent production of Piwi-associated small RNAs (piRNAs) in metazoans have evolved. One

process could represent the ancestral form of Piwi-associated small RNA biogenesis, or both could have evolved from a different ancestral RNAi mechanism. In this context, it would be interesting to know how Piwi-associated small RNAs are produced in another group of eukaryotes. Since some amoeba species have Piwi proteins,^{68, 69} investigation of their associated small RNAs is eagerly awaited. Also, because *Tetrahymena* has eleven Piwi proteins besides Twi1p,⁷⁰ studying their functions and the biogenesis of their interacting small RNAs would help to understand how Piwi proteins have evolved in eukaryotes.

The process of DNA elimination in *Tetrahymena* is similar to RNAi-directed heterochromatin formation in other eukaryotes such as yeasts, plants, flies and mammals.^{11, 71-73} The best characterized RNAi-directed heterochromatin formation process is centromeric heterochromatin formation in the fission yeast *S. pombe*.¹¹ In this process, siRNAs (20-22 nt) complementary to centromeric repeat sequences induce heterochromatin formation. The siRNAs are processed from long double-stranded RNA by the Dicer protein Dcr1 and interact with the Argonaute protein Ago1. The siRNA-Ago1 complex associates with centromeric repeat sequences through RNAPII-transcribed nascent transcripts. Subsequently, the complex recruits the H3K9 methyltransferase Clr4 to the target locus and induces H3K9me. This methylated histone recruits the chromodomain protein Swi6 to establish heterochromatin. The siRNA-Ago1 complex also recruits an RNA-directed RNA polymerase complex and Dcr1 to the target locus, leading to amplification of siRNA signals. As described in this chapter, the *Tetrahymena* scnRNA-Twi1p complex interacts with nascent ncRNAs and is required for Ezl1p-dependent accumulation of H3K9/K27me, which leads to heterochromatin formation. Therefore, RNAi-directed DNA elimination in *Tetrahymena* may be an evolutionary cousin of RNAi-directed heterochromatin formation in other eukaryotes. Since DNA elimination in *Tetrahymena* employs the E(z) ortholog Ezl1p for H3K9/K27me, it might be related to the Polycomb silencing mechanism. In this context, it is interesting to note that the RNAi pathway may also be involved in Polycomb silencing in flies and mice.^{74, 75}

Developmentally programmed DNA elimination, also called chromatin or chromosome diminution, has long been observed not only in ciliates⁷⁶ but also in several taxonomically diverged metazoans such as nematodes,^{77, 78} sciarid flies,⁷⁹ copepods,^{80, 81} lampreys⁸² and hagfish.^{83, 84} As in *Tetrahymena*, these DNA elimination events take place in the somatic lineage during early embryogenesis, and the eliminated DNAs are often transposon-like repetitive sequences. Interestingly, most eliminated DNA in these programmed elimination processes is at some point embedded into heterochromatin before it is eliminated. Moreover, as described earlier, the molecular mechanism of DNA elimination in *Tetrahymena* is related to RNA-directed heterochromatin formation in other eukaryotes. Therefore, programmed DNA elimination in general might have evolved from a conserved RNA-directed heterochromatin formation mechanism that removes harmful genetic elements from somatic lineages. Since DNA elimination occurs in several phylogenetically separated species, DNA excision processes might have independently arisen in each organism or conversely, have been preserved from the common ancestor in a few organisms which are currently exist. PiggyBac-like proteins, which may be domesticated transposases, play an essential role in removing heterochromatinized IESs in the ciliates *Paramecium*⁵⁸ and *Tetrahymena*.⁵⁹ Investigating how these PiggyBac-like proteins have been domesticated and how they interact with heterochromatin will help to understand how ciliates have acquired a DNA elimination mechanism during their evolution. Likewise, future studies on the mechanisms of DNA elimination processes in other organism may give us a broader framework for how these processes have evolved in multiple different taxa.

Future prospects

DNA elimination occurs precisely and reproducibly, and only minor variations of fewer than 10 bp from the elimination boundary have been observed. Although heterochromatin formation, including accumulations of H3K9/27me and the chromodomain protein Pdd1p, is necessary and sufficient to mark DNA sequences for elimination, it is not likely that mere changes in the nucleosome, which contains ~150 bp DNA, are sufficient to support this precision. We speculate that the enzyme (hypothetically called Excisase) that removes IESs may have some sequence preference that limits elimination boundaries to a small range. Future studies of the recently identified PiggyBac-like protein, which is a potential Excisase component, will help to clarify this issue.

Besides transposon silencing, DNA elimination is thought to be involved in mating-type determination. An individual *Tetrahymena* cell expresses one of seven mating types, and mating can occur between cells of any two different types. Since mating-type switching does not occur during vegetative growth, mating types are thought to be determined in the developing new macronucleus by alternative DNA rearrangements. Although a potential mating-type locus named *mat* has been identified,⁸⁵ the gene encoded by the *mat* locus has not yet been cloned. Identification of the *mat* locus-encoding gene, as well as a genome-wide comparison between micro- and macronuclear genome sequences from strains expressing different mating types, may enable us to identify the molecule(s) involved in mating-type determination and to understand how DNA rearrangements regulate gene expression.

Another area of interest for future research is the directed transportation of scnRNAs. scnRNAs are produced in the parental micronucleus and exported to the cytoplasm to form complexes with the Argonaute protein Twi1p, after which the complex migrates to the parental macronucleus and finally to the newly developing macronucleus (Fig. 6). Since this sequential localization of scnRNAs is essential for proper DNA elimination, the timing and direction of scnRNA-Twi1p translocations must be precisely regulated. Recently, micro- and macronucleus-specific importin alpha subunits and nucleoporins have been identified.^{86, 87} Selective interactions between the scnRNA-Twi1p complex and macronucleus-dedicated transporting systems may regulate the spatiotemporal behavior of scnRNAs. Further research into these nucleus-specific transportation machineries might elucidate how selective nuclear transport of scnRNA-Twi1p complexes is achieved.

In terms of the specific import of the scnRNA-Twi1p complex into the newly developing macronucleus, a more fundamental question arises. How are the fates of the somatic macronucleus and germline micronucleus determined? Because macronuclear differentiation (including expansion of the nucleus) starts before Twi1p localizes to the new macronucleus (KM, unpublished results), the scnRNA-Twi1p complex is not likely the fate determinant. In many metazoans, segregation of germline and somatic lineages depends on cytoplasmic determinants that are maternally preserved in the egg.⁸⁸ Although the molecular mechanism of nuclear fate determination in *Tetrahymena* has not been elucidated, it has long been known that the developmental fates of nuclei are related to their locations within the cell at a critical time.⁸⁹⁻⁹¹ Two consecutive mitoses produce four nuclei from a fertilized zygotic nucleus. Nuclei located in the anterior cytoplasm develop into macronuclei while others that are posteriorly localized become micronuclei (Fig. 3G). Thus, as in many metazoans, an asymmetrically localized molecule(s) (protein and/or RNA) along the anteroposterior axis may act as a determinant for macro- and/or micronuclear fates in *Tetrahymena*. Identification and characterization of this determinant would provide us not only with a mechanistic understanding of how nuclear fates and nucleus-specific transportation systems

are established in *Tetrahymena*, but also yield unique insights into how germline-soma segregation has evolved in non-metazoan eukaryotes.

Conclusion

An RNA infrastructure, a network of processes including small RNA biogenesis, post-transcriptional selection of small RNAs, two different nuclear transport processes for small RNAs and small RNA-induced heterochromatin formation, corporately conduct DNA elimination in *Tetrahymena*. Because the process leading to DNA elimination can readily be induced synchronously in several billion *Tetrahymena* cells⁹² and we are able to discriminately analyze the RNA-infrastructure in different developmental stages in this organism, DNA elimination in *Tetrahymena* is a useful model to study how RNA infrastructure regulates chromatin organization in eukaryotes.

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Figure 1.

Examples of small RNA-directed defense systems against molecular parasites in some prokaryotes and eukaryotes. (A) The CRISPR pathway, a prokaryotic defense system. CRISPR RNAs are processed by a set of Cas proteins and the complex targets phage DNA or RNA for degradation. (B) siRNA pathway in eukaryotes. Read-through transcripts from adjacent genes produce dsRNA containing a transposon sequence. The dsRNA is processed to siRNA by Dicer. Argonaute protein of the AGO subfamily associates with siRNA and induces degradation of transcripts from transposons as well as transcriptional silencing through alteration of chromatin structure. (C) The piRNA pathway in the metazoan germline. piRNA cluster produces single-stranded RNA that is processed to piRNAs,

probably by Piwi protein(s). Argonaute proteins of the Piwi subfamily associate with piRNAs and induce silencing of transposons complementary to the piRNAs at the post-transcriptional and transcriptional levels. (D) The scnRNA pathway in *Tetrahymena*. dsRNA produced from the micronuclear (Mic) genome is processed to scnRNA by the Dicer-like enzyme Dcl1p. scnRNA interacts with the Piwi subfamily protein Twi1p and promotes heterochromatin formation on IES in the newly developing macronucleus (New Dev. Mac). Eventually, IES is eliminated from the genome. Gray lines and waved lines represent genomes and transcripts, respectively.

Figure 2.

Nuclear dimorphism in *Tetrahymena thermophila*. *Tetrahymena* possesses two distinct nuclei within a single cell. A confocal microscopic view of *Tetrahymena* shows the larger macronucleus (Mac) containing the somatic genome and the smaller micronucleus (Mic) containing the germline genome. A cilium stained with anti-alpha tubulin antibody is shown in (A), a nucleus stained with DAPI in (B) and the merged image in (C). OA: oral apparatus. Scale bar, 5 μ m.

Figure 3.

Life cycle of *Tetrahymena*. (A) In nutrient-rich conditions *Tetrahymena* grows vegetatively by binary fission. (B) Starvation induces sexual reproduction by conjugation. Conjugation begins with pairing of complementary mating types; the parent expressing mating type I has nuclei in white and nuclei from mating type II are in black. (C) The micronucleus undergoes meiosis to produce four haploid nuclei, three of which degrade. (D) The remaining haploid micronucleus divides mitotically. (E-F) The haploid nuclei are exchanged reciprocally and fuse to make a zygotic diploid nucleus. (G) The zygotic nucleus, shown in gray, undergoes two mitotic divisions. From the four daughter nuclei of these divisions, the two located in the anterior cytoplasm differentiate into macronuclei and the two located posteriorly become

micronuclei. (H) The parental macronucleus is destroyed by an apoptosis-like DNA degradation process. (I) The cells separate and one of the two micronuclei is degraded. (J-K) The remaining micronucleus divides mitotically, and the subsequent cell division yields four progeny that are vegetative cells. The anterior to posterior axis of the cells is shown from top to bottom, respectively. Mac, macronucleus; Mic, micronucleus.

Figure 4.

The micro- and macronuclei differentiate from the same zygotic nucleus that is generated after meiosis of the parental micronucleus and subsequent karyogamy during sexual reproduction. The diploid micronucleus always maintains the entire genome. The chromosome contains both macronucleus-destined sequences (gray line) and micronucleus-limited sequences (IES; boxes). Approximately 6000 IESs (~0.5-20 kb in size) are excised from the developing macronuclear chromosomes during differentiation. Simultaneously, the chromosomes are fragmented at approximately 250 sites (white circles) by chromosome breakage coupled with addition of new telomeres (dark gray circles) and are endoreplicated ~50 times. In parallel, the parental macronucleus is degraded at the end of sexual reproduction. Mac, macronucleus; Mic, micronucleus.

Figure 5.

Epigenetic regulation of DNA elimination. (A) Genome rearrangement in a wild-type strain. IES-(a) and IES-(b) are precisely eliminated from the chromosome during new macronucleus development. During this process, the parental macronucleus is degraded. (B) Effect on an IES in the parental macronucleus. When IES-(a) is artificially introduced into the parental macronucleus, elimination of the endogenous IES homologous to that in the parental macronucleus is inhibited in the new macronucleus.³⁷ (C) Effect of an exogenous sequence in the parental micronucleus. When a bacterial *neo* sequence is artificially introduced into the parental micronucleus, this sequence is eliminated from the new macronucleus.^{38, 39} Mac, macronucleus; Mic, micronucleus.

Figure 6. Scan RNA model. Events occurring sequentially are shown from top to bottom. The approximate stages when events occur are indicated on the right by arrows. Stages correspond to those of Figure 3. See text for details. Mic: micronucleus, Mac: Macronucleus.