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DISSERTATION

Titel der Dissertation

A study of nuclear acting small RNA: Post-transcriptional modification and nuclear import

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Doktorin der Naturwissenschaften (Dr. rer.nat.)

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The four precepts that characterize the Method of Science

(Descartes, 1637)

The first was never to accept anything for true which I did not clearly know to be such; that is to say, carefully to avoid precipitancy and prejudice, and to comprise nothing more in my judgment than what was presented to my mind so clearly and distinctly as to exclude all ground of doubt.

The second, to divide each of the difficulties under examination into as many parts as possible, and as might be necessary for its adequate solution.

The third, to conduct my thoughts in such order that, by commencing with objects the simplest and easiest to know, I might ascend by little and little, and, as it were, step by step, to the knowledge of the more complex; assigning in thought a certain order even to those objects which in their own nature do not stand in a relation of antecedence and sequence.

And the last, in every case to make enumerations so complete, and reviews so general, that I might be assured that nothing was omitted.

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First and foremost, I would like to thank Kaz for teaching me the importance of the “Methods of Science” from Descartes, which he shared with me in the beginning of my PhD. Kaz has been a great supervisor and mentor and I cannot imagine a better place to my PhD than his lab.

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Summary

Small RNAs, ~20–30 nucleotides (nt) in length regulate gene expression at the transcriptional and post-transcriptional levels. In the plant *Arabidopsis*, all small RNAs are 3'-terminal 2'-*O*-methylated by HEN1, whereas only a subset of small RNAs carries this modification in metazoans. Methylation is known to stabilize small RNAs, but its biological significance remains unclear. In *Tetrahymena*, two classes of small RNAs have been identified: 28–29 nt RNAs (scnRNAs), that are expressed only during sexual reproduction, and constitutively expressed 23–24 nt siRNAs. In the first part of my PhD study, I have investigated the role of scnRNA methylation in *Tetrahymena*. I have demonstrated that scnRNAs, but not siRNAs, are 2'-O-methylated at their 3' ends. The *Tetrahymena* HEN1 homolog Hen1p is responsible for scnRNA 2'-*O*-methylation. Loss of Hen1p causes a gradual reduction in the level and length of scnRNAs, defects in programmed DNA elimination and inefficient production of sexual progeny. Therefore, Hen1p-mediated 2'-*O*-methylation stabilizes scnRNAs and ensures DNA elimination in *Tetrahymena*. My study shows that 3'-terminal 2'-O-methylation on a selected class of small RNAs regulates the function of a specific RNAi pathway.

scnRNAs are loaded onto the Argonaute protein Twi1p in the cytoplasm, but have been demonstrated to act in the nucleus. The process that transports loaded Argonaute proteins into the nucleus is generally poorly understood. In the second part of my PhD study, I was part of a project that investigated the nuclear import of Twi1p. We identified a novel protein, Giw1p, which binds to Twi1p and is required for its nuclear localization. Interestingly, Giw1p neither binds to unloaded Twi1p nor to Twi1p complexed with double stranded RNA. This suggests that Giw1p is able to sense the state of the Twi1p-scnRNA complex and selectively transports only the mature complex into the nucleus. The basis for this selective binding is most likely a conformational change of the Argonaute protein. This is the first report of a protein that can sense the loading state of an Argonaute.

Zusammenfassung

Kleine, ~20-30 Nukleotide (nt) lange RNAs können Genexpression sowohl auf transkriptioneller, als auch auf post-transkriptioneller Ebene regulieren. In *Arabidopsis* sind alle kleinen RNAs am 3' Ende 2'-*O*-methyliert. In Metazoen hingegen trägt nur ein Teil der kleinen RNAs diese Modifikation. Es ist zwar erwiesen, dass kleine RNAs durch Methylierung stabilisiert werden, die biologische Bedeutung dieser Modifikation ist jedoch unzureichend verstanden. *Tetrahymena thermophila* exprimiert zwei Klassen von kleinen RNAs. Die erste Klasse setzt sich aus ständig exprimierten ~23–24 nt siRNAs zusammen, während ~28–29 nt scnRNAs die zweite Klasse bilden. Die Funktion der Methylierung von kleinen RNAs in *Tetrahymena* untersuchte ich im ersten Teil meiner Doktorarbeit. Ich konnte zeigen, dass scnRNAs, aber nicht siRNAs, an ihren 3' Enden methyliert sind. Das *Tetrahymena* HEN1 Homolog Hen1p katalysiert diese Reaktion. Das Fehlen von Hen1p hat eine Verkürzung, sowie einen teilweisen Abbau der scnRNAs zur Folge. Diese Destabilisierung führt zu einem Defekt in der DNA Eliminierung, wodurch die Anzahl sexueller Nachkommen stark reduziert wird. Unsere Studie zeigt, dass 2'-*O*-Methylierung von kleinen RNAs einen spezifischen RNAi Stoffwechselweg in Eukaryoten regulieren kann.

Das Argonautprotein Twi1p wird im Zytoplasma mit scnRNA beladen, entfaltet seine Funktion aber im Zellkern. Im zweiten Teil meiner Doktorarbeit untersuchten wir, wie beladene Argonautproteine erkannt und in den Zellkern transportiert werden. Wir identifizierten ein neues Protein, Giw1p, welches mit Twi1p interagiert und für dessen Lokalisation im Zellkern verantwortlich ist. Giw1p bindet weder an unbeladenes, noch an mit doppelsträngiger RNA beladenes Twi1p. Dies deutet darauf hin, dass Giw1p den Zustand des Argonaut-scnRNA Komplexes erkennt und nur den reifen Komplex in den Zellkern transportiert. Ein möglicher Grund für diesen selektiven Transport ist eine Konformationsänderung von Twi1p. Dies ist die erste Beschreibung eines Proteins, welches die Beladung eines Argonauts erkennen kann.

Introduction

1 History of small RNA research

Small RNAs, 20 – 30 nt in length, play an unexpectedly large role in regulating gene expression and chromatin dynamics in most eukaryotic organisms. Despite their huge cellular impact, the first small RNA was only discovered in 1993 by genetic screens in nematode worms (Wightman et al., 1993; Lee et al., 1993). Due to their small size, this class of RNAs had simply been missed in earlier analyses of regulatory RNAs (reviewed in Grosshans and Filipowicz, 2008).

The first endogenous small RNA discovered in eukaryotes was the micro RNA (miRNA) lin-4 (Lee et al., 1993; Wightman et al., 1993). It had already been known that the locus lin-4 was essential for the control of postembryonic developmental events in *C. elegans* by negatively regulating the level of LIN-14 protein (Arasu et al., 1991). Subsequent chromosomal walking and transformation rescue unravelled that lin-4 does not encode a protein. Instead, it gives rise to two small transcripts of 22 and 61 nt (Lee et al., 1993). These RNAs contain sequences complementary to the 3' untranslated region of the lin-14 messenger RNA (mRNA). Based on these results, it was proposed for the first time, that small RNA might regulate mRNA via anti-sense RNA-RNA interaction.

A parallel line of research investigated the mechanisms of post-transcriptional gene silencing by transgenes (reviewed in Hammond et al., 2001). It had been found that introduction of transgenic copies of a gene into *Petunia* did not result in the expected rise of gene expression levels but instead, led to gene silencing (Napoli et al., 1990). Similar observations were made in *C. elegans*, where antisense RNA was introduced to probe gene function. This generated the expected phenotype but surprisingly, injection of the control sense strand created the identical phenotype (Guo and Kemphues, 1995). The breakthrough came when a mixture of sense and

antisense RNA was injected into worms (Fire et al., 1998). This showed that the double-stranded (ds) RNA silenced the expression of the target gene tenfold more efficiently than either strand alone. This process was called RNA interference (RNAi) and was awarded in 2006, only eight years after its discovery, the Nobel Prize. Today, scientists routinely use small RNAs to silence genes of interest in a variety of model organisms and in cell culture. Research is ongoing to also use RNA interference in the treatment of human disease (reviewed in Grimm, 2009).

The components that mediate RNA interference triggered by both endogenous small RNAs and double stranded RNA are overlapping and highly conserved. All small RNAs act in complex with Argonaute (Agos) proteins (see section 3). It is the small RNA that confers the specificity of targeting. Small RNAs are currently grouped into three main classes: micro RNAs, small interfering RNAs (siRNAs) and Piwi interacting RNAs (piRNAs).

2 Three classes of small RNAs

2.1 micro RNA

Since the discovery of the founding members of the class of miRNAs, lin-4 and let-7 (Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993), hundreds of miRNAs have been identified in plants, animals and viruses. These endogenous small RNAs are 21-23 nt in length and are generated from imperfectly base-paired hairpin-shaped transcripts (Fig.1A) (reviewed in Bartel, 2004). Most miRNA genes are transcribed by RNA polymerase II (Pol II) and like messenger RNAs they contain 5' cap structures, are polyadenylated and might be spliced. The primary transcript is initially cleaved by a nuclear RNase III enzyme (Drosha in animals; Dicer in plants). The resulting precursor miRNA (pre-miRNA) is then recognized by an Exportin and is transported into the cytoplasm. The final step of miRNA biogenesis is cleavage of the pre-miRNA into the mature miRNA duplex by the cytoplasmic RNase III enzyme Dicer. The resulting bulged double stranded ~22 nt RNA has 5' monophosphates and 2 nt 3' overhangs. Like all small RNAs, miRNAs are eventually loaded onto Argonaute proteins. After the removal of the passenger strand, the complex specifically binds to target mRNAs by Watson-Crick base pairing to induce post-transcriptional gene silencing (see section 4).

2.2 small interfering RNAs

The class of 20-25 nt small interfering RNAs can be subdivided into exo- and endogenous small interfering RNAs. Exogenous siRNAs are derived from experimentally introduced double-stranded RNAs or viral RNAs (Hamilton, 1999), while endogenous siRNAs (endo-siRNAs) are derived from transposon transcripts, sense-antisense transcript pairs, inverted repeats or long stem loop structures. In organisms that possess an RNA - dependent RNA polymerase, the targeted single-stranded RNA can be converted into long

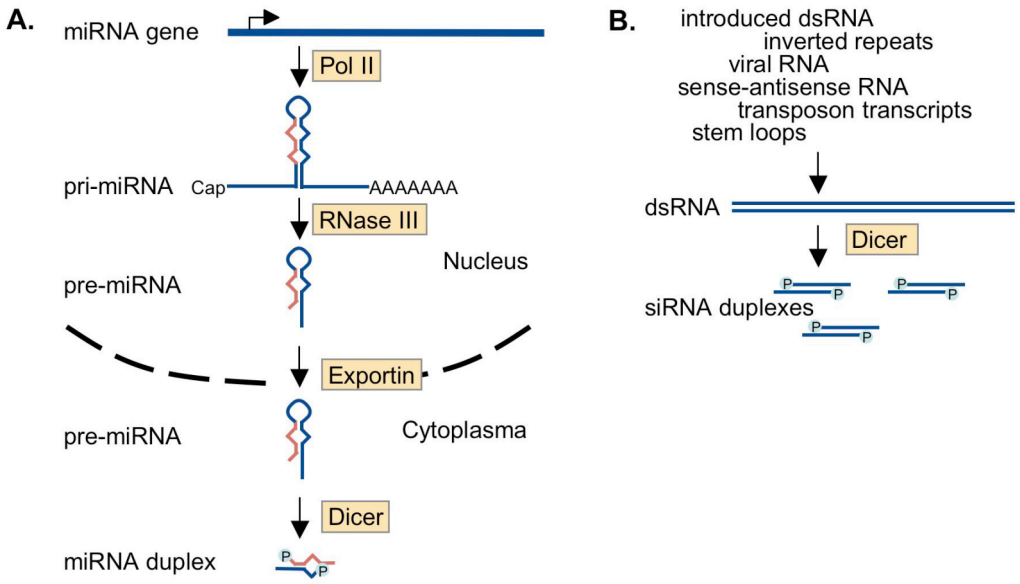

Figure 1. Biogenesis of miRNAs and siRNAs.

A. Biogenesis of miRNAs. Most miRNA genes are transcribed by RNA polymerase II (Pol II). The capped and polyadenylated primary microRNA (pri-miRNA) forms a stem loop structure that contains bulges and is processed by a nuclear RNase III enzyme into a precursor miRNA (pre-miRNA). Its structure is recognized by an Exportin and transported out of the nucleus. The pre-miRNA is subsequently cleaved by Dicer which results in a miRNA duplex with 5' phosphates and 3' 2 nt overhangs that contains bulges. **B. Biogenesis of siRNAs.** Dicer cleaves double stranded RNA into small interfering RNAs (siRNAs). These have perfect complementarity and display, like all Dicer products, 5' phosphates and 3' 2 nt overhangs. Adapted from Kim et al., 2009.

dsRNA to serve as a Dicer substrate. This leads to an amplification of the RNAi signal (reviewed in Baulcombe, 2007). Endogenous siRNAs have first been identified in plants and in *C. elegans* (Ambros et al., 2003; Hamilton et al., 2002). Only recently they have been also described in *Drosophil*a, mouse oocytes and embryonic stem cells (Tam et al, 2008; Babiarz et al., 2008; Czech et al., 2008). In contrast to miRNAs, the processing of siRNAs is only dependent on the RNase III enzyme, Dicer, which sequentially cleaves the

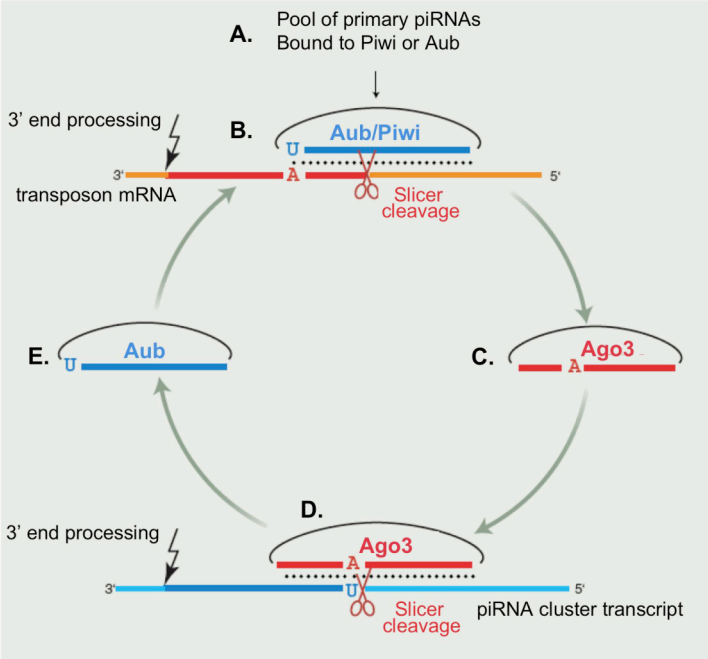

Figure 2. The ping-pong cycle of piRNA production in *Drosophila*.

A. A pool of primary piRNAs, in complex with either Aubergine (Aub) or Piwi, trigger the initiation of the ping-pong cycle. These piRNAs are produced by an unknown mechanism and/or are maternally deposited. **B.** The piRNA-Aub/Piwi complex recognizes and cleaves complementary sequences in transposon RNA, which generates the 5' end of a new piRNA, partially complementary to the primary piRNA. Its 3' end is processed by an unknown nuclease. **C.** The new piRNA is loaded onto Ago3. **D.** This complex is then able to target and slice piRNA cluster transcripts, which again produce new piRNAs that are loaded onto Aub (**E.**) and are able to target transposon mRNA. From Aravin et al., 2007.

long dsRNA (Fig.1B) (reviewed in Kim, 2009). The generated siRNAs are perfectly matched and like miRNAs, have 5' monophosphates and 3' 2 nt overhangs. Depending on the organism, endogenous siRNAs either target complementary RNA transcripts for destruction, translational inhibition and/or mediate changes on the chromatin level (see section 4 and 5).

2.3 Piwi interacting RNAs

Argonaute proteins can be subdivided into the Argonaute and the Piwi (P-element induced wimpy testis) clade (see section 3). While miRNAs and siRNAs are both associated with proteins of the Argonaute familiy, the animal specific Piwi-interacting RNAs associate with members of the Piwi protein family (Cox et al., 2000; Lau et al., 2006; Aravin et al., 2006; Brennecke et al., 2007). piRNAs are longer (~26-30 nt) than siRNAs and miRNAs, and their production is Dicer independent (Vagin et al., 2006; Houwing et al., 2007). Small RNA sequencing studies in *Drosophila* have indicated that piRNA biogenesis involved the nuclease activity of the Piwi proteins themselves (see section 3) (Brennecke et al., 2007; Gunawardane et al., 2007). In this so-called ping-pong model (Fig. 2), one type of Piwi proteins that is associated with anti-sense piRNAs cleaves sense transcripts and this cleavage generates the 5' ends of sense piRNAs that associate with a second type of Piwi protein. These newly generated complexes then cleave anti-sense transcripts, which in turn generate the 5' ends of anti-sense piRNAs. In this way, the piRNA population is amplified (Brennecke et al., 2007; Gunawardane et al., 2007). Factors that are necessary for the formation of the 3' ends of piRNAs have so far not been identified. Piwi proteins together with their associated piRNAs have been implicated in transcriptional and posttranscriptional transposon control (Aravin et al., 2008; Aravin et al., 2007; Kuramochi-Miyagawa et al., 2008; Brennecke et al., 2007; Houwing et al., 2007; Saito et al., 2006). However, the role of piRNAs that are not derived from transposable or repetitive elements is unknown (Robine et al., 2009).

3 Argonaute proteins

3.1 Argonaute and Piwi proteins

All small RNAs are inevitably bound by Argonautes. This highly conserved protein family was first identified in plants, and named after its characteristic squid-shaped leaf mutant phenotype (Bohmert et al., 1998). Its members are defined by the presence of a PAZ (named after three proteins – Piwi, Argonaute and Zwille) and a Piwi domain (Fig. 3A). Argonaute protein can be phylogenetically subdivided into two clades: the Argonaute clade based on *Arabidopsis thaliana* Ago1 and the Piwi clade based on *Drosophila melanogaster* Piwi (Fig. 3B) (Lin and Spradling, 1997; Bohmert et al., 1998). While plants only encode Argonaute proteins and ciliates only have Piwis, insects and mammals ubiquitously express Argonaute proteins but additionally express Piwi proteins in the germline. Also the number of encoded Argonaute genes per organism ranges, from 1 in *S. pombe* to 27 in *C. elegans* (reviewed in Hock, 2008). It is becoming more and more apparent that Argonaute proteins are key regulators in many, if not all, cellular processes. The function of an Argonaute protein depends on both, its biochemical properties (e.g. localisation signals, sites for interaction with other proteins, slicing activity) and the sequence of its bound small RNA, which mediates target specificity.

3.2 Structure of Argonaute proteins

All Argonaute proteins show a similar domain structure (Fig. 3A), consisting of an amino-terminal, a PAZ, a mid and a Piwi domain. Except for the amino-terminal domain, each domain has been ascribed with a specific function. The PAZ domain contains conserved aromatic residues that anchor the 3' end, while the mid domain specifically recognizes the 5' phosphate of the guide strand (reviewed in Nowotny and Yang, 2009). The mid domain additionally has been implicated in protein–protein interactions. Ago interactors, like Tas3

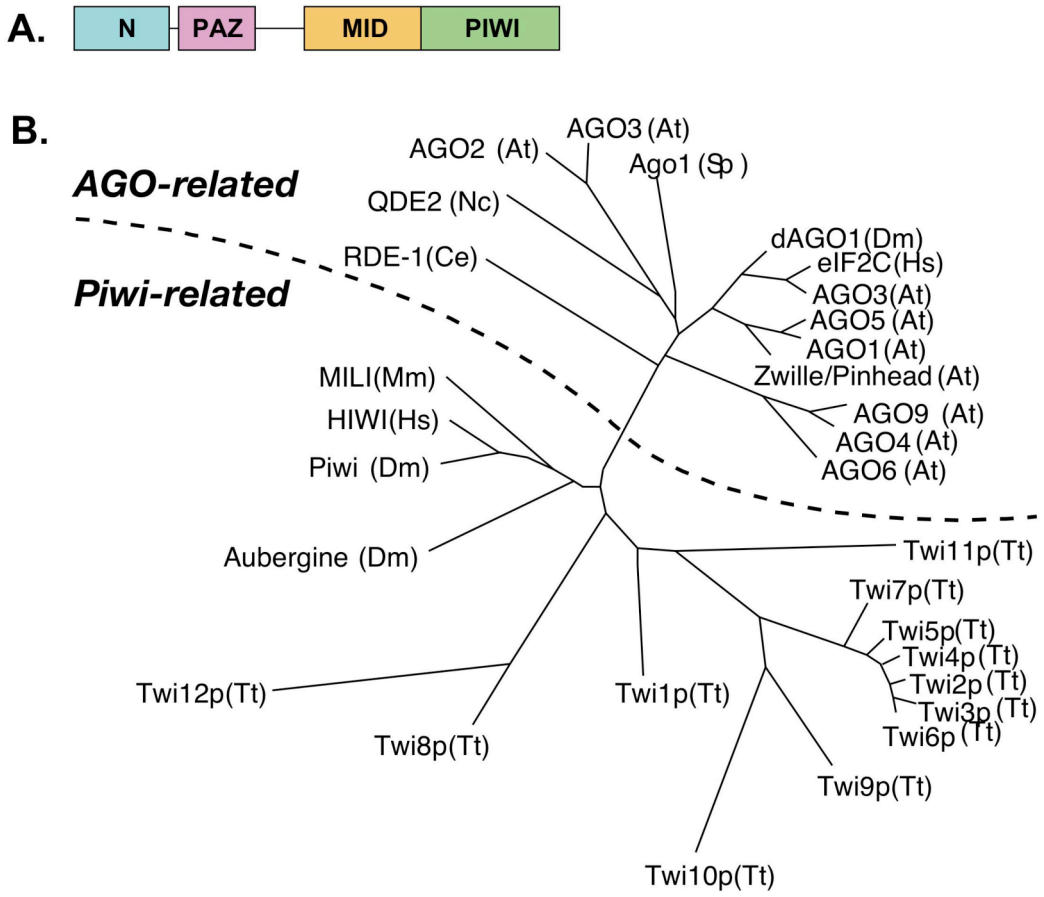

Figure 3. Argonaute proteins.

A. Secondary structure of Argonaute proteins. All Argonaute proteins display the same domain composition. They all consist of an N-terminal, a PAZ, a mid and a Piwi domain that are connected with linkers. **B. phylogenetic tree of selected Argonaute proteins.** Members of the Argonaute family are phylogenetically classified into two subfamilies: the Argonaute and the Piwi clade. (Ce: C. elegans; Nc: Neurospora crassa; At: A. thaliana; Sp: S. pombe; Dm: D. melanogaster; Hs: Homo sapiens; Tt: Tetrahymena; Mm: M. musculus)

in *S.pombe*, form so called 'Ago-hooks' that bind the mid domain (Till et al., 2007). The Piwi domain has a tertiary structure belonging to the RNase H family of enzymes. Indeed, some Argonautes, that contain the conserved catalytic core composed of an Asp-Asp-His (DDH) motif, have intrinsic endonucleolytic activity (Song et al., 2004; Rivas et al., 2005; Martinez and Tuschl, 2004). This so-called 'slicer' activity can destroy target mRNA (see

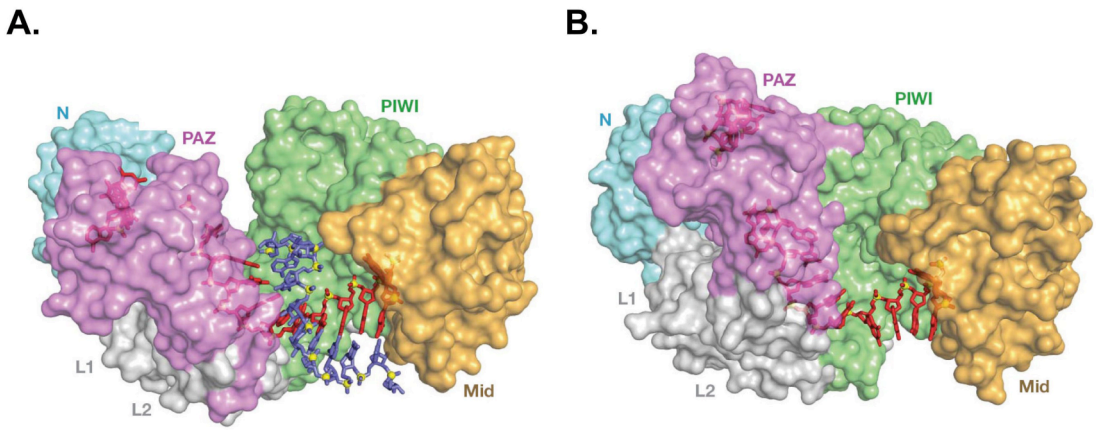

Figure 4. The Argonaute protein undergoes a marked conformational change when proceeding from the tertiary to the binary complex.

Thermus thermophilus Argonaute protein shown in a space-filling representation with the different domains coded in colours as in 3A. **A. Ternary complex.** Argonaute complexed with guide DNA (red) and passenger RNA (blue) duplex. PAZ- and Piwi-containing lobes form a wide-opened nucleic acid binding channel. **B. Binary complex.** Argonaute after removal of the passenger RNA. Rotation of the N- and PAZ-domain-containing lobe results in a closed conformation, bringing the PAZ and Piwi domain into proximity. From Wang et al., 2008b.

section 4.1) (Song et al., 2004) and has been implicated in piRNA biogenesis (see section 2.3) (Brennecke et al., 2007). The distance from the 5' end of the siRNA directs the cleavage site (Martinez and Tuschl, 2004; Elbashir et al., 2001). Mammalian Ago2, for example, cuts the phosphodiester bond in the target RNA opposite the 10^{th} an 11^{th} residue of the siRNA (Liu et al., 2004). However, slicing is not an absolute requirement for Argonaute function. Argonautes without the catalytic triad silence genes by bringing other effector complexes to the target (see section 4).

Crystal structures of eubacterial and archaeal Argonaute proteins revealed a bilobal structure, with the PAZ containing (N, linker and PAZ) and the Piwi containing (Mid and Piwi) lobes on either side of the nucleic acid (Fig. 4A) (Song et al., 2004; Wang et al., 2008a; Wang et al., 2008b; Wang et al.,

2009). Interestingly, there is a marked conformational difference in the relative alignments of the PAZ- and the Piwi- containing lobes between the open, tertiary (Argonaute plus ds nucleic acid) and the closed, binary complex (Argonaute plus guide strand) (Fig. 4B). This switch of the Argonaute structure has several mechanistic implications. First, the tertiary form seems to favour positioning of the cleavage site of the target RNA close to the catalytic site of the Piwi domain (Wang et al., 2009). Second, the structural switch might underlie the observed allosteric regulation of Argonaute proteins after binding to miRNAs (Djuranovic at al., 2010). Third, there might be proteins that specifically bind only one of the two structures. Unfortunately, crystallization of a complete Argonaute protein from higher eukaryotes has not been yet successful.

3.3 Sorting of small RNAs into Argonaute proteins

How newly generated small RNAs are specifically sorted into the large number of different Argonaute proteins is not well understood. In *Arabidopsis* sorting in many cases seems to be directed by the 5' terminal nucleotide (Mi et al., 2008). However, numerous classes of small RNAs in *Tetrahymena* and many classes of siRNA, miRNA and piRNAs in other eukaryotes show a 5' Uracil bias as a common feature (Ghildiyal et al., 2008; Couvillion et al., 2009; Girard et al., 2006; Xie et al., 2005). Therefore, additional selection mechanisms must be at work. This is best studied in *Drosophila* where post-biogenesis sorting depends on the structure of the small RNA duplex. The position of central mismatches seems to be the dominant determinant for this (Tomari et al., 2007; Czech et al., 2009; Steiner et al., 2007). However, small RNAs with a similar sequence composition can also be targeted to specific Argonaute pathways. The length of the small RNA might be a critical determinant, but also coupling small RNA biogenesis to the loading of specific Argonautes seems to be important (Tomari et al., 2007). It has been demonstrated that Dicer is critical for the loading of miRNAs onto the Argonaute protein in *Drosophila* egg extracts (Liu et al., 2007a). How proteins of the Piwi clade are loaded has not been described yet.

miRNAs and siRNAs are intitially loaded onto Argonautes in their

double-stranded form. But only one strand, the guide strand, is retained in the functional ribonucleoprotein complex. The selection of this strand is governed by the thermodynamic profile of the siRNA duplex termini (Khvorova et al., 2003; Schwarz et al., 2003). The other strand, the passenger strand, is removed during the assembly process. Argonaute proteins with an active catalytic triad seem to use slicing activity for passenger strand removal (Steiner et al., 2009; Leuschner et al., 2006; Rand et al., 2005; Matranga et al., 2005; Miyoshi et al., 2005), while other Argonautes most likely depend on additional factors like exonucleases or RNA helicases (Liu et al., 2009; Maiti et al., 2007; Robb et al., 2007).

Argonaute proteins in complex with single-stranded guide RNA can regulate transcription. One can distinguish between post-transcriptional mechanisms (see section 4) and regulations on the transcriptional level (see section 5).

4 Post-transcriptional silencing mechanisms

4.1 Degradation of RNAs

Small RNAs can mediate sequence specific destruction of target RNAs by two means. First, siRNAs, piRNAs and miRNAs that have perfect complementarity to a RNA target can direct endonucleolytic cleavage within the base-paired region (Fig. 5A) (reviewed in Wu and Belasco, 2008; reviewed in Malone and Hannon, 2009b). At least in vitro, the minimal effector complex consists only of the Argonaute and the small RNA. The resulting fragments are quickly degraded, probably because they bear unprotected ends that are susceptible to exonuclease attacks. Slicing of RNAs is mainly used by miRNAs in plants to regulate genes (reviewed in Jones-Rhoades et al., 2006), by piRNAs in metazoans to combat transposons (reviewed in Malone and Hannon, 2009), and by siRNAs in plants, flies and worms to destroy viral RNA reviewed in (reviewed in Stram and Kuzntzova, 2006).

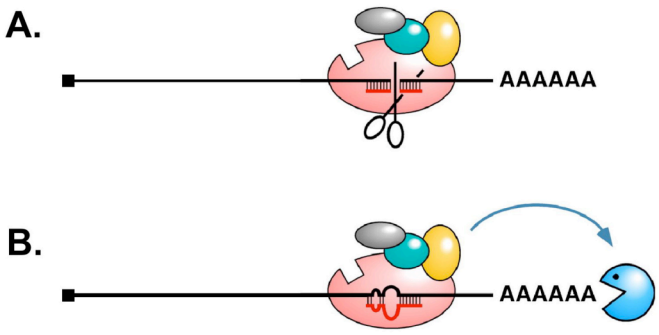

Figure 5. Argonaute mediated degradation of mRNAs.
A. mRNA cleavage. RNAs can be endonucleolytically cleaved by catalytic Argonaute proteins if the small RNA has perfect complementarity to the target. (m7G cap: black square; poly(A) tail: AAAAAA; Argonaute: pink; associated proteins: grey, petrol, yellow; scissor: slicing activity of the Argonaute) **B. polyA removal.** Small RNAs partially complementary to the messenger RNA in complex with Argonaute proteins meditate mRNA decay by recruiting enzymes (blue) that remove the polyA tail. Adapted from Wu and Belasco, 2008.

The second form of targeted RNA destruction is slicer independent. In this case, small RNAs mediate RNA degradation by recruiting deadenylating and decapping enzymes to the target. (Fig. 5B) (reviewed in Wu and Belasco, 2008). The respective RNA is then also degraded by exoribonucleases.

4.2 Translational inhibition by miRNPs

mRNA destruction is not the only mechanism that small RNAs use to regulate gene expression. miRNAs can also inhibit gene expression at the level of

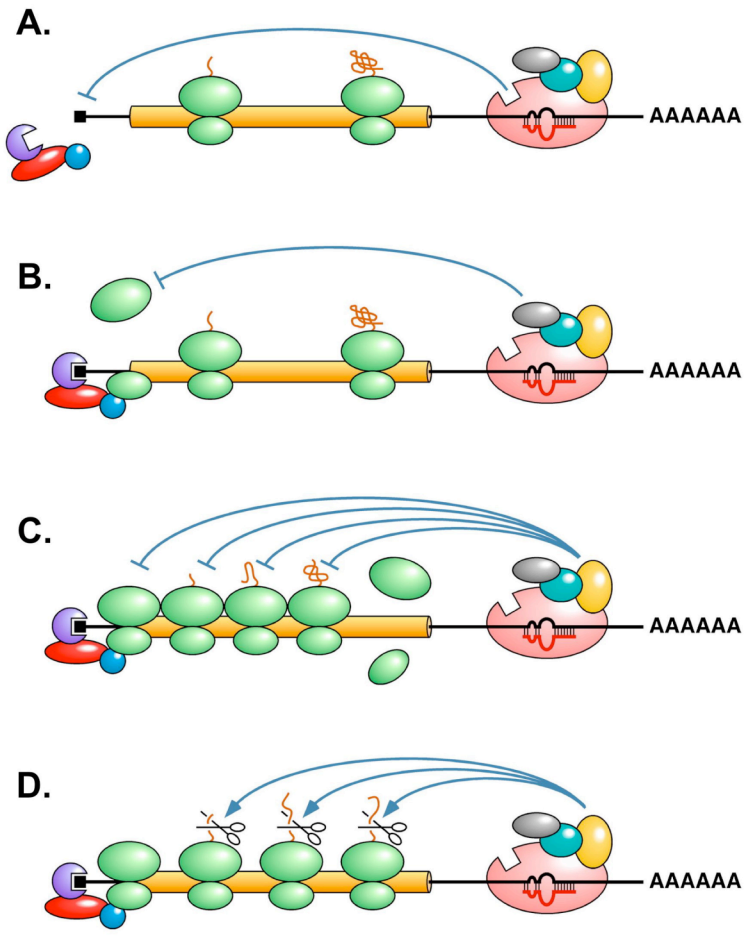

Figure 6. Hypothetical mechanisms of translational repression by miRNAs.
A. Competition between RISC and eIF4E. RISC, instead of eIF4E, might bind to the mRNA cap, thereby preventing the formation of the translation initiation complex. (Black square: m7G cap; yellow box: protein-coding region; AAAAAA: poly(A) tail; green: ribosomes; brown: nascent polypeptides; violet: eIF4E subunit of the cap-binding complex; pink: Argonaute; grey, petrol, yellow: associated proteins) **B. Inhibition after cap recognition**. Argonaute-small RNA complexes are reported to impede the association of small and large ribosomal subunits. **C. Ribosome drop-off.** RISC might inhibit elongation of translation by initiating premature termination. **D. Cotranslational degradation of nascent polypeptides.** RISC has also been reported to recruit enzymes that degrade nascent polypeptides. Adapted from Wu and Belasco, 2008.

translation. The mechanisms by which miRNA containing ribonucleoprotein complexes (miRNPs) inhibit translation of target mRNAs are still highly controversial. To date, at least six different models have been proposed. (1) It has been suggested that the mid domain of human Ago2 and the translation initiation factor eIF4E compete for cap binding (Fig. 6A) (Kiriakidou et al., 2007). This idea however has recently been refuted (Kinch and Grishin, 2009). Nevertheless, a cap-dependent mechanism is supported by evidence that cap-independent translation under the control of internal ribosome entry sites seems to be insensitive to miRNA regulation (Fig. 6B) (reviewed in Wu and Belasco, 2008). In addition, some Argonaute proteins seem to bind to eIF4E, thereby blocking the interaction with other initiation factors (2) (reviewed in Iwasaki, 2009). Also, the fact that the number of ribosomes on the mRNA is reduced upon miRNP binding underpins this idea (Pillai et al., 2005). However, this could be explained by (3) 'ribosome drop-off' after translational initiation (Fig. 6C). Other proposed mechanisms of translational inhibition are (4) retarded elongation by translating ribosomes or (5) cotranslational degradation of nascent polypeptides (Fig. 6D) (reviewed in Wu and Belasco, 2008). Another way to repress translation is (6) the sequestration of mRNAs to discrete cytoplasmic foci, called processing bodies or P-bodies (reviewed in Iwasaki et al., 2009).

All these models are not mutually exclusive and on the basis of current evidence, it seems reasonable that miRNAs employ multiple mechanisms to repress translation of targeted messages (reviewed in Wu and Belasco, 2008). Effects might depend on particular cellular conditions or might be specific for individual targets. Interestingly, it has even been indicated that under certain conditions some small RNAs can translationally activate their target RNA (Vasudevan et al., 2007).

4.3 Advantages of small RNA mediated post-transcriptional control

The main role of cytoplasmic Argonaute complexes is the negative regulation of mRNAs. This serves as an additional regulatory level on top of transcriptional control. It further enables cells to react faster to changing needs in protein expression than the half-life of mRNAs originally would allow.

Using small RNAs rather than proteins to target the regulation of cytoplasmic RNAs has several advantages. First, it might be more practical for a cell to produce different adapter RNAs that are specific for their targets than to develop hundreds of proteins that exclusively recognize particular RNA molecules. Second, it allows the system to react quickly, as it requires no protein synthesis. Instead, all protein components are ready for action in the cytoplasm and await the small RNAs as adaptive elements to connect to their target transcripts. All together, this results in lower cost for the organism, as one system is able to target many different mRNAs.

5 Nuclear acting small RNA pathways

5.1 Biological functions of nuclear RNA interference

Nuclear and perinuclear Argonaute pathways, seem to have evolved mainly to inactivate harmful sequences. Uncontrolled transposon activity can disrupt genes, alter regulatory networks and can cause chromosomal abnormalities (reviewed in Malone and Hannon, 2009). A tight regulation of mobile elements is especially important in the germline as it prevents both short-term reductions in fertility due to germ cell loss and long-term reductions in fitness through accumulation of mutations (Aravin et al., 2008).

The discrimination of mobile elements from the cell's own DNA is not trivial, as transposons fall into many classes and only have little overall resemblance. Although mechanisms like regulated splicing patterns or sequence specific proteins can reduce the activity of some transposons, small RNAs have been shown to be the key mediators in the fight against transposons (reviewed in Malone and Hannon, 2009). The benefits of using nuclear small RNAs to combat mobile elements are evident. As in the cytoplasm, nuclear small RNAs are versatile adapters that allow the connection of the protein machinery to the target sequence. The nuclear small RNA systems are in addition extremely adaptive. Since targeted transcripts in many cases give rise to new small RNAs, either with the help of RNA-dependent RNA polymerases (RdRPs) (Axtell et al., 2006; Pak and Fire, 2007) or in the course of the ping-pong cycle (Brennecke et al., 2007; Gunawardane et al., 2007), minor changes in sequence are immediately incorporated. Moreover, small RNAs serve as an epigenetic memory. It has been demonstrated that they can be maternally deposited into fly and fish embryos (Blumenstiel and Hartl, 2005; Houwing et al., 2007). Inherited piRNAs are important for mounting an effective silencing response. The lack of maternal piRNA inheritance underlies the phenomenon of hybrid dysgenesis, where offspring from *Drosophila* strains that differ in the presence of a particular transposon are sterile (Brennecke et al., 2008).

Nuclear small RNAs not only target transposon sequences but also mediate heterochromatin formation of repetitive elements to avoid genome instability (Peng et al., 2008). A related function for nuclear small RNAs is the formation of centromeric heterochromatin. Centromeric DNA typically consists of large arrays of rapidly evolving satellite repeats. Deep sequencing studies have identified abundant endogenous siRNAs that map to centromere sequences in *S. pombe*, plants, *Drosophila* and vertebrates. It has been demonstrated in yeast and mouse that Dicer is essential for formation of functional centromers (Kanellopoulou et al., 2005; Volpe et al., 2003).

5.2 Target recognition by small RNAs in the nucleus

The connection between small RNAs and transcriptional silencing was first drawn in plants, where dsRNA directed sequence specific *de novo* methylation of promoters (Wassenegger et al., 1994) Interestingly, this DNA methylation was strictly limited to sequences homologous to the dsRNA. The fact that the targeted sequence was smaller than a nucleosome suggested a small RNA-DNA interaction (Pelissier and Wassenegger, 2000). More recent results, however, indicate that small RNAs might target DNA methylation and heterochromatin formation via small RNA–nascent transcript interactions. Among the first evidences for this was the finding that miR165/166, which is responsible for specific asymmetric DNA methylation in *Arabidopsis*, only has a recognition site on the target after RNA processing (Bao et al., 2004). Also, the involvement of a specialized DNA-dependent RNA Polymerase (Pol V) that interacts with AGO4 and is necessary to induce heterochromatic marks at several loci supports this notion (Wierzbicki et al., 2008; Li et al., 2006). It is however possible, that Pol V is needed only to open up the chromatin to allow small RNA-DNA base pairing (Wierzbicki et al., 2008).

Research in single cellular organisms also speaks in favour of target recognition via small RNA-RNA interactions. In *S.pombe*, RNA polymerase II is required for RNAi-dependent heterochromatin assembly. Tethering of the Argonaute containing RNA-Induced Transcriptional Silencing (RITS) to a nascent RNA initiates RNAi and heterochromatin dependent silencing (Buhler et al., 2006; Kato et al., 2005). Also the identification of an RNA helicase

required for the chromatin-Argonaute interaction in *Tetrahymena* implicates small RNA-noncoding RNA rather than RNA-DNA interactions prior to heterochromatin formation in ciliates (Aronica, 2008) (see **attachment 3** for a point of view article on this subject).

Small RNA dependent heterochromatin formation has also been reported in *C. elegans*, *D. melanogaster* and in mammals (see section 5.3) (reviewed in Djupedal, 2009). While the nature of small RNA mediated nuclear target recognition in *C. elegans* has not yet been described, there is genetic and biochemical evidence for an interaction between RNA polymerase II and the small RNA machinery in heterochromatic silencing in *Drosophila* (Kavi et al., 2009). Also in mammals, RNA Pol II transcripts seem to be required for siRNA-mediated heterochromatin formation (Han et al., 2007; Weinberg et al., 2006).

Taken together, it seems possible that small RNAs generally bind to nascent transcripts and not directly to DNA to establish and maintain heterochromatin. This was rather unexpected, as heterochromatin is generally considered transcriptionally silent. Recent studies provide a solution to this paradox (Chen et al., 2008; Kloc et al., 2008). At least in *S. pombe*, transcription of heterochromatin seems to depend on the stage of the cell cycle. The heterochromatic structure is proposed to break up during S-phase, enabling its transcription by RNA Pol II, with subsequent reassembly of heterochromatin and gene silencing. Whether a similar cell-cycle dependent heterochromatin transcription produces nascent transcripts for siRNA-chromatin interaction in other eukaryotes remains unknown.

5.3 Mechanisms of small RNA directed transcriptional silencing

After target recognition (see section 5.2), the Argonaute complex mediates heterochromatin formation by recruiting effector proteins. This mechanism is best understood at the centromeres of fission yeast (Fig. 7), where a self-reinforcing loop couples heterochromatin assembly and siRNA production (reviewed in Djupedal and Ekwall, 2009; reviewed in Ekwall, 2007; reviewed in Kloc and Martienssen, 2008). In *S. pombe*, centromeric heterochromatin

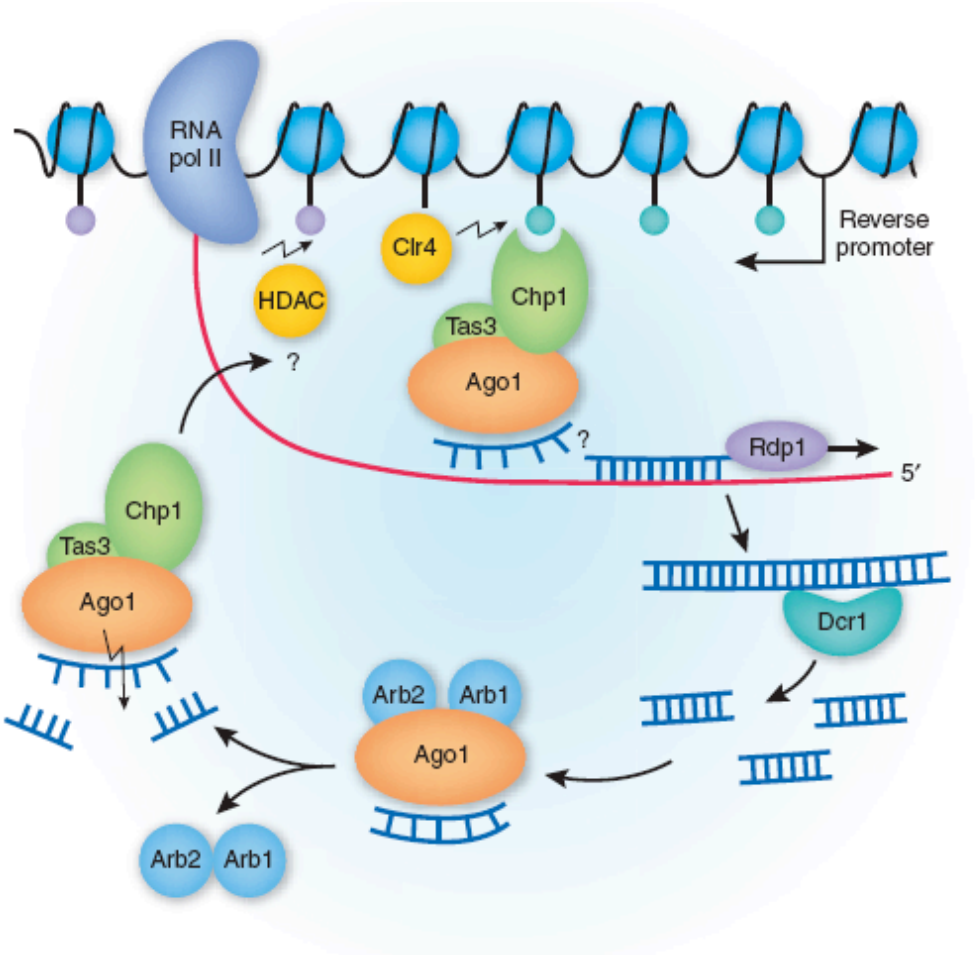

Figure 7. Model for RNAi-directed heterochromatin formation in *S. pombe*. Transcripts are generated by RNA Pol II from a centromeric promoter. siRNAs complementary to this transcript allow the RNA-dependent RNA polymerase (Rdp1) its conversion to dsRNA. Dicer (Dcr1) subsequently cleaves it into short ds siRNAs. These small RNA duplexes are loaded onto Ago1, currently complexed with Arb1 and Arb2. Following the dissociation of Ago1 from these two proteins, Ago1 associates with Chp1 and Tas3 to form the RITS. After passenger strand removal, Ago1 uses the siRNA as a guide to target RITS to homologous nascent RNA Pol II transcripts in the centromeric region. The RITS complex then stimulates histone methylation of the centromere at H3-K9 by the Clr4 histone methyltransferase enzyme. Histone methylation has to be preceded by histone deacetylation of acetylated H3K9, carried out by HDAC enzymes. (Question marks: unknown mechanisms; arrows: Ago1 slicer activity and nucleosome modifications; purple 'lollipops': acetylated H3-K9; teal lollipops: dimethylated H3-K9). From Ekwall, 2007.

formation depends on local transcription in S-phase. Ago1, complexed with a centromere specific siRNA, recognizes and cleaves the nascent transcripts. It is believed that this cleavage creates entry sites for the RNA-directed RNA polymerase complex (RDRC) (Irvine et al., 2006). The RDRC generates double stranded RNA which is processed by Dicer (Dcr1) into 21-24 nt small RNAs. These small RNA duplexes are loaded onto Ago1, which is at that time complexed with Arb1 and Arb2. These Argonaute siRNA chaperones are involved in siRNA maturation and required for siRNA generation and heterochromatin assembly (Buker et al., 2007). Following the dissociation of Ago1 from these two proteins, Ago1 associates with Tas3, a GW-repeat protein with unknown function and the chromo domain protein Chp1, forming the RITS complex. After the passenger strand of the small RNA has been removed by slicing, RITS is targeted to nascent transcripts that are homologous to the guide strand. The RITS complex then stimulates histone deacetylation of H3K9 followed by H3K9 methylation by the recruited Clr4 histone methyltransferase. This leads to a strengthened binding of the RITS complex to chromatin as Chp1 binds methylated histones (Schalch et al., 2009). Interestingly, mutations that interfere with Chp1 recruitment to RITS lead to a failure of heterochromatin formation. This means that the recruitment of RITS depends on both the guidance by siRNA and H3K9 methylation. This co-dependency has been proposed to ensure a stringent control of small RNA mediated heterochromatin formation at the correct locations. The molecular mechanisms of small RNA-directed heterochromatin formation in *Tetrahymena* are to some extend similar (see section 9 for details).

Nuclear Argonaute-dependent small RNA pathways also exist in metazoans. The mechanistic details, however, have not been described so far. In *C.elegans*, there is genetic evidence that Argonaute complexes are involved in chromosome segregation, meiotic silencing of unpaired DNA and transcriptional gene silencing of transgenes (Grishok et al., 2005; She et al., 2009; Robert et al., 2005; Claycomb et al., 2009). The situation in *Drosophila* is more extensively studied but highly controversial. As in *S. pombe*, heterochromatin in *Drosophila* is found in pericentromeric regions, mostly consisting of satellite repeats and transposable elements and is associated with H3K9 methylation. Despite these similarities, the evidence supporting a

role of small RNA mediated heterochromatin formation and transcriptional gene silencing remains indirect (reviewed in Fagegaltier, 2009). Theoretically, there are two systems that could contribute to heterochromatin formation in flies: the endogenous siRNA and the piRNA pathway. Most studies have investigated the role of the latter. The following evidence supports a role for the nuclear protein Piwi and the piRNA pathway in somatic heterochromatin formation: First, multiple copies of a transgene induce Piwi and Polycomb dependent transcriptional silencing of the transgene as well as of the endogeneous gene (Pal-Bhadra et al., 2004a). Second, mutations in the *Drosophila* Piwi proteins Piwi and Aubergine modify position effect variegation (PEV) (Pal-Bhadra et al., 2004b). Third, *Drosophila* Piwi directly interacts with the heterochromatin protein HP1a and loss of Piwi results in a partial loss of HP1 staining on polytene chromosomes (Pal-Bhadra et al., 2004b; Brower-Toland et al., 2007). piRNA-Piwi complexes might therefore direct heterochromatin formation in somatic cells in *Drosophila*. The difficulty with the described results is that piRNAs have not been detected outside of the reproductive tract (Malone et al., 2009b). As a result, the mechanisms underlying the observed Piwi-dependent somatic silencing have remained elusive. It is possible that piRNAs play an initiator role in heterochromatin establishment in the germline, resulting in the observed phenotypic changes in somatic cells. piRNAs and Piwi proteins are expressed in the germline (Brennecke et al., 2007; Kalmykova et al., 2005). However, also their direct involvement in heterochromatin formation remains to be demonstrated.

Mammals and plants do not only use histone, but also DNA methylation for the epigenetic repression of genes and transposons (Aravin et al., 2008; reviewed in Verdel et al., 2009). In mammalian cell lines synthetic siRNAs have been shown to induce transcriptional silencing on target promoter DNA in rare cases. It has been established that this is dependent on transcription of the targeted site and on recruitment of histone and/or *de novo* DNA methyltransferases (reviewed in Verdel et al., 2009). Several mechanisms seem to contribute, since changes either in DNA or in histone methylation or in both, have been detected after silencing of different loci (Hawkins et al., 2009). It is worth mentioning that even an Argonaute

dependent activation of transcription by synthetic small RNAs has been reported (Hawkins et al., 2009; Schwartz et al., 2008).

Mammalian endogenous siRNAs have so far only been identified in oocytes and mouse embryonic stem cells (Babiarz et al., 2008; Tam et al., 2008; Watanabe et al., 2008). Interestingly, a fraction of these maps to genic regions and depletion of Dicer significantly increased the expression of regions targeted by these small RNAs. It is possible that this is a result of defective small RNA dependent heterochromatin formation. A substantial fraction of endo-siRNAs and of the Dicer independent and germline specific piRNAs matches annotated transposons in mammals (Aravin et al., 2007). Interestingly, in mice a homozygous mutation in any single Piwi gene causes male sterility, while females bearing homozygous mutations in individual Piwi genes are viable and fertile (Carmell et al., 2007; Kuramochi-Miyagawaet al., 2004). This is likely because in female mice transposon rich loci give rise to both siRNAs and piRNAs, which probably act redundantly, while males seem to depend on piRNAs only to restrict transposon activity. How endo-siRNAs target transposons is unclear, but mammalian piRNAs in association with Piwi proteins act twofold. First, they destroy transposon transcripts by slicing, which additionally leads to an amplification of piRNAs (see section 2.3). Second, they guide DNA methylation, which is crucial for transposon silencing. In mice no direct recruitment the *de novo* methylation machinery by Piwi proteins could be detected but it has been proposed that DNA methylation might be preceded by specific histone modifications (Aravin et al., 2008).

Also in plants, where small RNA mediated mechanisms were first discovered, the RNA interference machinery has been demonstrated to promote methylation of histones as well as of DNA. Numerous reverse and forward genetic screens identified components of the responsible machineries. It turned out that plants and *S. pombe* have a large set of RNAi proteins, chromatin modifying proteins and their associated activities in common (reviewed in Verdel, 2009). Thus, the basic mechanisms seem to be highly conserved. The biggest differences between the systems are the presence of RNA-directed DNA methylation (RdDM) in addition to histone methylation and the existence of specialized polymerases in plants. Two

additional RNA polymerase complexes, Pol IV and Pol V, are responsible for generating transcripts essential for siRNA production and targeting of RdDM, respectively. The evolution of two distinct protein complexes for the production of distinct non-coding transcripts emphasizes this dual role of non-coding RNAs during transcriptional silencing. Another difference to yeast is the larger number of proteins involved in RNAi and additionally the high level of interconnectivity between different RNAi pathways (reviewed in Djupedal and Ekwall, 2009). A common result of nuclear small RNAi pathways in plants, however, is the methylation of cytosines. As in mammals, DNA methylation and histone modifications seem to be interconnected in self-reinforcing feedback loops. Unlike heterochromatin, RdDM does not seem to spread substantially into adjacent sequences (reviewed in Matzke and Birchler, 2005). Although certain levels of DNA methylation are present throughout the genome, it seems to be concentrated on repeat regions and centromers, implicating small RNAs in transposon silencing and centromere function. Small RNAs in plants are also involved in gene regulation and insertion of inverted repeats leads to small RNAs that are able to silence targeted promoters (reviewed in Matzke and Birchler, 2005; reviewed in Verdel and Ekwall, 2009).

In summary, the ability of nuclear Argonaute proteins to recognize and thereby target homologous sequences is highly conserved. It is clear that Argonaute proteins are not only central proteins in cytoplasmic RNA regulation but are also key players in nuclear small RNA mediated silencing mechanisms.

6 Transport of Argonaute proteins into the nucleus

As most organisms differentiate between cytoplasmic and nuclear RNA interference pathways, nuclear acting Argonaute proteins need to be specifically recognized and transported into the nucleus. This seems to depend on the canonical nuclear import machinery, as Importin 8 directly binds human Argonaute proteins and knockdown of Importin 8 reduces the nuclear Ago2 pool in HeLa cells (Weinmann et al., 2009). Moreover, the removal of the putative nuclear localisation signal (NLS) from *Drosophila* Piwi resulted in its cytoplasmic localization in the mutant (Saito et al., 2009). Also, the *C. elegans* Argonaute protein NRDE-3 depends on a functional NLS for nuclear localization (Guang et al., 2008). Interestingly, the nuclear import of NRDE-3 additionally depends on the loading of the protein with small RNA. This might be a common necessity for Argonaute proteins that are loaded in the cytoplasm but act in the nucleus. Also in mouse and *Drosophila* unloaded Argonaute proteins cannot enter the nucleus (Aravin et al., 2008; Malone et al., 2009a). How the state of the Argonaute proteins can be recognized is not understood. On one hand, it is possible that the NLS is only accessible for the import machinery if the Argonaute protein is complexed with RNA. On the other hand, it is also feasible that additional adapter proteins are necessary, which detect the difference between unloaded and loaded Argonautes to mediate the translocation of the latter. Another possibility is that factors quickly transport unloaded Argonautes out of the nucleus or that unloaded Argonautes are actively retained for loading in the cytoplasm by proteins covering the NLS. Evidence for this comes from studies of *Drosophila* Piwi, which normally localizes to the nuclei of germline cells. Mutants in the putative RNA helicase *armitage* have a defect in small RNA loading of Piwi and this results in a loss of Piwi from germ cell nuclei (Malone et al., 2009a). In contrast, Piwi expressed by transfection Schneider 2 (S2) cells localized to the nucleus, although not loaded under these conditions (Siomi et al., 2010). Since Piwi is normally not expressed in S2 cells it is possible that a factor,

coexpressed in the germline with Piwi, is missing in S2 cells, leading to the observed nuclear localisation. Future research will reveal how organisms distinguish between unloaded and mature Argonaute complexes and to what extent these mechanisms are conserved.

7 Chemical modifications of small RNA

Small RNAs do not only differ in their biogenesis pathway, their length and sometimes their 5' nucleotide bias, but also in their chemical modifications. RNA modifying enzymes are able to alter the genetically encoded nucleotides by either adding a chemical group (such as a methyl-, formyl-, acetyl-, isopentenyl-, etc. group) or by deamination, reduction or thiolation (reviewed in Grosjean, 2007). More than 120 distinct nucleotide modifications have been identified, of which 100 can be found in tRNAs. The situation is less complex in regard to small RNAs, as no more than one chemical modification has been reported.

7.1 Methylation of small RNAs

Some classes of small RNAs are modified at their 3' terminus. These carry a methyl group on the oxygen of the second 2' C of the ribose of the 3'-most nucleotide (Fig. 8A) (Yang et al., 2006). Terminal 2'-*O*-methylation of small RNAs in all studied cases depends on homologs of the methyltransferase HEN1 (HUA ENHANCER 1). This protein was first identified in *Arabidopsis*, where *hen1* mutants enhanced the phenotype of plants with mutations in *hua1* and *hua 2*, which are weakly comprised in stamen and carpel identities (Chen et al., 2002). As many aspects of the mutant phenotype are similar to those in Dicer (*dcl1*) mutants, it was proposed that HEN1 acted in small RNA metabolism (Park et al., 2002). Indeed, HEN1 encodes for the methyltransferase (MTase) that is responsible for small RNA modification in plants (Yu et al., 2005). The *S*-adenosylmethionine-dependent MTase domain is highly conserved among HEN1 homologs (Fig. 8B) (Park et al., 2002). Despite the conservation of the MTase activity of Hen1 from bacteria to mammals (Kirino and Mourelatos, 2007b; Saito et al., 2007; Yu et al., 2005; Chan et al., 2009), the specificity and biological function of the enzyme differs between species. Three subfamilies of Hen1 seem to exist and, with the

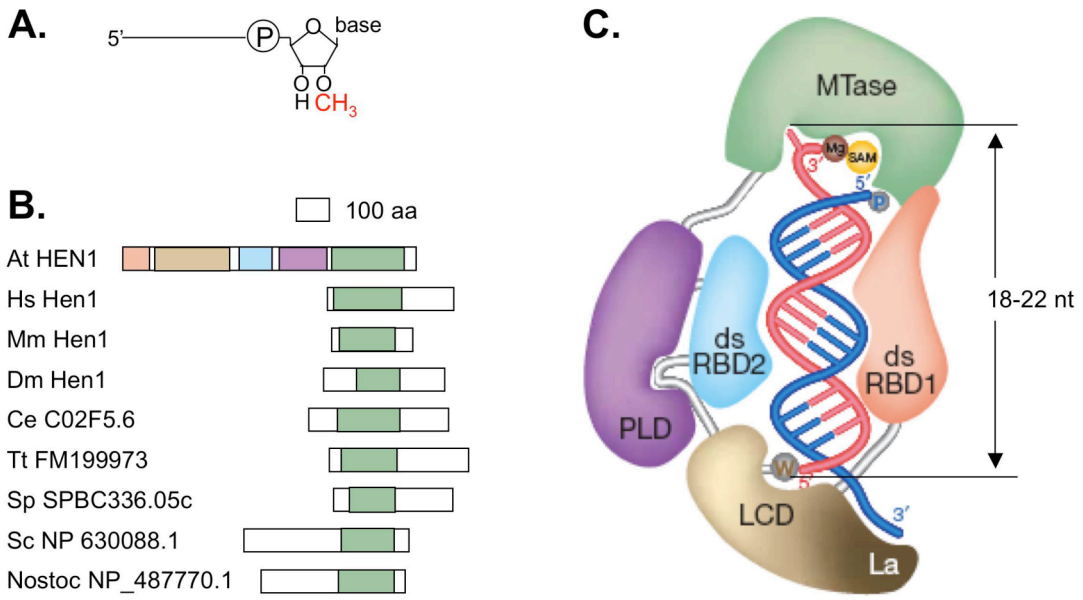

Figure 8. Small RNA methylation

A. Schematic view of a 2'-*O* methylated small RNA. Methyl group on th eterminal ribose of the RNA in red. **B. Schematic representation of HEN1 and its homologs.** The position of the conserved methyltransferase (MTase) domain is indicated as green boxes. *Arabidopsis* HEN1 additionally has four other domains: dsRNA binding domain (dsRBD) dsRBD1, orange; LA-motif containg domain (LCD), beige; dsRBD2, blue; PPIase-like domain (PLD), purple. At, *Arabidopsis thaliana*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Tt, *Tetrahymena thermophila*; Sp, *Schizosaccharomyces pombe*; Sc, *Streptomyces coelicolor*. Nostoc, Nostoc sp. PCC 7120. GenBank accession numbers of the proteins are shown after the species abbreviation Adapted from Park et al., 2002. **C. Structural model of At HEN1 complexed with substrate RNA.** Same color code as B. The RNA is gripped by the two dsRBD domains. The distance between the 5' end binding LCD and the 3' end modifying MTase domain determines the plant-specific size specificity of HEN1. Adapted from Huang et al., 2009.

exception of the MTase domain, Hen1 from plants, animals, and bacteria have nothing in common in terms of domain arrangement and size (Fig. 8B) (Mui Chan et al., 2009). In bacteria, Hen1 is part of an RNA repair system (Chan et al., 2009). Bacterial Hen1 seems to act on the 3' terminus of single stranded RNA after cleavage of RNA by ribotoxins. The 2'-*O* methylation at the cleavage site likely protects repaired tRNA against recutting. In

metazoans and plants, Hen1 is involved in 3' terminal 2'-*O* methylation of small RNAs. In plants, all classes of small RNAs seem to carry this modification (Li et al., 2005; Yu et al., 2005). Plant small RNAs are methylated before their loading onto Argonaute proteins reviewed in (reviewed in Chen, 2005). HEN1 recognizes small RNA duplexes by plant-specific N-terminal dsRNA-binding domains (Fig. 8B and 8C) (Huang et al., 2009) and requires 2 nt 3' overhangs which is the pattern produced by Dicers (Yu et al., 2005). Plant HEN1 additionally has a strict size requirement and only methylates 21-24 nt small RNA duplexes (Fig. 8C) (Yang et al., 2006). In contrast to this, methylation of small RNA is restricted to particular classes of small RNAs in metazoans. piRNAs and siRNAs but not miRNAs are 2'-*O*-methylated at their 3' termini (Horwich et al., 2007; Houwing et al., 2007; Kirino and Mourelatos, 2007a; Ohara et al., 2007; Ruby et al., 2006; Vagin et al., 2006). Hen1 homologues also mediate these reactions. However, the specificity of the enzymes is different. Animal Hen1 can only bind to certain Argonautes and small RNAs are modified on the respective proteins, after removal of the passenger strand (Horwich et al., 2007; Kirino and Mourelatos, 2007b; Saito et al., 2007). In accordance with this, recombinant *Drosophila* Hen1 has no size specificity (Saito et al., 2007). The Argonaute dependent RNA methylation in metazoans allows restriction of the methylation to specific classes of small RNAs.

7.2 Function of small RNA methylation

One definite function of small RNA methylation in *Arabidopsis*, is the protection of small RNA from a 3'-end uridylation activity (Li et al., 2005). As U tailing of RNA correlates with exonucleolytic degradation, unmethylated small RNAs in plants are reduced in abundance or completely absent. This also explains the phenotypic similarity of *hen1* and *dcl1* mutants. Stabilisation of small RNAs in plants might be necessary as plants are able to convey silencing information, which is probably mediated by small RNAs over long distances (Hamilton et al. 2002; Klahre et al. 2002; Yoo et al. 2004).

The role of small RNA methylation in animals is less clear. In *Drosophila*, steady state levels of piRNAs are barely reduced compared to

wild type and the expression level of most retrotransposons remains unchanged (Saito et al., 2007). Only HetA, an element highly sensitive to mutations that disrupt piRNAs function, quadrupled its expression in the *hen1* mutant (Horwich et al., 2007). However, *hen1* mutant flies are viable and fertile. Thus, the function of 3' end methylation in animals is unclear.

It is likely that terminal methylation of small RNAs generally impedes the activities of enzymes that target hydroxyle groups of the last nucleotide such as ligases, terminal nucleotidyl transferases or polymerases. This has been demonstrated *in vitro* as 2'-*O* methylation reduces the activity of t4 RNA ligase and yeast poly(A) polymerase on small RNA (Yang et al., 2006). An inhibition of poly(U) polymerases and 3'-5'-exonucleases by small RNA methylation in metazoans might have a similar stabilizing effect as in plants. This would also explain the choice of methylated small RNAs. siRNAs and piRNAs in animals seem to be mainly involved in silencing of transposons, which is a constant thread. A high turnover rate of these small RNAs is therefore unnecessary and might even be harmful for the cell, especially in transcriptionally inactive stages. In contrast, miRNAs are involved in gene regulation. As animals need to respond quickly to changing conditions, a higher turnover rate of miRNAs might provide an evolutionary advantage.

Alternatively, it has been proposed that certain primer dependent RdRPs might be able to distinguish between methylated and unmethylated small RNAs and might only use the latter for extension (reviewed in Chen et al., 2005). However, so far this has not been demonstrated in any organism.

Another very exciting function for the methyl group on small RNA might be its direct involvement in DNA or histone methylation. In this case, the methyl group would be directly transferred from the small RNA. Again, this remains to be demonstrated.

8 *Tetrahymena thermophila*

8.1 The model organism *Tetrahymena*

Tetrahymena thermophila (Fig. 9A) is a ciliated protozoan that occupies a key position in the branch of alveolates in eukaryotic evolution (Fig. 9B). The highly developed cells are motile and phagotropic, and although unicellular, *Tetrahymena* displays germline and somatic differentiation.

Despite its relative evolutionary distance to animals and plants, there is an amazing degree of conservation of macromolecules and mechanisms (reviewed in Frankel 1999). The cultivation on simple media and its rapid growth made it an attractive model organism for molecular and cellular biology since the 1940s. Landmark discoveries have been made in *Tetrahymena* since. These include the discovery of dynein motors (Gibbons and Rowe, 1965), self-splicing (Cech et al., 1981), telomers and telomerases (Blackburn and Gall, 1978; Greider and Blackburn, 1985) and the function of histone acetyltransferases (Brownell et al., 1996). The research on self-splicing and telomers and telomerases was rewarded with the Nobel prize in 1989 and 2009 respectivly.

The development of genetic tools for *Tetrahymena* is well advanced and has maintained this organism at the forefront of fundamental research. Genes can be exogenously expressed from rDNA minichromosomes or can be relatively easy endogenously tagged or knocked out by homologous recombination. The use of gene suppression by RNAi has also been established (Howard-Till and Yao, 2006). Constructs are introduced by biolistic bombardement with DNA coated particles, electroporation or microinjection. The ability to explore the role of recessive deleterious or lethal mutations, which can be propagated in the homozygous state in the transcriptionally silent micronucleus (Mic) (see section 8.2), further strengthens the role of *Tetrahymena* as a model organism (reviewed in Turkewitz, 2002).

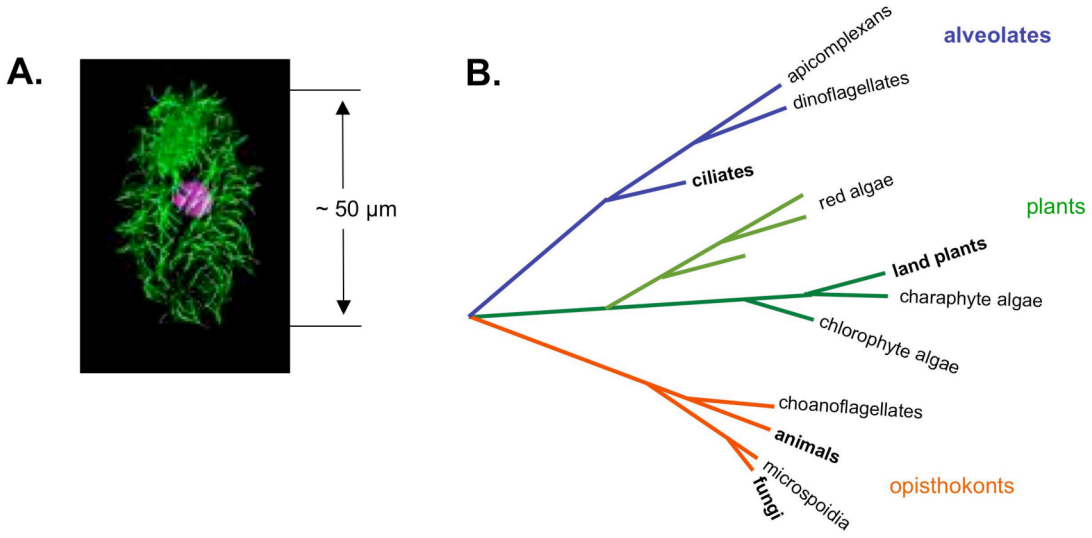

Figure 9. *Tetrahymena thermophila* is a ciliated protozoan and displays nuclear dimorphism.

A. Microscopic image of a *Tetrahymena*. The cilia are immunofluorescently stained against tubulin in green, the DNA of the two nuclei is shown in pink. **B. Phylogenetic tree.** Three of eight major eukaryotic groups are depicted in this phylogenetic tree. Alveolates, blue; plants, green; opisthokonts, red. Subgroups containing organisms mentioned in this work are in bold. This tree is based on a consensus of molecular and ultrastructural data. Adapted from Baldauf, 2003.

The 104 Mb macronuclear (Mac) genome sequence is available on the *Tetrahymena* genome database (www.ciliate.org) (Eisen et al., 2006). ~27,000 open reading frames have been predicted and sequencing of the Mic genome is under progress. The recent release of the *Tetrahymena genome expression database* (http://tged.ihb.ac.cn), which contains information about the genome-wide expression of *Tetrahymena* genes during growth, starvation and conjugation, supplies the *Tetrahymena* community with yet another powerful tool (Miao et al., 2009).

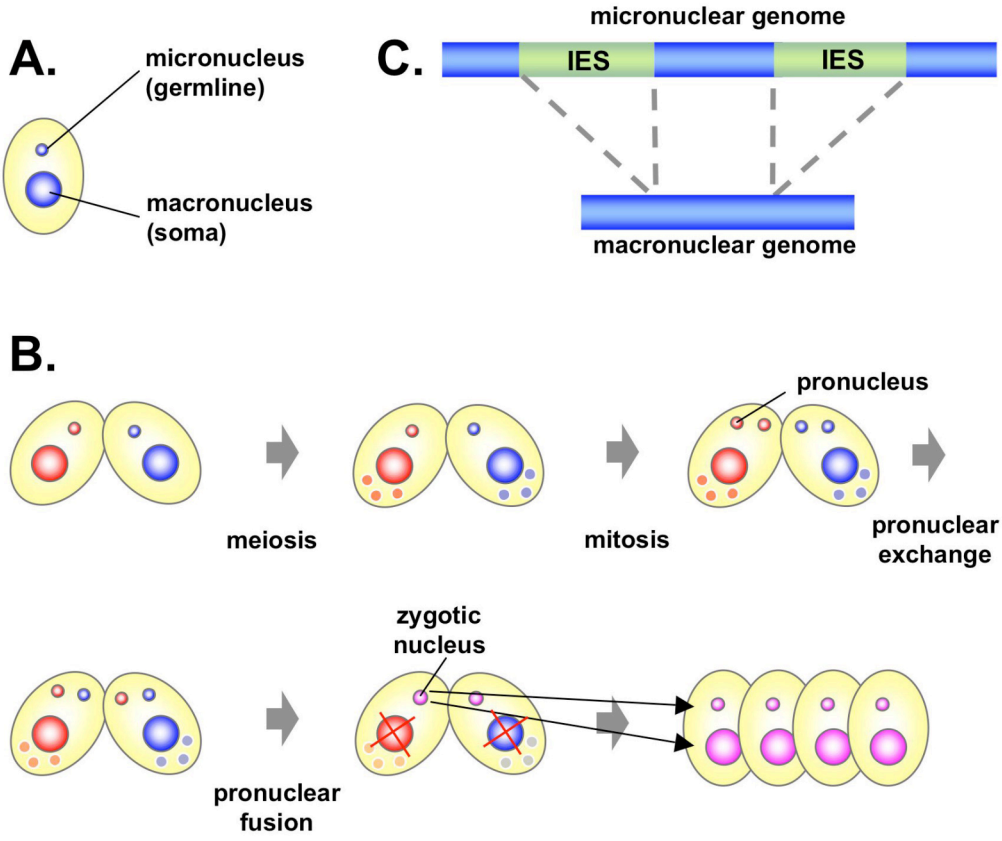

Figure 10. Nuclear dimorphism in *Tetrahymena thermophila*.

A. Schematic illustration of nuclear dimorphism. *Tetrahymena* contains two different nuclei in each single cell. The smaller one (micronucleus) represents the germline. It is transcriptionally inactive during vegetative growth and displays its function only during sexual reproduction. The bigger one (macronucleus) contains the somatic genome and is transcriptionally active. **B. Conjugation process.** Mating is initiated when cells of different mating types are starved and mixed. When two cells conjugate, the micronucleus displays its germline properties. It undergoes meiosis, mitosis and cross-fertilization. The zygotic nucleus gives rise to new micro- and macronuclei. **C. DNA elimination.** Internal eliminated sequences (IES, green) are precisely removed during the development of the new macronucleus. Macronucleus destined sequences (blue) are re-ligated.

8.2 Nuclear Dimorphism

Tetrahymena displays nuclear dimorphism (Fig. 9A and 10A) (reviewed in Frankel, 1999). The diploid Mic performs germline function and is transcriptionally silent during vegetative growth. The soma-like Mac contains multiple copies of the rearranged micronuclear genome and is the centre of gene expression. During vegetative growth, the Mic divides mitotically, whereas the Mac chromosomes are distributed randomly by amitosis. The generation time of vegetatively growing *Tetrahymena* in rich medium is typically 2-3 hours. Upon starvation, however, cells undergo a sexual reproduction event, called conjugation (Fig. 10B). Conjugation starts with pairing of two cells of different mating type and involves a succession of nuclear processes. The germline micronucleus displays its germline properties: It undergoes meiosis, followed by a postmeiotic mitosis. This leads to the formation of gametic nuclei (pronuclei) that are exchanged between the cells. After reciprocal cross-fertilization, new Mics and Macs develop from the zygotic nucleus, while the parental Mac is destroyed. The cells separate and, upon supply of nutritions, divide to form new vegetatively growing cells. Although the zygote gives rise to new Mics and new Macs, these nuclei differ in their DNA content, since DNA elimination occurs during the development of the new Mac (Fig. 10B). The mechanisms underlying this process include small RNA mediated histone modifications and programmed DNA rearrangements and will be discussed in detail later (see section 9).

8.3 Small RNA pathways in *Tetrahymena*

RNAi pathways are predicted to exist in *Tetrahymena*. It expresses three Dicer-related proteins, one RdRP and eleven Argonaute proteins of the Piwi clade (Twis) (Lee and Collins, 2007; Mochizuki and Gorovsky, 2005; reviewed in Seto, 2007).

The diversity of small RNAs in *Tetrahymena* is accordingly, extremely high. Profiling of vegetatively expressed small RNAs yielded numerous small RNA (sRNA) classes (Couvillion et al., 2009). These derive from pseudogene families, distinct types of repeats, predicted stem loop structures and RNAs that are anti-sense to introns and exons of predicted protein-coding genes.

The best-studied class of small RNAs that are expressed throughout

the life cycle consists of 23-24 nt siRNAs associated with Twi2p. Two essential enzymes, RNA-dependent RNA polymerase 1 (Rdr1) and Dicer 2 (Dcr2), cooperate in their production (Lee and Collins, 2007; Lee et al., 2009). A high percentage of these small RNAs maps to clusters in the Mac genome (Couvillion et al., 2009). Interestingly, transcripts from these loci are hardly detectable (Lee and Collins, 2006). The abundance of these small RNAs, together with the absence of target RNAs and the cytoplasmic localization of Twi2p, suggests it to be involved in post-transcriptional gene silencing (Couvillion et al., 2009). The existence of such a pathway in *Tetrahymena* has been experimentally demonstrated by overexpression of hairpin RNAs. This leads to the production of 23-24 nt RNAs and to a large reduction of the targeted transcripts (Howard-Till and Yao, 2006).

Small RNAs associated with the nuclear Argonaute protein Twi8p originate from mRNA producing loci. It has been proposed that they mediate co-transcriptional regulation. They might be involved in reducing the mRNA production from convergent genes (Couvillion et al., 2009). The function of other vegetatively expressed classes of small RNAs, like low-copy repeat sRNAs associated with Twi7p or telo-sRNAs associated with Twi10p, is not understood.

Another class of small RNAs are the 26-31 nt scan RNAs (scnRNAs). These Mic-derived conjugation-specific small RNAs are the central players of the small RNA directed DNA elimination pathway in *Tetrahymena*, and will be discussed in more detail.

9 Small RNA directed DNA elimination in *Tetrahymena*

During the sexual reproduction of *Tetrahymena*, the DNA of the developing new macronucleus undergoes extensive rearrangements. Breakage of the five large chromosomes, inherited from the micronucleus, fragments the genome into approximately 250 macronuclear chromosomes (reviewed in Chalker, 2008). In addition, 15% of the genome is eliminated. Approximately 6000 IES (internal eliminated sequences), of 0.5 to >20 kb, are excised and their flanking sequences are religated. The DNA elimination pathway involves the comparison of three nuclei (the meiotic Mic, the parental Mac and the developing new Mac) using scnRNAs. This ensures, that sequences that have not been present in the parental Mac will not be present in the new Mac either.

9.1 The scnRNA model

To explain how IES are specifically eliminated during Mac development, the scnRNA model has been developed (Mochizuki et al., 2002; Mochizuki, 2004a). The pathway starts with the acquisition of TATA-binding protein, the histone variant H2A.Z and histone acetylation, to the otherwise silent Mic, in early conjugation (Stargell et al., 1993; Stargell and Gorovsky, 1994). This leads to the bidirectional transcription of the micronuclear genome (Chalker and Yao, 2001). The generated, non-coding transcripts are capped but not polyadenylated and seem to be generated by RNA Pol II (Mochizuki and Gorovsky, 2004c; Chalker and Yao, 2001). Transcription start and end are not well defined and transcripts starting in flanking DNA and continuing into IESs have been detected (Fig. 11A) (Aronica et al., 2008; Chalker and Yao, 2001; Schöberl and Mochizuki, unpublished). The transcripts presumably form dsRNA, which is cleaved by a Dicer-like protein (Dcl1p) into 28-30 nt long scnRNAs (Fig. 11B) (Malone et al., 2005; Mochizuki and Gorovsky, 2005). scnRNAs have been shown to associate with the

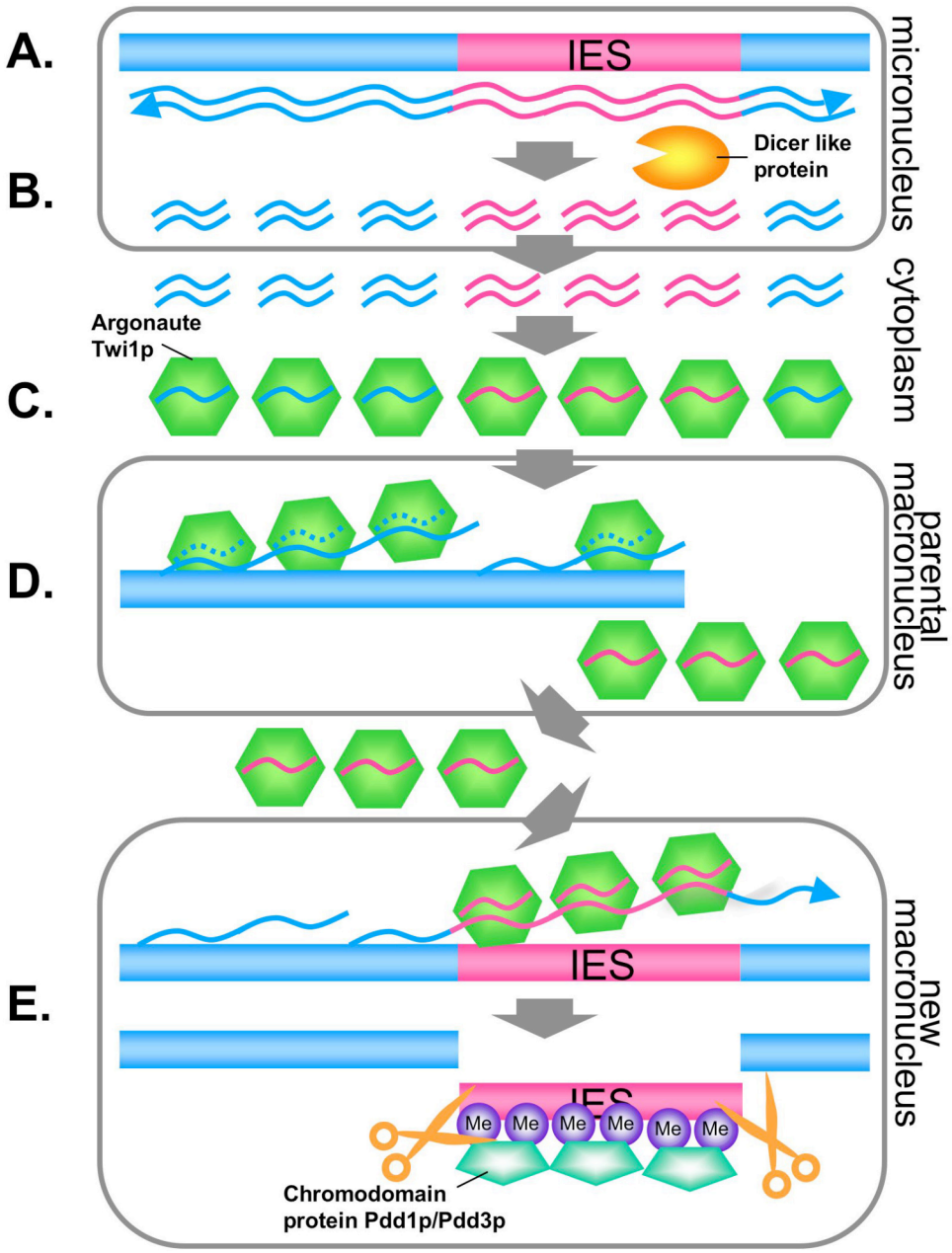

Figure 11. The scanning model

Consecutively occurring events are shown from top to bottom. **A. Production of double stranded RNA** (thin lines) by bidirectional transcription of genomic DNA (thick line). **B. production of scnRNAs** by dicer-like protein Dcl1p (yellow). **C. Association of scnRNAs with Twi1p** (green). **D. Scanning.** scnRNA is compared with non-coding RNA. **E. Heterochromatin formation and IES elimination.** (histone methylation: purple; excisase: orange).

conjugation-specific Argonaute protein Twi1p (Fig. 11C) (Mochizuki and Gorovsky, 2004a). As micronuclear Twi1p localisation was never observed, it has been proposed that scnRNA is loaded onto Twi1p in the cytoplasm (Mochizuki et al., 2002). Since scnRNAs are derived from promiscuous transcription of the micronuclear genome, they are not specific for the IES sequences they are supposed to target for elimination in the developing Mac. Therefore, scnRNAs need to be selected for IES specificity. For that reason, the Twi1p-scnRNA complex is transported into the parental macronucleus, where scnRNAs are compared to the genome by base pairing with nascent transcripts (Fig. 11D) (Aronica et al., 2008). scnRNAs complementary to the parental Mac DNA are removed from the pool of small RNAs during a mechanistically undefined process. On the contrary, sequences homologous for IES are enriched (Mochizuki and Gorovsky, 2004a). The Mic-specific scnRNAs, in complex with Twi1p, are then transported into the developing new Mac where they induce heterochromatin formation on the IES (Fig. 11E). Nascent noncoding transcripts seem to mediate the interaction between Twi1p-scnRNA complexes and chromatin (Aronica et al., 2008). The scnRNAs target H3K27 and H3K9 trimethylation (Taverna et al., 2002; Liu et al., 2007b), which leads to the recruitment of the chromodomain proteins Pdd1p and Pdd3p (Madireddi et al., 1996; Nikiforov et al., 1999). The sequences targeted in this process are subsequently packaged into heterochromatic, nuclear substructures, called ‘dumposomes’ and are eventually deleted (Madireddi et al., 1996).

9.2 Remaining questions in small RNA mediated DNA elimination

Although this small RNA mediated DNA elimination pathway has been intensively studied in the past years, there are still many exciting open questions to be answered.

First, it is unknown how the promiscuous transcription in the meiotic micronucleus is regulated. During sexual conjugation, RNA Pol II is temporarily transported into this otherwise silent nucleus (Mochizuki and Gorovsky, 2004c) where it localizes with other hallmarks of active transcription (see section 9.1). We believe that there might be an activating

factor that either associates with the RNA polymerase itself or binds to the DNA to allow this promiscuous transcription. The same activator might also be required for the production of the detected non-coding transcripts in the parental and the developing new Mac (Aronica et al., 2008).

Second, it has been observed that scnRNAs have a 5' Uracil bias (Lee and Collins, 2006), yet, the mechanism underlying this bias is unknown. It is possible that it is generated by Dicer-like protein (Dcl1p), which is responsible for scnRNA biogenesis. As Dcl1p has no recognizable RNA terminus-binding domain, it might not be processive. Alternatively, the 5' bias might be the result of a 5' nucleotide specificity of the Twi1p loading machinery or of Twi1p itself as it has been described for *Arabidopsis* Argonautes (Mi et al., 2008).

Third, another puzzling aspect of the RNA-directed DNA elimination pathway in *Tetrahymena* is the transport of the involved proteins into the different nuclei. RNA polymerase II localizes to the Mac in vegetative cells. However, upon mating, it is transiently transported into the micronucleus (Mochizuki and Gorovsky, 2004c). It has been demonstrated that micro- and macronuclear pore complexes diverge in their protein composition (Malone et al., 2008; Iwamoto et al., 2009). Differential expression of import receptors might therefore explain the specific targeting of proteins to either the Mic or the Mac. It is also unknown how the scnRNAs are exported from the micronucleus and the factors that help their loading onto Twi1p remain elusive.

Fourth, the role of the slicing activity of Twi1p is not clear. Slicing of mRNA in the cytoplasm is well studied, and also its role during piRNA production is established. However, the function of slicing activity of nuclear Argonaute proteins is poorly understood. It has been demonstrated in *S. pombe* and in *Arabidopsis* that the slicing activity of nuclear Argonaute proteins is required to induce heterochromatin formation (Qi et al., 2006; Irvine et al., 2006). Whether small RNA-nascent RNA pairing leads to slicing and whether this transcript cleavage functions in silencing is currently unclear. However, it has been shown in other organisms that slicing is required for passenger strand removal after the incorporation of the small dsRNA into the Argonaute protein (Rand et al., 2005; Steiner et al., 2009). As the elimination of the passenger strand is a prerequisite of target recognition, inefficient

passenger strand elimination instead of absent transcript slicing might be the reason for impaired heterochromatin formation. Thus, the role of the slicing activity of nuclear Argonaute proteins requires further investigation.

Fifth, one of the least understood mechanisms of the pathway is the scanning process. How is it possible that small RNAs, probably homologous to the entire Mic genome, are compared to 104 Mb of the Mac genome? And how does the cell designate the pools of homologous and non-homologous small RNAs, so that only the latter move into the developing new Mac? It is likely that homologous small RNAs are targeted for degradation by specific nucleases. Active turnover of small RNAs has been described in plants and *C. elegans* (Chatterjee and Grosshans, 2009; Ramachandran and Chen, 2008). Uridylation has been proposed to target RNAs for exonucleolytic cleavage, while 3' adenylation and 3' *O* methylation of the terminal nucleotide can have stabilizing effects on small RNAs (Li et al., 2005; Ramachandran and Chen, 2008; van Wolfswinkel et al., 2009). Another possibility is that the non-coding transcripts from the parental macronucleus serve as molecular sponges that sequester scnRNAs homologous to sequences in the parental macronucleus (Lepere et al., 2008). A similar sequestering mechanism has been described in plants, where the activity of miRNAs is regulated by the expression of RNAs that mimic their targets and thereby reduce the amount of available miRNA (Franco-Zorrilla et al., 2007). The sequestration model, however, does not account for the observed loss of scnRNAs homologous to the macronuclear genome during conjugation in *Tetrahymena* (Aronica et al., 2008; Mochizuki and Gorovsky, 2004a). It has also not been observed that a large fraction of Twi1p is detained in the parental Mac after scanning.

Seventh, Twi1p-scnRNA complexes induce heterochromatin formation on the eliminated DNA in the developing Mac. The IES are packaged into heterochromatic nuclear substructures, called dumposomes. How this compaction of heterochromatin works is not well studied. It might be related to the formation of higher eukaryotic heterochromatin structures at the periphery of nuclei.

Eighth, IES are subsequently removed and the Mac destined sequences are religated. How the exact IES boundaries are established is unknown. The DNA elimination machinery is thought to recognize the very

loose consensus 5'-ANNNNT-3' (Saveliev and Cox, 1996). Indeed, *in vitro* studies performed in our lab indicate that a PiggyBac transposase recognizes this sequence (Cheng et al., in press). However, additional factors are necessary to determine which site is cleaved. Small RNAs are probably not able to target heterochromatin very precisely as target recognition occurs via noncoding transcripts (Aronica et al., 2008). Therefore, some DNA features might additionally define the cleavage site. A cis-acting polypurine tract (A_5G_5) that has been shown to be necessary for the removal of the M element and a similar sequence has been found for the R element. These sequences might lead to nucleosome exclusion to allow binding of the elimination machinery (reviewed in Segal and Widom, 2009). However, since a polypurine tract is not found in all IES, other sequences might influence the cleavage. The enzymatic machinery that carries out the DNA destruction and end-joining reaction remains also unknown.

Aims of the thesis

Small RNAs ~20–30 nt in length regulate gene expression at transcriptional and post-transcriptional levels. In the plant *Arabidopsis*, all small RNAs are 3'-terminal 2'-*O*-methylated by HEN1, whereas only a subset of small RNAs carries this modification in metazoans. This methylation is known to stabilize small RNAs, but its biological significance remained unclear. *Tetrahymena* expresses two classes of small RNAs: the constitutively expressed 23–24 nt siRNAs and the 28–29 nt scnRNAs that are expressed only during sexual reproduction. We decided to study whether these classes of small RNAs are also 3' terminally modified. If so, we wanted to understand the function of small RNA methylation in *Tetrahymena*.

→ Please see Attachment 1

Some Argonaute proteins are loaded with small RNAs in the cytoplasm but act in the nucleus. Regulatory mechanisms must exist that initiate only the transport of loaded Argonautes. In the second part of my PhD study, I was involved in a project that investigated how loaded Argonaute proteins are recognized in the cytoplasm. The *Tetrahymena* Argonaute protein Twi1p is loaded with scnRNAs before its nuclear import. We identified a novel protein, Giw1p, which binds to Twi1p and initiates the selective transport of only the mature Twi1p-scnRNA complex into the nucleus. My aim was to understand in vitro how Giw1p detects the state of the Twi1p-small RNA complex.

→ Please see Attachment 2

Summary and Discussion of publications

This thesis describes work carried out from November 2006 to December 2009 in the laboratory of Dr. Kazufumi Mochizuki at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA) in Vienna, Austria. The thesis is written as a cumulative dissertation.

Publication 1:

2'-O-methylation stabilizes Piwi-associated small RNAs and ensures DNA elimination in *Tetrahymena*

Henriette M. Kurth and Kazufumi Mochizuki. **RNA**. 2009 Apr;15(4):675-85.

Tetrahymena expresses 23-24 nt siRNAs constitutively and 28-29 nt scnRNAs specifically during conjugation. We found that scnRNAs, but not 23-24 nt siRNAs carry a methyl group at their 3' terminus. The *Tetrahymena* HEN1 homologue, Hen1p, is responsible for this modification. Hen1p localizes to the parental macronucleus and is only detectable during a short window of time early in conjugation, when the Argonaute protein Twi1p localizes to this nucleus. Hen1p directly interacts with Twi1p, which indicates that scnRNAs are methylated when they have already complexed with Twi1p. *Tetrahymena HEN1* knockout (KO) strains have no obvious defects during vegetative growth and the accumulation and the length of the constitutively expressed siRNAs is unaffected. On the contrary, the viability of sexually produced progeny is greatly reduced and DNA elimination is partially affected. This is most likely due to destabilization of scnRNAs in the absence 2'-*O*-methylation. Although scnRNA levels are similar between the wildtype and the KO strains early in conjugation, the levels drop in KO cells in later stages. This

demonstrates that Hen1p-mediated 2'-*O*-methylation specifically regulates the turnover of scnRNAs.

We believe that the main role of 2'-*O*-methylation is scnRNA stabilization. The mechanism of how small RNA methylation prevents degradation, however, remains unknown. It either may protect from exoribonuclease attacks or increase the binding affinity of the small RNA to the Argonaute protein. The 3' terminus of small RNAs is anchored in the PAZ domain methylation might change the affinity of the small RNA to this domain. A reduced binding affinity of the small RNA would lead to degradation, as scnRNA is unstable in the absence of Twi1p (Mochizuki and Gorovsky, 2004a).

The degradation of small RNAs is also part of a physiological mechanism in the DNA elimination pathway. scnRNAs homologous to sequences in the macronucleus are reduced during scanning. We initially speculated that scnRNA methylation might play a direct role in this process. The methyl group might be actively removed in case scnRNAs associate with complementary sequences to initiate degradation. Alternatively, scnRNAs could be only methylated if they are not homologous to the macronuclear DNA and only then be transported into the new developing macronucleus. Mutations in essential components of the small RNA-mediated DNA elimination pathway, however, completely block the production of sexual progeny. The partial defect observed in *HEN1* KO cells therefore indicates that the methylation of scnRNA is not essential for the scanning process. The nuclease(s) responsible for the observed reduction of scnRNAs during scanning must work independently of RNA methylation.

It has recently also been reported that small RNAs associated with Twi8p are modified (Couvillion et al., 2009), most likely by Hen1p mediated 2'-*O*-methylation. Twi8p is expressed throughout the *Tetrahymena* life cycle and its associated small RNAs have been implicated in co-transcriptional gene regulation (Couvillion et al., 2009). It is likely that small RNA methylation also stabilizes these RNAs. In spite of this, *HEN1* KO cells do not show any obvious defects during vegetative growth. This either means that other vegetatively expressed small RNAs have redundant functions to Twi8p-associated small RNAs or that they are subjected to a feedback regulation

that ensures stable levels. In these cases, stabilisation of Twi9p-associated RNAs is not essential. It is probably more economic for cells to improve the stability of small RNAs. In contrast, *Tetrahymena* scnRNAs need to be stable for a certain time period. They are produced in the micronucleus early in conjugation (Malone et al., 2005; Mochizuki and Gorovsky, 2005) and induce heterochromatin formation approximately seven to eight hours later. No secondary scnRNA production has been described so far. scnRNA methylation is therefore necessary for efficient DNA elimination.

Small RNAs associated with other Argonaute proteins in *Tetrahymena* are not modified at their 3' termini (Couvillion et al., 2009). This implies that Hen1p specifically recognizes only Twi1p and Twi8p and methylates only their associated RNAs. This is reminiscent of *Drosophila* and mouse Hen1p activity, which is also restricted to a subgroup of Argonaute proteins. In metazoans, only small RNAs associated with Argonautes from the Piwi clade are methylated (Saito et al., 2007). This suggested that Hen1 recognizes all Piwis but no Argonaute proteins. However, in *Tetrahymena,* all twelve Argonaute proteins belong to the Piwi clade. Our finding, that 23-24 nt siRNAs are unmodified was the first report describing Piwi-associated small RNAs that are not modified by 2'-*O*-methylation. How *Tetrahymena* Hen1p distinguishes between different Piwi proteins is unknown. Structures of Piwi proteins are so far not available. Interestingly, Twi1p, Twi8p and Hen1p localize to the parental Mac (Couvillion et al., 2009; Mochizuki et al., 2002). Only small RNAs that localize to the nucleus may therefore be 2'-O-methylated in *Tetrahymena*. Consistent with this idea, GFP-Twi2p and GFP-Twi12p, which both associate with non-modified small RNAs, localize to the cytoplasm (Couvillion et al., 2009).

Publication 2:

The *Tetrahymena* Argonaute-binding protein Giw1p directs a mature Argonaute-siRNA complex to the nucleus

Tomoko Noto[1], **Henriette M. Kurth**[1], Kensuke Kataoka, Lucia Aronica, Leroi DeSouza, Michael Siu, Ronald Pearlman, Martin Gorovsky, Kazufumi Mochizuki. **Cell**. 2010 Mar 5;140(5):692-703.

[1] with equal contribution

How nuclear-acting Argonaute proteins that are loaded in the cytoplasm are recognized and transported into the nucleus is not well understood. We find that the nuclear localization of the *Tetrahymena* Argonaute protein Twi1p depends on small RNAs. Neither unloaded Twi1p nor Twi1p associated with double-stranded RNA can enter the parental macronucleus. Only the mature complex, consisting of Twi1p and single-stranded scnRNA is imported.

We identified a novel Twi1p-interacting protein, Giw1p (gentleman-in-waiting). Giw1p is expressed specifically during sexual conjugation and localizes to both the cytoplasm and the nuclei. DNA elimination is inhibited in the absence of Giw1p and *GIW1* KO strains do not produce any viable progeny. In addition, the strains display a developmental arrest and 70% of cells abort mating in mid conjugation. Giw1p is dispensable for scnRNA production, loading of the scnRNA onto Twi1p and for passenger strand removal, but is necessary for the nuclear transport of the mature Twi1p complex. Giw1p does not seem to be a general nuclear transporter but rather is specific for Twi1p. Giw1p directly interacts with the PAZ and the Piwi domain and initiates the nuclear import of only the mature Twi1p-scnRNA complex. This is the first report of a protein that can sense the loading-state of an Argonaute protein.

It had been observed that the nuclear localization of Argonaut proteins depends on the presence of their small RNA cargos (Aravin et al., 2008;

Guang et al., 2008), but the mechanistic details underlying these findings were not understood. We identified the novel Twi1p interaction partner Giw1p that seems to be responsible for the selective nuclear import of the mature Twi1p complex. Giw1p binds to wild type Twip, which is associated with single stranded RNA, but not to slicer dead Twi1p, which is complexed with a scnRNA duplex. In vitro experiments confirmed that the presence of dsRNA inhibits the Giw1p-Twi1p interaction. Giw1p binds to the PAZ and the Piwi domain of Twi1p. These domains line the small RNA binding groove in the structure of archaeal Argonautes (Song et al., 2004). Interestingly, the conformation of a bacterial Argonaute protein changes upon transition from the tertiary to the secondary complex (see Fig. 4 of Introduction) (Wang et al., 2008b). The space between the PAZ and the Piwi domain is wider when the protein is associated with both guide- and passenger strand (open conformation) than when it is associated with single-stranded RNA only (closed conformation). We propose that the scnRNA passenger strand removal similarly alters the distance between the PAZ and the Piwi domain in Twi1p, allowing Giw1p to bind only the mature complex. This model explains how Giw1p might distinguish between the tertiary and the secondary Twi1p complex to target only the latter into the nucleus.

The model, however, does not explain why unloaded Twi1p is not imported into the nucleus. In vitro, Giw1p binds to Twip even in the absence of RNA. Also removal of RNA from lysate does not completely abolish the binding between endogenous Twi1p and Giw1p. In this experiment, however, Giw1p is already associated with Twi1p when the scnRNA is removed, which might distort the result. Unfortunately, we were not able to do pull down Twi1p from *DCL1* KO strains, which do not generate scnRNAs, because of a mating defect. It has been proposed that unligated Argonaute is flexible and probably switches between the open and the closed conformation in vitro (Rashid et al., 2007). The flexibility might be restricted only upon formation of the Argonaute-siRNA complex. I therefore propose a model in which unloaded Twi1p is kept in the open conformation in vivo by unidentified cytoplasmic chaperones or Twi1p loading factors, so that only the mature complex is recognized by

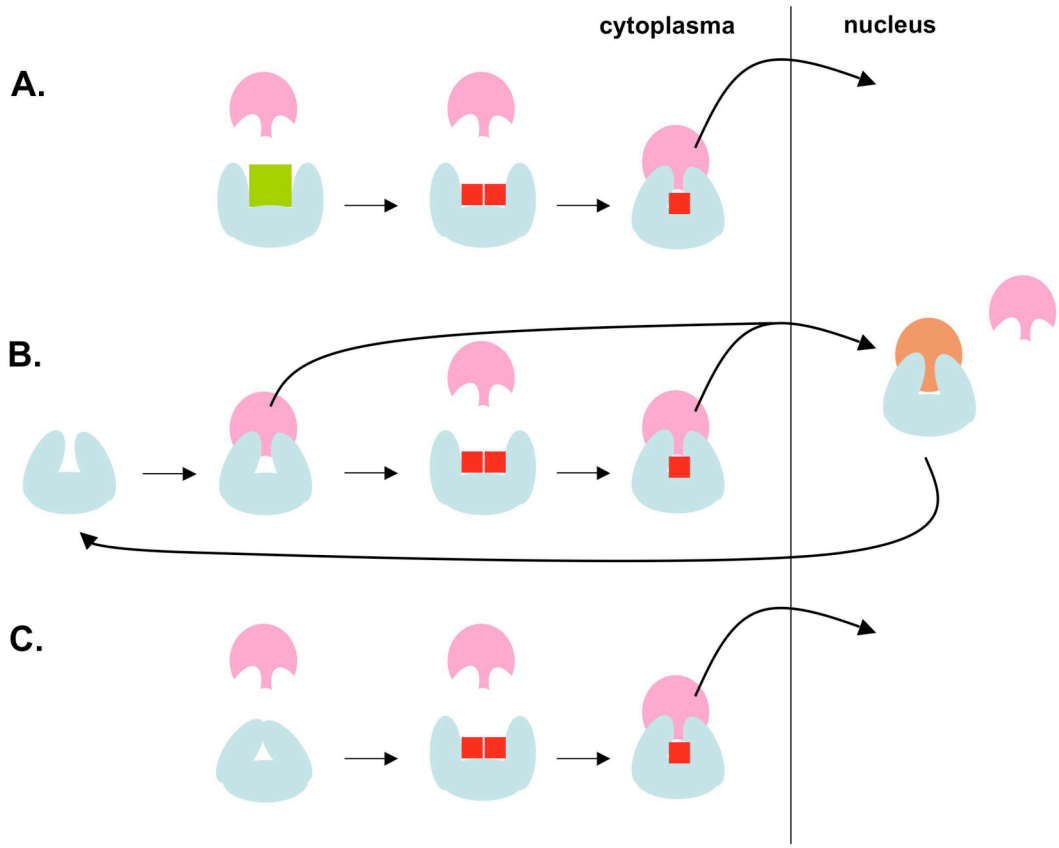

Figure 12. Models of Giw1p dependent nuclear import of Twi1p

A. Giw1p recognizes only Twi1p loaded with single stranded RNA because unloaded Tw1ip is kept in an open conformation. Giw1p might recognize the closed conformation of both unloaded Twi1p and Twi1p loaded with a single stranded RNA. However, chaperones might keep the unloaded Twi1p in an open conformation. This would prevent binding of Giw1p and only the mature complex would be transported into the nucleus. (Giw1p, pink; Twi1p, grey; RNA, red squares (two squares, double-stranded; one square, single stranded); chaperone, green) **B. Giw1p recognizes both unloaded Twi1p and Twi1p loaded with single stranded RNA.** In this case, both complexes would be imported into the nucleus. Another mechanism (orange) must recognize unloaded Twi1p in the nucleus to transport it out to the cytoplasm where it again would have a chance to be loaded. **C. Giw1p can distinguish between the three loading states of Twi1p.** Giw1p would transport only the mature Twi1p-scnRNA complex into the nucleus.

Giw1p and is transported into the nucleus (Fig. 12A). Alternatively it is possible that Giw1p binds unloaded Twi1p in vivo and mediates its nuclear import. If this is the case, a mechanism must exist that quickly transports unloaded Twi1p out of the nucleus (Fig. 12B). It is a third possibility that Giw1p can distinguish between the three states of Twi1p - unloaded, associated with double-stranded and associated with single-stranded RNA - in vivo (Fig. 12C). The structures of eukaryotic Argonautes in the different loading-states are unfortunately not available.

Giw1p has no obvious similarity with any previously identified protein from other organisms. However, it has been reported that the nuclear localization of some Argonaute proteins also depends on small RNAs in other organisms (Aravin et al., 2008; Guang et al., 2008). It can be assumed that similar mechanisms are at work in higher eukaryotes.

The mechanism how Giw1p mediates the transport of Twi1p into the nucleus is unknown. Unfortunately, the *Tetrahymena* nuclear localisation signal is unidentified. It seems reasonable to speculate that Twi1p has no NLS and therefore depends on binding of Giw1p for nuclear import.

Giw1p can be co-precipitated with Twi1p between 4 and 8 hours post mixing. Giw1p might therefore be not only important for the transport of Twi1p-scnRNA into the parental macronucleus, but also for the subsequent import of the complex into the new developing macronucleus. It is unlikely that Giw1p stays complexed with Twi1p throughout conjugation, since Twi1p binds to nascent noncoding transcripts via its associated scnRNA both in the parental and the newly developing macronucleus (Aronica et al., 2008). Giw1p would probably interfere with this base pairing as it bridges the RNA binding cleft.

The bridging of the binding cleft might however hint to an additional, cytoplasmic function of Giw1p. It has been proposed that the entire micronuclear genome is transcribed early in conjugation to produce scnRNAs. These would therefore be homologous to both nongenic and genic sequences. Twi1p has slicing activity and mature, single-strand associated Twi1ps could target virtually all mRNAs in the cytoplasm for degradation before scanning. This idea is consistent with the phenotype of *GIW1* KO cells, which is much more severe than the phenotype of *TWI1* KO or *TWI1* slicer-

dead strains. Whether Twi1p really slices mRNAs in the absence of Giw1p remains to be demonstrated.

Publication 3:

Non-coding RNA: A bridge between small RNA and DNA

(point of view)

Henriette M. Kurth and Kazufumi Mochizuki. **RNA Biol**. 2009 Apr 7;6(2).

An important question in RNA biology is how the nuclear RNAi machinery interacts sequence-specifically with the genome to establish chromatin or DNA modifications. Small RNAs might either recognize the target DNA directly, or alternatively might interact indirectly by base-pairing with nascent transcripts. In this point of view, we discuss recent work that sheds light onto this question in ciliates (Aronica et al., 2008; Lepere et al., 2008). Both articles suggest that non-coding RNAs mediate the interaction between chromatin and small RNAs. We further outline future research directions in the study of the function of these non-coding RNAs.

Contribution

Publication 1:

2'-O-methylation stabilizes Piwi-associated small RNAs and ensures DNA elimination in *Tetrahymena*

Henriette M. Kurth and Kazufumi Mochizuki. **RNA**. 2009 Apr;15(4):675-85.

HK carried out the experimental work. HK and KM designed the experiments, interpreted the results and prepared the manuscript.

Publication 2:

The *Tetrahymena* Argonaute-binding protein Giw1p directs a mature Argonaute-siRNA complex to the nucleus

Tomoko Noto[1], **Henriette M. Kurth**[1], Kensuke Kataoka, Lucia Aronica, Leroi DeSouza, Michael Siu, Ronald Pearlman, Martin Gorovsky, Kazufumi Mochizuki. **Cell.** 2010 Mar 5;140(5):692-703.

[1] with equal contribution

TN carried out the experiments related to Fig. 1, Fig. 2, Fig. 6 and Fig. 7. HMK carried out the experiments related to Fig. 3 and Fig. 6. KK carried out the experiment related to Fig. S4. LA carried out the experiment related to Fig. 7. KM carried out experiment related to Fig. 4, Fig. 5 and Fig. 7. LVD, KWMS and REP identified Giw1p by mass-spectrometry. TN, HMK, MAG and KM

designed the experiments. TN, HMK, and KM interpreted the results and prepared the manuscript.

Publication 3:

Non-coding RNA: A bridge between small RNA and DNA (review)

Henriette M. Kurth and Kazufumi Mochizuki. **RNA Biol**. 2009 Apr 7;6(2).

HK wrote the point of view article independently. MK reviewed the draft and changes were discussed in depth.

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März 2010

Abbreviations

A	Adenosine
Ago	Argonaute
C	carbon
DDH	Asparagin-Asparagin-Histidin
ds	double-stranded
endo-siRNA	endogenous small interfering RNA
G	Guanosine
H3K9	methylation of Lysine 9 on histone 3
IES	internal eliminated sequences
KO	knock out
Mac	macronucleus
Mic	micronucleus
miRNA	micro RNA
miRNP	miRNA containing ribonucleoprotein complex
mRNA	messenger RNA
MTase	methyltransferase
NLS	nuclear localisation signal
nt	nucleotide
O	oxygen
PAZ	Piwi Argonaute Zwille
pCp	[5'-^{32}P]-cytidine-5',3'-bisphosphate
PEV	position effect variegation
Piwi	P-element induced wimpy *t*estis
piRNA	Piwi-interacting RNA
Pol	DNA-dependent RNA polymerase
pre-miRNA	precursor miRNA
RdDM	RNA-directed DNA methylation
rDNA	circular plasmids containing ribosomal RNA gene
RDRC	RNA-directed RNA polymerase complex
RdRP	RNA-dependent RNA polymerase
RITS	RNA-induced transcriptional silencing
RNAi	RNA interference
S2	*Drosophila* Schneider cells
S-phase	DNA synthesis phase of the cell cycle
scnRNA	scan RNA
siRNA	small interfering RNA
sRNA	small RNA
telo-sRNAs	small RNAs complementary to telomeric repeats
Twi	Tetrahymena Piwi
U	Uridine

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Appendix 1

2'-O-methylation stabilizes Piwi-associated small RNAs and ensures DNA elimination in *Tetrahymena*

Henriette M. Kurth and Kazufumi Mochizuki. **RNA**. 2009 Apr;15(4):675-85.

Appendix 2

The *Tetrahymena* Argonaute-binding protein Giw1p directs a mature Argonaute-siRNA complex to the nucleus

Tomoko Noto[1], **Henriette M. Kurth**[1], Kensuke Kataoka, Lucia Aronica, Leroi DeSouza, Michael Siu, Ronald Pearlman, Martin Gorovsky, Kazufumi Mochizuki. **Cell.** 2010 Mar 5;140(5):692-703.

[1] with equal contribution

Appendix 3

Non-coding RNA: A bridge between small RNA and DNA

(point of view)

Henriette M. Kurth and Kazufumi Mochizuki. **RNA Biol**. 2009 Apr 7;6(2).

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2′-O-methylation stabilizes Piwi-associated small RNAs and ensures DNA elimination in Tetrahymena

Henriette M. Kurth and Kazufumi Mochizuki

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2′-*O*-methylation stabilizes Piwi-associated small RNAs and ensures DNA elimination in *Tetrahymena*

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ABSTRACT

Small RNAs ~20–30 nucleotides (nt) in length regulate gene expression at the transcriptional and post-transcriptional levels. In the plant *Arabidopsis*, all small RNAs are 3′-terminal 2′-*O*-methylated by HEN1, whereas only a subset of small RNAs carry this modification in metazoans. This methylation is known to stabilize small RNAs, but its biological significance remains unclear. In the ciliated protozoan *Tetrahymena thermophila*, two classes of small RNAs have been identified: RNAs ~28–29 nt long (scnRNAs) that are expressed only during sexual reproduction, and constitutively expressed ~23–24 nt siRNAs. In this study, we demonstrate that scnRNAs, but not siRNAs, are 2′-*O*-methylated at their 3′ ends. The *Tetrahymena* HEN1 homolog Hen1p is responsible for scnRNA 2′-*O*-methylation. Loss of Hen1p causes a gradual reduction in the level and length of scnRNAs, defects in programmed DNA elimination, and inefficient production of sexual progeny. Therefore, Hen1p-mediated 2′-*O*-methylation stabilizes scnRNA and ensures DNA elimination in *Tetrahymena*. This study clearly shows that 3′-terminal 2′-*O*-methylation on a selected class of small RNAs regulates the function of a specific RNAi pathway.

Keywords: HEN1; 2′-*O*-methylation; Piwi; DNA elimination; *Tetrahymena*

INTRODUCTION

Small noncoding RNAs produced by RNAi-related mechanisms regulate gene expression. They form functional effecter complexes with conserved Argonaute proteins, which can be divided into the Ago and Piwi subfamilies. Most eukaryotes possess a number of different Argonaute–RNA complexes, which are engaged in a wide variety of functions, including mRNA degradation, translational repression, and chromatin remodeling (for review, see Chu and Rana 2007).

Expression of small RNA is tightly regulated, mostly at the level of precursor RNA transcription (for example, see Johnson et al. 2003). In some cases, however, small RNA expression is post-transcriptionally regulated through control of precursor RNA processing (Obernosterer et a. 2006; Thomson et al. 2006; Yang et al. 2006a; Viswanathan et al. 2008). In addition, RNA degradation is likely to regulate the accumulation of small RNAs. One potential mechanism is the protection of small RNAs from degradation by 2′-*O*-methylation at their 3′ terminus (Li et al. 2005; Horwich et al. 2007).

In the plant *Arabidopsis*, all small RNAs (both si- and miRNAs) that have been studied are 2′-*O*-methylated at their 3′ ends. In contrast, in metazoans, only a subset of small RNAs carries 2′-*O*-methylation at their 3′ ends. In vertebrates, this modification has yet to be found on any miRNAs associated with Ago proteins that have been studied to date (Houwing et al. 2007; Kirino and Mourelatos 2007a; Ohara et al. 2007). In *Drosophila*, Ago2-associated small RNAs (mainly siRNAs) are modified at their 3′ ends, most likely by 2′-*O*-methylation, whereas Ago1-associated small RNAs (mainly miRNAs) are largely unmodified (Horwich et al. 2007; Kawamura et al. 2008). Interestingly, most if not all Piwi-associated small RNAs (piRNAs) are 2′-*O*-methylated at their 3′ ends in all metazoans that have been studied so far (Vagin et al. 2006; Horwich et al. 2007; Houwing et al. 2007; Kirino and Mourelatos 2007a; Ohara et al. 2007; Saito et al. 2007). Therefore, 3′-terminal 2′-*O*-methylation occurs on selected small RNAs, and it may regulate specific RNAi pathways in metazoans.

The RNA methyltransferase HEN1 is responsible for 3′-terminal 2′-*O*-methylation of small RNAs in *Arabidopsis*

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(Yu et al. 2005) and *Drosophila* (Horwich et al. 2007; Saito et al. 2007). Mouse HEN1 is also able to add 2′-*O*-methyl groups to small RNAs in vitro (Kirino and Mourelatos 2007b), but its role in vivo has yet to be studied. *Arabidopsis* HEN1 methylates double-stranded small RNAs (Yu et al. 2005; Yang et al. 2006b), probably before they are loaded into Argonaute proteins. This is consistent with the fact that all small RNAs are 2′-*O*-methylated, regardless of the Argonaute proteins to which they bind. In contrast, the HEN1 enzymes from *Drosophila* and mouse add 2′-*O*-methyl groups only to single-stranded small RNAs (Horwich et al. 2007; Saito et al. 2007). Thus, in these animals, 2′-*O*-methylation most likely occurs on small RNAs that have already complexed with Argonaute proteins. *Drosophila* HEN1 interacts with Piwi proteins (Piwi, Aubergine, and Ago3), whose binding partners (piRNAs) are 2′-*O*-methylated, but not with the Ago protein Ago1, whose small RNA partners are not modified (Saito et al. 2007). Therefore, the substrate specificity of HEN1 homologs in metazoans probably reflects the fact that HEN1 proteins interact with a specific class of Argonaute proteins.

The role of 3′-terminal 2′-*O*-methylation of small RNAs is well studied in *Arabidopsis*. *Arabidopsis* HEN1 mutants phenocopy the loss of activity of the general RNAi machinery, including Dicers and Argonautes (Chen et al. 2002; Boutet et al. 2003). This is because 2′-*O*-methylation protects small RNAs from degradation by exonucleases (Ramachandran and Chen 2008). The loss of this modification causes a general reduction in the levels of small RNAs in plants (Boutet et al. 2003; Li et al. 2005). Therefore, generally all small RNAs in *Arabidopsis* depend on 2′-*O*-methylation for their stability. Thus, this modification does not seem to control a specific RNAi pathway in plants.

In *Drosophila* mutant strains that lack the activity of the HEN1 homolog DmHEN1 (Pimet), the length and abundance of piRNAs, which are normally 2′-*O*-methylated at the 3′ end, are lower than in wild-type flies. In contrast, the levels of miRNAs, which are unmodified also in wild-type flies, are the same in both mutant and wild-type *Drosophila* (Horwich et al. 2007). Therefore, selective 2′-*O*-methylation of small RNAs regulates the turnover of a specific class of small RNAs, at least in flies. Horwich et al. (2007) reported that the HeT-A transposon mRNA was increased in the absence of *DmHEN1*. Nevertheless, because the *Drosophila* mutant strain lacking DmHEN1 is viable and fertile (Saito et al. 2007), the significance of this regulation remains unclear. Moreover, the functions of 2′-*O*-methylation of small RNAs in other eukaryotes have not been reported. Thus, despite the attractiveness of the idea that 3′-terminal 2′-*O*-methylation of a selected class of small RNAs regulates a specific RNAi pathway, this regulation has yet to be proven necessary for any biological process.

The ciliated protozoan *Tetrahymena thermophila* has 12 Piwi proteins, but no Ago proteins have been identified in the sequenced *Tetrahymena* genome. *Tetrahymena* expresses at least two classes of small RNAs generated by RNAi-related mechanisms. The first class comprises small RNAs of ~28–29 nucleotides (nt) (scnRNAs) that are expressed exclusively during sexual reproduction (conjugation) and are probably synthesized from many different genome loci (Chalker and Yao 2001; Mochizuki et al. 2002). scnRNAs bind specifically to the Piwi protein Twi1p (Mochizuki and Gorovsky 2004), and the scnRNA–Twi1p complex plays a pivotal role in developmentally programmed DNA elimination (Mochizuki et al. 2002). The other class of small RNAs consists of constitutively expressed siRNAs of ~23–24 nt that map to a small number of genomic loci (Lee and Collins 2006, 2007). The function of these siRNAs has not yet been identified.

In this study, we demonstrate that scnRNAs, but not siRNAs of ~23–24 nt, are 2′-*O*-methylated by the *Tetrahymena* HEN1 homolog Hen1p. Hen1p-mediated 2′-*O*-methylation specifically stabilizes scnRNAs and ensures DNA elimination and the consequent survival of *Tetrahymena* progeny. Our findings lend support to the view that 3′-terminal 2′-*O*-methylation of a selected class of small RNAs regulates a specific RNAi pathway in eukaryotes.

RESULTS

scnRNA, but not ~23–24 nt siRNA, is 2′-*O*-methylated

T. thermophila expresses two classes of small RNAs made by RNAi-related mechanisms: RNAs of ~28–29 nt (scnRNAs) that are expressed only during sexual reproduction (Mochizuki et al. 2002) and constitutively expressed siRNAs of ~23–24 nt (Lee and Collins 2006).

In order to analyze whether scnRNAs are modified at their 3′ ends, total RNA from conjugating *Tetrahymena* cells enriched for scnRNAs was subjected to periodate oxidation/β-elimination (Akbergenov et al. 2006). After these reactions, RNAs containing both 2′- and 3′-hydroxyl groups at their 3′ ends are shorter by one nucleotide and they have an extra negative charge from the phosphate group left after the nucleotide removal. Modifications in 2′- and/or 3′-hydroxyl groups prevent the periodate oxidation reaction and keep the RNA intact. As a result, unmodified RNA migrates as if it was ~1.5 nt shorter than the modified RNA in a denaturing polyacrylamide gel after the treatment. The mobility of the scnRNAs isolated at different stages of conjugation did not increase after periodate oxidation/β-elimination, while unmodified control synthetic RNAs in the same reactions did (Fig. 1A). This result indicates that most, if not all, scnRNAs are modified at their 3′ ends throughout the process of conjugation.

To test whether the siRNAs of ~23–24 nt are modified at their 3′ ends, total RNA enriched for small RNAs was

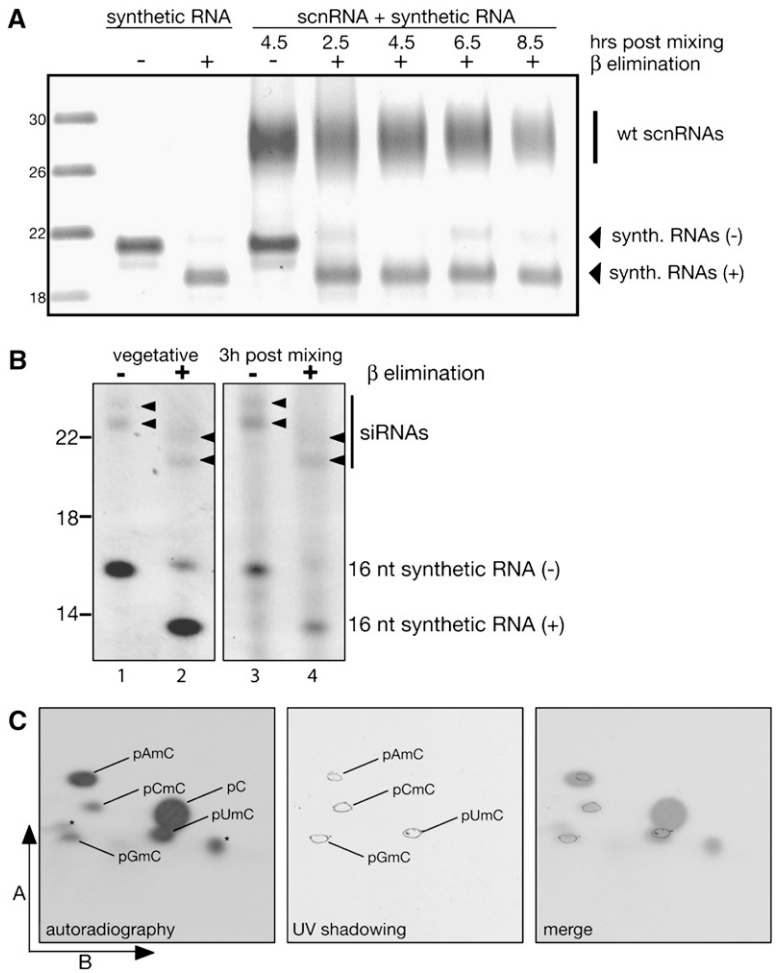

FIGURE 1. *Tetrahymena* scnRNAs are 2′-*O*-methylated at their 3′ ends. (*A*) scnRNAs are modified at their 3′ ends throughout conjugation. Small RNAs isolated at different time points of conjugation (2.5, 4.5, 6.5, and 8.5 h post-mixing) were combined with a synthetic, 21-nt RNA and subjected to periodate oxidation/β-elimination. The reactions were subjected to denaturing gel electrophoresis and visualized using a nucleic acid-specific fluorescent dye. Untreated RNAs isolated from cells at 4.5 h post-mixing were analyzed in the same gel. The positions of the RNA markers are shown on the *left*. At all the stages tested, the mobility of the scnRNAs did not increase after the reaction, whereas that of the unmodified, synthetic 21-nt RNA did, indicating that scnRNAs were modified at their terminal nucleotides. (*B*) The constitutively expressed small RNAs in *Tetrahymena* are not methylated. Small RNAs from vegetative (lane *2*) and mating (lane *4*) cells were subjected to periodate oxidation/β-elimination. The reactions were separated in a denaturing gel, and the RNA was visualized using a nucleic acid staining dye. Untreated small RNAs (lane *1*, vegetative cells; lane *3*, mating cells) were analyzed on the same gel. The constitutively expressed siRNAs of ~23–24 nt (arrowheads) displayed a shift after the reaction. (*C*) scnRNAs are 2′-*O*-methylated. Gel-purified scnRNAs were radiolabeled by pCp ligation and treated with P1 nuclease. The reactants and the synthetic unlabeled dinucleotide standards (pCmpC, pGmpC, pAmpC, and pUmpC) were resolved by 2D-TLC on the same plate and visualized by autoradiography (*left*) and UV shadowing (*middle*). The four spots detected by UV shadowing are circled (*middle*), and their identities were determined by comparing the relative positions of these spots using a dinucleotide mobility map described previously (Keith 1995). Four radiolabeled spots comigrated with the dinucleotide standards (*right*), suggesting that scnRNAs were 2′-*O*-methylated. Asterisks in the *left* panel indicate unidentified reaction products.

isolated from vegetatively growing and mating *Tetrahymena* and subjected to periodate oxidation/β-elimination. As shown in Figure 1B, the mobility of endogenously expressed siRNAs of ~23–24 nt increased after this treatment, indicating that most, if not all, siRNAs of ~23–24 nt are not modified at their 3′ ends in vivo.

scnRNA is 2′-*O*-methylated

Because the presence of a 3′-hydroxyl terminus on scnRNAs has been demonstrated previously (Mochizuki et al. 2002), the above result indicated a modification of the 2′-hydroxyl termini of scnRNAs. We next tested whether this modification was 2′-*O*-methylation. PAGE-purified scnRNAs were ligated to [5′-^{32}P]cytidine 5′,3′ bisphosphate (pCp) at the 3′-hydroxyl terminus, and the reaction products were digested with nuclease P1. Because the presence of a bulky modification at the 2′-position reduces the rate at which nuclease P1 hydrolyzes the neighboring phosphodiester bond (Grosjean et al. 2007), RNA lacking such a modification produces cytidine 5′-monophosphates (pC) as a [^{32}P]-labeled product; in contrast, modified RNA produces labeled dinucleotides as well as pC. The digested scnRNA was resolved by two-dimensional thin-layer chromatography (2D-TLC) and analyzed by autoradiography (Fig. 1C, left). Synthetic 2′-*O*-methylated dinucleotides (pAmpC, pCmpC, pGmpC, and pUmpC) were separated on the same plate and visualized by UV shadowing (Fig. 1C, middle). The migration distance of the four spots detected by autoradiography matched those of the synthetic 2′-*O*-methylated dinucleotide standards (Fig. 1C, right), indicating that the 3′ termini of the scnRNAs were indeed modified by 2′-*O*-methylation.

Hen1p has scnRNA methyltransferase activity in vitro

In *Arabidopsis*, the RNA methyltransferase HEN1 modifies the terminal 2′ hydroxyl group of siRNAs and miRNAs (Yu et al. 2005). In the fly and mouse, HEN1 homologs methylate terminal 2′ hydroxyl groups of piRNAs (Horwich et al. 2007; Houwing et al. 2007; Kirino and Mourelatos 2007b; Ohara et al. 2007; Saito et al. 2007). The *Tetrahymena* genome possesses a single gene (TTHERM_00433810) that encodes a protein

with strong similarity to these HEN1 homologs (Supplemental Fig. S1A,B). We named this gene *HEN1*. Hen1p, the protein predicted from the *HEN1* mRNA sequence (DDBJ/EMBL/GenBank FM199973), has a conserved methyltransferase domain (Supplemental Fig. S1B). Although *Arabidopsis* HEN1 has a double-stranded RNA-binding domain, *Tetrahymena* Hen1p lacks this domain, as do metazoan HEN1 proteins (Supplemental Fig. S1A).

To test whether Hen1p has scnRNA methyltransferase activity, recombinant Hen1p (rHen1p) was expressed in *Escherichia coli*. Purified rHen1p was incubated with synthetic RNAs of 29 nt whose 3′ ends carried either a 2′-hydroxyl or a 2′-*O*-methyl and S-adenosyl-L[methyl-^{14}C] methionine, which served as a methyl group donor. rHen1p methylated the RNA with the 2′-hydroxyl terminus but did not methylate the RNA with a 2′-*O*-methyl terminus (Fig. 2A). These results indicate that Hen1p can methylate the terminal 2′-hydroxyl group of scnRNAs.

rHen1p does not methylate double-stranded synthetic scnRNAs that possess blunt ends or 3′-overhangs of 2 nt, which mimic Dicer-processed RNA (Fig. 2A). This suggests that rHen1p methylates only single-stranded scnRNAs. Consistent with this, the gel mobility of scnRNAs in a *TWI1* mutant strain, which is incapable of dissociating

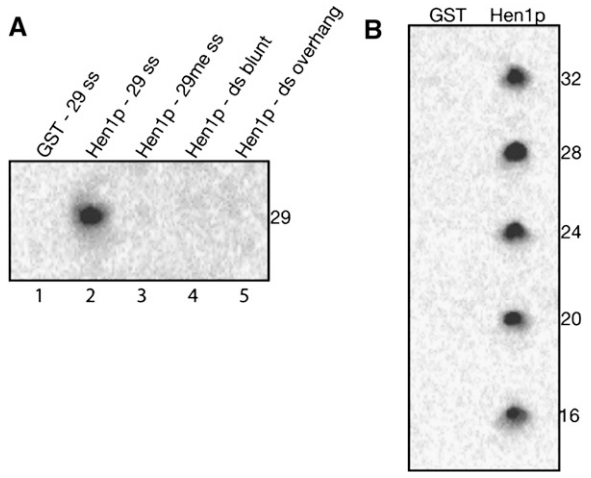

FIGURE 2. *Tetrahymena* Hen1p methylates scnRNAs. (*A*) Recombinant *Tetrahymena* Hen1p methylates single-stranded scnRNAs in vitro. Recombinant *Tetrahymena* Hen1p (lanes *2–5*) and control GST protein (lane *1*) were incubated with S-adenosyl-L[methyl-^{14}C] methionine ([^{14}C]SAM) as a methyl donor and synthetic, single-stranded RNA of 29 nt (lanes *1*,*2*), single-stranded RNA of 29 nt with a 2′-O-methyl group at the terminal nucleotide (lane *3*), and double-stranded RNAs of 29 bp with blunt ends (lane *4*) or 3′ 2-nt overhangs (lane *5*). The reactions were analyzed by denaturing gel electrophoresis followed by autoradiography. Hen1p transferred methyl groups only to single-stranded unmodified RNA (lane *2*). (*B*) The activity of Hen1p is not size-specific. Recombinant *Tetrahymena* Hen1p was incubated with [^{14}C]SAM and synthetic RNAs of 16, 20, 24, 28, and 32 nt. The reaction was analyzed by denaturing gel electrophoresis followed by autoradiography. Hen1p transferred methyl groups to RNA substrates of all sizes tested.

double-stranded scnRNAs (T. Noto and K. Mochizuki, unpubl.), increased after periodate oxidation/β-elimination treatment (Supplemental Fig. S3). We conclude that Hen1p 2′-*O*-methylates only single-stranded scnRNAs both in vitro and in vivo. This also indicates that 2′-*O*-methylation of scnRNAs occurs after they are loaded into Twi1p and the "passenger" strand of double-stranded scnRNA is removed.

rHen1p also transferred methyl groups to synthetic RNAs of 16, 20, 24, 28, and 32 nt (Fig. 2B). Therefore, at least in vitro, Hen1p can methylate not only scnRNAs of ~28–29 nt but also small RNAs that are the size of the siRNAs of ~23–24 nt constitutively expressed in *Tetrahymena*.

Hen1p and Twi1p colocalize and interact

An anti-Hen1p antibody was produced and used to study the expression and localization of Hen1p. Hen1p was not detected by Western blotting in either vegetatively growing or starved cells (Fig. 3A), but *HEN1* mRNA was detected in growing cells by RT-PCR (Supplemental Fig. S4). This suggests that vegetative cells may express a small amount of Hen1p. Hen1p was detected at a very early stage of conjugation at 2 h post-mixing, but the protein quickly disappeared thereafter (Fig. 3A). A similar result was obtained using immunofluorescent staining. Hen1p was not detected in starved cells (Fig. 3B) but was first detected in the macronucleus when the micronucleus was in meiotic prophase at ~2 h post-mixing (Fig. 3C,D). Hen1p subsequently became undetectable in post-meiotic stages at ~4 h post-mixing (Fig. 3E). In conclusion, Hen1p accumulation is developmentally regulated and specifically occurs in the macronucleus.

Both Hen1p and the Piwi protein Twi1p, which associates with scnRNA (Mochizuki and Gorovsky 2004), localize to the macronucleus during micronuclear meiotic prophase (Fig. 3C,F). We therefore examined whether Twi1p and Hen1p interact. Recombinant GST-Twi1p was mixed with recombinant Hen1p, and a pull-down assay was performed using glutathione beads. As a negative control, recombinant GST protein was used instead of GST-Twi1p. Hen1p copurified with GST-Twi1p, but not with GST (Fig. 3G), indicating that Hen1p and Twi1p directly interact.

Hen1p is essential for scnRNA methylation in *Tetrahymena*

To address whether Hen1p is required for scnRNA methylation, *HEN1* knockout (KO) *Tetrahymena* strains were constructed. The entire Hen1p coding sequence of all polyploid macronuclear copies was replaced with a drug resistance marker in all *HEN1* loci (Supplemental Fig. S5A,B). scnRNAs from *HEN1* KO strains showed increased

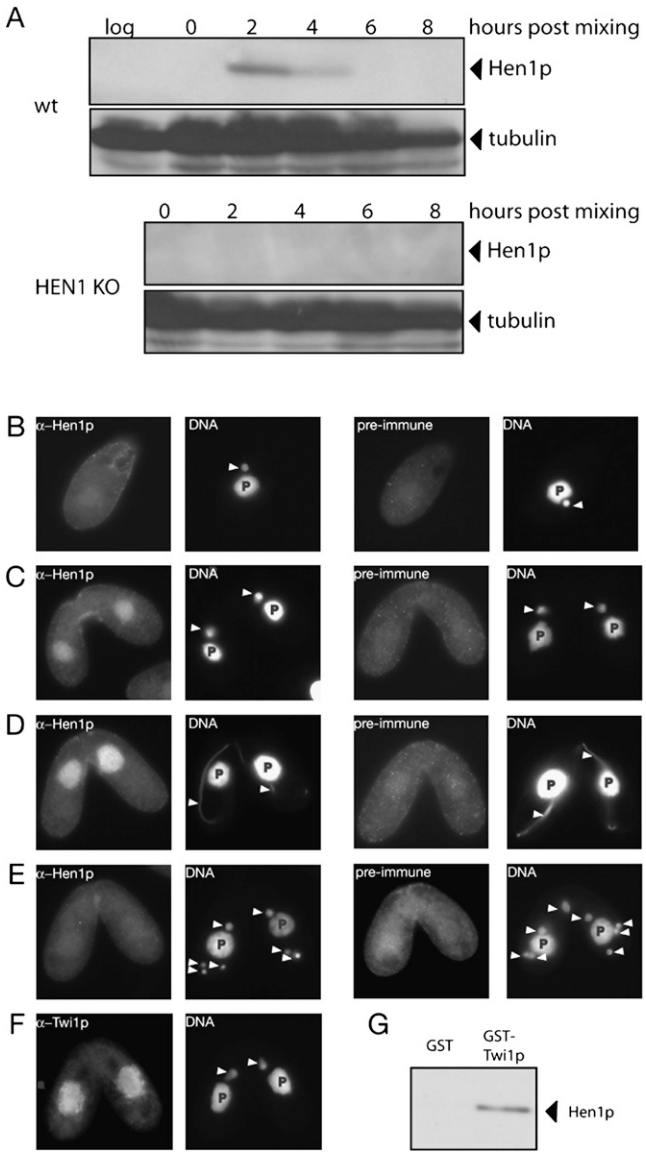

FIGURE 3. Hen1p colocalizes and interacts with Twi1p. (*A*) Hen1p is expressed early in conjugation. Cell lysates were prepared at the vegetative growing stage (log) and at different developmental stages of sexual reproduction (0 h post-mixing, before mating; 2 h, Mic premeiosis; 4 h, Mic meiosis; 6 h, post-zygotic mitosis-Mac anlagen; 8 h, new macronuclear development). For detailed developmental stages, see Cole et al. (1997). The lysate was analyzed by Western blotting using antibodies against Hen1p (*top*) and α-tubulin (*bottom*). Hen1p was detected only during the early conjugation stage (2–4 h post-mixing). (*B–F*) Hen1p and Twi1p localize in the parental macronucleus early in conjugation. Starved (*B*) or mating (premeiosis, *C*,*D*,*F*; post-second meiosis, *E*) wild-type cells were processed for immunofluorescence using an anti-Hen1p antibody (*leftmost* columns in *B–E*), preimmune serum (third column from the *left* of *B–E*), or an anti-Twi1p antibody (*F*, *left*). Cells were counterstained with DAPI (second and fourth columns from the *left*) to visualize the nuclei. Arrowheads indicate micronuclei; P, parental macronuclei. (*G*) Association between Hen1p and Twi1p. Recombinant GST (*left*) or GST-Twi1p (*right*) was immobilized on glutathione beads and incubated with recombinant Hen1p. After washing, proteins on the beads were eluted and analyzed by Western blotting using an anti-Hen1p antibody. Hen1p specifically bound to GST-Twi1p but not to GST alone.

gel mobility after periodate oxidation/β-elimination treatment (Fig. 4A), indicating that scnRNAs are not modified at their 3′ termini in the absence of *HEN1*. Therefore, *HEN1* is essential for 2′-*O*-methylation of scnRNAs in *Tetrahymena*.

Hen1p is essential for stable accumulation of scnRNAs

Total RNA was extracted from wild-type and *HEN1* KO cells, and scnRNA accumulation was analyzed at several different time points during conjugation. Although comparable levels of scnRNAs were detected in the early stages of conjugation (4 h post-mixing) in wild-type and *HEN1* KO cells, the amount of scnRNAs from *HEN1* KO cells in late stages of conjugation (6–10 h post-mixing) was substantially lower than in wild-type cells. In addition, the scnRNAs were gradually shortened by a few bases in the absence of *HEN1* (Fig. 4B). In contrast, the accumulation of constitutively expressed siRNAs of ~23–24 nt was unaffected by the absence of *HEN1* (Fig. 4B). Therefore, the turnover of scnRNAs and siRNAs of ~23–24 nt is independently regulated, and only scnRNAs are destabilized in the absence of Hen1p. Since scnRNAs are synthesized only during the early stages of conjugation, these results suggest that Hen1p protects scnRNA from degradation by 2′-*O*-methylating their 3′ ends.

Viability of progeny is greatly reduced in *HEN1* KO strains

The *HEN1* KO strains showed no obvious defects during vegetative growth (data not shown). They mated normally and produced exconjugants (progeny) containing new micro- and macronuclei (data not shown). Thus, at the cytological level, nuclear differentiation appeared to occur normally without *HEN1*.

To test the viability of progeny from the *HEN1* KO strains, single mating pairs were placed into nutrient medium and their growth was analyzed. In wild-type cells, ~40% of isolated pairs produced viable sexual progeny, whereas only ~3% of *HEN1* KO pairs gave rise to viable sexual progeny (Supplemental Fig. S5C). These data indicate that Hen1p is not essential for the production of functional sexual progeny, but it does play an important role in the efficiency of this process.

DNA elimination is inhibited in *HEN1* KO strains

In the sexual reproduction of *Tetrahymena*, the micronucleus produces both new micro- and macronuclei. During macronuclear development, ~6000 internal eliminated sequences (IESs) are removed, and the remaining macronuclear-destined sequences are religated (for review, see Yao and Chao 2005). scnRNAs play essential roles in DNA elimination

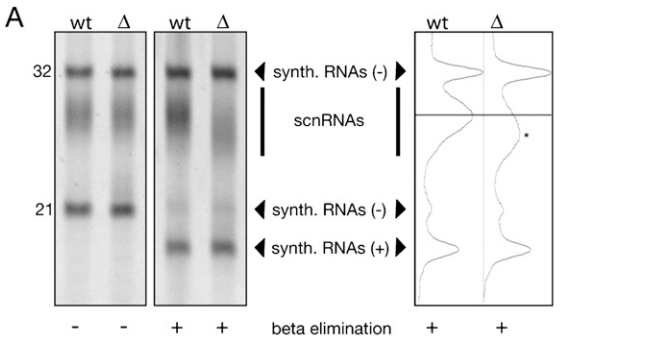

FIGURE 4. *HEN1*-mediated 2′-*O*-methylation stabilizes scnRNAs in *Tetrahymena*. (*A*) scnRNAs in *HEN1* knockout (Δ*HEN1*) cells are not modified. Small RNAs from wild-type (wt) and Δ*HEN1* (Δ) strains extracted at 4 h post-mixing were combined with synthetic, unmodified RNA of 21 nt and were treated with (+) or without (−) periodate oxidation/β-elimination. The reactions were then mixed with synthetic RNA of 32 nt and analyzed by denaturing gel electrophoresis followed by staining with a nucleic acid–specific fluorescent dye (*left*). Densitometric analysis of the fluorescent signal is shown on the *right*. The gel mobility of scnRNAs from Δ*HEN1* strains increased after the treatment, indicating that they have free 3′ hydroxyl groups. (*B*) scnRNAs are destabilized in Δ*HEN1* strains. Total RNA from wild-type (wt) and Δ*HEN1* (Δ) strains was extracted at the indicated time points of conjugation, run on a sequencing gel, and stained with a nucleic acid–specific fluorescent dye. As conjugation proceeded, scnRNAs in Δ*HEN1* cells became shorter and less abundant than wt scnRNAs at the same time points.

(for review, see Meyer and Chalker 2006). Therefore, we examined whether 2′-*O*-methylation of scnRNA plays a role in eliminating DNA in the new macronucleus.

We analyzed the elimination of four different IESs (M, R, Cal, and Tlr1 elements) (Austerberry et al. 1989; Katoh et al. 1993; Wells et al. 1994) in single sexual progeny (Fig. 5A–C). In 18 of 56 progeny of the *HEN1* KO strains, one or more of the four examined IES loci were not eliminated (Fig. 5C). In contrast, all of these loci were eliminated in the progeny of the control strain (Fig. 5C). Thus, we conclude that DNA elimination is affected in the absence of Hen1p. Although 38 of 56 progeny of the *HEN1* KO strains showed complete or only reduced elimination of the four IESs, the removal of many of the other ~6000 IESs was likely to be affected in these progeny. Since DNA elimination is believed to be essential for producing viable sexual progeny (Coyne et al. 1999; Nikiforov et al. 1999; Mochizuki et al. 2002; Liu et al. 2007; Aronica et al. 2008), the reduced production of viable offspring in the *HEN1* KO strains (Supplemental Fig. S5C) is likely due to this partial DNA elimination defect.

DISCUSSION

Function of 2′-*O*-methylation with respect to scnRNA

In this study, we demonstrated that *Tetrahymena* scnRNAs are 2′-*O*-methylated by the RNA methyltransferase Hen1p.

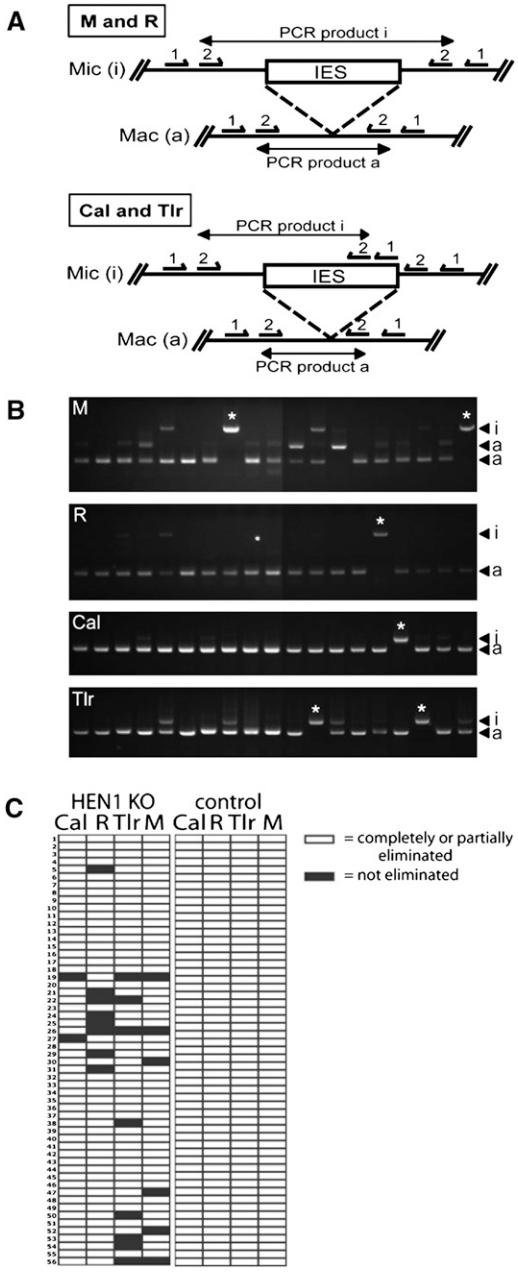

FIGURE 5. (Legend on next page)

Loss of Hen1p renders scnRNAs unstable, partially disables DNA elimination, and makes the production of sexual progeny inefficient. Since the defect in DNA elimination and production of sexual progeny can be explained by the instability of scnRNAs, we believe that the main function of 2′-*O*-methylation is scnRNA stabilization.

The manner by which 2′-*O*-methylation stabilizes scnRNAs remains unknown. It may directly protect scnRNA from exoribonuclease attack. Alternatively, scnRNAs lacking a 2′-*O*-methyl group may associate less tightly with Twi1p, and dissociated scnRNAs may be more susceptible to ribonuclease degradation. In light of this suggestion, it is worth noting that the 3′ terminus of siRNA provides a binding site for the PAZ domains of human Ago1 and Ago2 (Ma et al. 2004; Lingel et al. 2004) and that scnRNAs are unstable in the absence of Twi1p (Mochizuki and Gorovsky 2004).

Similar to the effects reported here for scnRNAs in *Tetrahymena*, HEN1-mediated 2′-*O*-methylation was reported to stabilize small RNAs in *Arabidopsis* and *Drosophila* (Chen et al. 2002; Li et al. 2005; Horwich et al. 2007). Plants can convey silencing information, which is probably mediated by siRNAs, over long distances (Hamilton et al. 2002; Klahre et al. 2002; Yoo et al. 2004). 2′-*O*-Methylation of siRNAs in plants may enable them to travel long distances, thus enhancing the defense against viruses (Boutet et al. 2003). In all metazoans studied to date, piRNAs are 2′-*O*-methylated (Vagin et al. 2006; Horwich et al. 2007; Houwing et al. 2007; Kirino and Mourelatos 2007a; Ohara et al. 2007; Saito et al. 2007) and Piwi proteins function primarily in germline cells (Cox et al. 1998; Deng and Lin 2002; Das et al. 2008). Since germline cells are transcriptionally inactive at certain developmental stages in many animals (Zalokar 1976; Newport and Kirschner 1982; Seydoux et al. 1996; Leatherman et al. 2002), piRNAs may have to be 2′-*O*-methylated to increase their turnover time and ensure transposon silencing, even in the absence of their de novo production. Also, scnRNAs in *Tetrahymena* must be stable because they are produced at early stages of conjugation (Malone et al. 2005; Mochizuki and Gorovsky 2005) but they play a role in DNA elimination during late stages of conjugation. Thus, 2′-*O*-methylation appears to have evolved as a way to protect small RNAs from degradation in pathways that require their stable accumulation.

FIGURE 5. DNA elimination is affected in *ΔHEN1* progeny. (*A*) Schematic diagram of the IES elimination assay. Four primers per locus (M and R loci, *top*) or six primers per locus (Cal and Tlr1 loci, *bottom*) were used for nested PCR. Primers marked 1 were used first, followed by those marked 2. Shorter Mac-form products (a) indicate that the locus was rearranged, whereas longer Mic-form products (i) indicate a defect in DNA elimination. (*B*) An example of the results of DNA elimination assays in *ΔHEN1* progeny. Single cells were picked at 30 h post-mixing and used in nested PCR reactions as shown in *A*. M, R, Cal, and Tlr1 loci were tested in each progeny cell for DNA elimination. Representative PCR results of 19 progenies of *ΔHEN1* cells are shown. The positions of PCR products from unrearranged (i) and rearranged (a) loci are indicated. The progeny that showed only an unrearranged PCR product (highlighted with asterisks) in at least one of the four loci were counted as cells with defective DNA elimination. (*C*) Summary of the results obtained from 56 progeny cells of the *ΔHEN1* and control cells. M, R, Cal, and Tlr1 loci were tested in each progeny cell (1–56) for DNA elimination. Open boxes indicate rearranged (a) DNA; filled boxes, unrearranged (i) DNA. Eighteen of 56 progeny cells from *ΔHEN1* had DNA elimination defects in at least one of the four loci examined, whereas none of the progeny from control cells showed such defects.

A specific class of small RNAs is 2′-*O*-methylated in *Tetrahymena*

We show in this study that scnRNAs, but not siRNAs of ~23–24 nt, are 2′-*O*-methylated at their 3′ ends. Therefore, only this specific class of small RNAs is 2′-*O*-methylated in the ciliate *Tetrahymena*. Since all 12 Argonaute proteins identified in *Tetrahymena* belong to the Piwi subfamily, the siRNAs of ~23–24 nt are most likely associated with some of those Piwi proteins. If this is the case, this is the first report describing Piwi-associated small RNAs that are not modified by 2′-*O*-methylation. This suggests that 2′-*O*-methylation is not a universal feature of Piwi-associated small RNAs.

Tetrahymena Hen1p can methylate various sizes of small, single-stranded RNAs in vitro (Fig. 2B), whereas only scnRNAs seem to be methylated in vivo (Figs. 1A, 2C). This specific methylation of scnRNAs is likely ensured by (1) the restricted accumulation of Hen1p early in conjugation (Fig. 3A), when scnRNAs begin to accumulate (Mochizuki et al. 2002); (2) the localization of Hen1p to the parental macronucleus (Fig. 3B), where Twi1p is also localized (see also Fig. 3F; Mochizuki et al. 2002); and (3) the interaction of Hen1p with Twi1p (Fig. 3G), which specifically binds to scnRNA (Mochizuki and Gorovsky 2004). The selective interaction between HEN1 protein and Piwi proteins has also been reported in *Drosophila* ovaries (Saito et al. 2007). In addition to this HEN1–Argonaute interaction, the specificity of small RNA 2′-*O*-methylation in metazoans may be controlled by the spatial and temporal regulation of HEN1 homolog expression, as in *Tetrahymena*.

Evolution of piRNA biogenesis

Although all detectable scnRNAs are methylated at their 3′ ends throughout conjugation (Fig. 1A), Hen1p, which is responsible for methylating the 3′ end (Fig. 4A), only transiently appears during early conjugation (Fig. 3A–E). Therefore, scnRNAs that are present in the middle and later stages must have been methylated by Hen1p during the early stages of conjugation. This argues that most, if not all, scnRNAs are produced during the early stages of conjugation, when the Dicer homolog Dcl1p processes micronuclear noncoding RNA to produce scnRNAs (Malone et al. 2005; Mochizuki and Gorovsky 2005). This also indicates that little or no secondary Dicer-independent scnRNA synthesis

occurs during the later stages of conjugation. In addition, siRNAs of ~23–24 nt in *Tetrahymena*, which likely associate with Piwi proteins, are produced through a Dicer-dependent mechanism (Lee and Collins 2007). These processes differ markedly from the Dicer-independent production of piRNA in metazoans (Vagin et al. 2006; Houwing et al. 2007; Das et al. 2008; Wang and Reinke 2008). In *Drosophila*, it has been postulated that piRNAs are produced by sequential actions of the endoribonuclease (slicer) activities of Piwi proteins (Brennecke et al. 2007; Gunawardane et al. 2007). At this time, we cannot conclude whether the Dicer- or Piwi-dependent form of piRNA production is the ancestral form. It would be interesting to know how piRNAs are produced and whether all of these are 2′-*O*-methylated in *Dictyostelium*, which is an amoeba more closely related to metazoans than to ciliates.

MATERIALS AND METHODS

Cell lines and culture conditions

The wild-type CU428 and B2086 strains of *T. thermophila* were provided by Dr. P. J. Bruns (Cornell University). These strains are also available at the *Tetrahymena* Stock Center (Cornell University, http://tetrahymena.vet.cornell.edu). The cells were grown at 30°C in SPP medium (Gorovsky et al. 1975) containing 2% proteose peptone. Before mating, the cells were washed and resuspended (5×10^5 cells/mL) in 10 mM Tris buffer (pH 7.5). After 12–18 h of incubation at 30°C, equal numbers of cells were mixed and mated. In the experiments shown in Figures 3A and 4B, the cultures were refed at 2 and 4 h post-mixing, respectively, by adding one-third volume of 4× SPP medium to limit the initiation of mating after that period.

Analysis of small RNAs

For the experiments shown in Figures 1A and 4A and Supplemental Figure S3, total RNA enriched in small RNAs was extracted using the mirVana Kit (Ambion). For the experiment shown in Figure 1B, total RNA was first extracted with Trizol (Invitrogen), and the small RNA fraction was enriched according to the method described by Lee and Collins (2006). For the experiment shown in Figure 4B, total RNA was extracted with Trizol. Oligo ribonucleotides were synthesized by Dharmacon. The periodate oxidation/β-elimination reactions were performed as described (Akbergenov et al. 2006). RNA was separated in 12%–20% polyacrylamide-urea sequencing gels and was visualized using Gel Red (Biotium).

Two-dimensional thin-layer chromatography

Total RNA was isolated with Trizol (Invitrogen) at 4 h post-mixing and separated in a preparative sequencing gel; the scnRNAs (~26–31 nt) were purified from the gel. The purified scnRNAs were ligated to [5′-^{32}P]pCp using T4 RNA ligase (Amersham). The ligated scnRNAs–pCp were gel purified and digested for 20 min at 37°C using P1 nuclease (400 mU/mL final) (Sigma). The digest was mixed with synthetic 2′-*O*-methylated dinucleotide standards (pAmpC, pCmpC, pGmpC, and pUmpC), spotted onto HPTLC cellulose glass plates (10 × 10 cm; Merck), and resolved with isobutyric acid/25% ammonium hydroxide/water (66:1:33 by volume) in the first dimension and then with isopropanol/HCl/water (70:15:15 by volume) in the second dimension. The labeled dinucleotides were visualized by autoradiography, while the synthetic dinucleotide standards were visualized by UV shadowing.

Expression and purification of Hen1p protein from *E. coli*

Because of the differential codon usages in *Tetrahymena*, the entire Hen1p coding sequence was synthesized (GenScript) for expression in *E. coli*. The sequence of the synthetic *HEN1* gene is available upon request. The synthetic gene was subcloned into a modified pGEX-4T-1 vector (pGEX4T1-TEV), which has a TEV protease recognition sequence at the C terminus of the GST (gift from T. Clausen, IMP, Vienna) to allow production of a GST-TEV-Hen1p fusion protein. The construct was expressed in the *E. coli* BL21 (DE3) strain overnight at 25°C in the presence of 50 μM IPTG. The proteins were purified using glutathione Sepharose 4B resin (GE Healthcare), and the recombinant Hen1p was eluted from the beads by cleaving the TEV sequence with AcTEV protease (Invitrogen).

Methyltransferase assay

In vitro methyltransferase assays were performed as described previously (Yu et al. 2005) with slight modifications. Hen1p (~35 μg) was incubated for 5 h at 37°C with 0.6 nmol of synthetic oligoribonucleotides (Dharmacon) (for sequences, see Supplemental Fig. S2) and 1.5 μCi S-adenosyl-L[methyl-^{14}C] methionine (Amersham). After proteinase K treatment, followed by phenol/chloroform extraction and ethanol precipitation, half of the sample was separated in a thin (0.5-mm) 12% polyacrylamide-urea sequencing gel. The gel was dried, wrapped in Saran wrap, and exposed overnight to a storage phosphor screen (GE Healthcare). Images were captured using a Typhoon Imager (GE Healthcare) and analyzed using ImageQuant software (GE Healthcare).

Indirect immunofluorescent staining

Cells were fixed in 3.7% formaldehyde and 0.5% Triton-X 100 for 30 min at room temperature (RT), resuspended in 3.7% formaldehyde and 3.4% sucrose, and dried on poly-L-lysine (Sigma)–coated cover slips. The samples were blocked for 1 h with 3% BSA (Sigma), 10% normal goat serum (Invitrogen), and 0.1% Tween 20 in PBS followed by overnight incubation at 4°C in blocking solution containing a 1:500 dilution of anti-Hen1p or a 1:200 dilution of anti-Twi1p (Aronica et al. 2008). The anti-Hen1p antibody was obtained by immunizing a rabbit with recombinant Hen1p. After washes with PBT, samples were incubated with a 1:1000 dilution of anti-rabbit antibody conjugated with Alexa 488 (Invitrogen) for 1 h at RT. The samples were washed, incubated with 10 ng/mL DAPI (Sigma) in PBS, mounted with ProLong Gold (Invitrogen), and observed by fluorescent microscopy.

RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) from the wild-type strains B2086 and CU428, and residual genomic DNA was eliminated using the Turbo DNase Kit (Ambion). cDNA was

synthesized from 5 μg of total RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) and a random hexamer as a primer. cDNA from 12.5 ng of total RNA was amplified by PCR (30 sec at 94°C, 30 sec at 51°C, 40 sec at 72°C; 32 cycles) with primers 5′-AAAATTAATTCGAAGATGGTTCATAC-3′ and 5′-TAGGAGAATAAGTTATCTGCAGTGG-3′.

GST pull-down assay

GST, GST-Twi1p, and Hen1p were expressed in *E. coli* and purified as described above. GST or GST-Twi1p (0.1 μg) were incubated for 30 min at 4°C with glutathione Sepharose 4B resin (GE Healthcare) in GST pull-down buffer (GPB) (20 mM Tris at pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10% glycerol, 0.1 mM EDTA, 1 mM DTT). The beads were washed once with 1% BSA in GPB and once with 0.1% BSA in GPB. Hen1p (0.1 μg) was added, and the samples were incubated for 2 h at 4°C. The beads were washed five times with 0.1% BSA in GPB and boiled in SDS loading buffer. The samples were loaded onto an SDS-PAGE gel, and the presence of Hen1p was monitored by Western blotting using the anti-Hen1p antibody.

Construction of *HEN1* KO strains

The *HEN1* KO construct (see Supplemental Fig. S5A) was generated by overlapping PCR. First, the 5′UTR and 3′UTR were amplified using the following primers (sequences overlapping with a *neo4* cassette are underlined):

5_UTR_fw, 5′-GGACTCGAGTGATAAAAATGAGTTGTTTGCTTATT-3′;
5_UTR_rv, 5′-GTCTATCGAATTCCTGCAGCCCAAACCGGCTAGTTTTTACTTAG-3′;
3_UTR_fw, 5′-CTGGAAAAATGCAGCCCTTGAAGCATTACAAAATAAATGG-3′; and
3_UTR_rv, 5′-GGAATTCTCAACAACTAAATTCAAAC-3′.

The products were combined with the *neo4* cassette (Mochizuki 2008) using 5_UTR_fw and 3_UTR_rv and the PCR extender system (5PRIME). The B2086 and CU428 wild-type strains were transformed with the PCR product as described (Cassidy-Hanley et al. 1997), and the transformants were selected with 100 μg/mL paromomycin in the presence of 1 μg/mL cadmium chloride. The endogenous Mac *HEN1* loci were replaced by phenotypic assortment and selection using increasing concentrations of paromomycin. Complete replacement was confirmed by Southern hybridization (Supplemental Fig. S5B).

DNA elimination assay

Elimination of M, R, Cal, and Tlr1 IES elements (Austerberry et al. 1989; Katoh et al. 1993; Wells et al. 1994) was performed as described previously (see also Fig. 5A; Aronica et al. 2008) with a slight modification. In this assay, the control cells were wild-type cells possessing a *neo4* marker at the 3′ flanking region of the *HHT2*-mCherry fusion gene in the macronuclei (K. Kataoka and K. Mochizuki, unpubl.). Single pairs of control and *HEN1* KO strains were placed into drops of SPP medium at ~8 h post-mixing and were allowed to complete conjugation. The separated exconjugants were analyzed at 30 h post-mixing by nested PCR. In the first PCR, all five sets of primers—the outer primers for the four IES regions and the outer *neo4* primers—were used in a single reaction. In the second PCR, only one set of inner primers was used per reaction. In this way, DNA elimination of all four IES loci in a single exconjugant could be analyzed. The control and *HEN1* KO strains contain *neo4* genes in their parental Macs, but their progeny lack *neo4* genes in their micronuclei and new macronuclei. Samples that provided *neo4* amplification products, indicating that the parental cells aborted conjugation, were excluded from the study.

Progeny viability test

Viability of progeny was analyzed as described previously (Mochizuki et al. 2002) with a slight modification: The mating pairs were isolated at 6–8 h post-mixing, and the growth of the cells was examined ~60 h after cloning.

SUPPLEMENTAL MATERIAL

Supplemental material can be found at http://www.rnajournal.org.

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Supplementary Materials

Legends for Supplementary Figures

Figure S1. Comparison of HEN1 homologues

(A) Alignment of the conserved domain of HEN1 homologues. The methyltransferase domain is indicated by a black bar. Amino acids that are identical are highlighted in black, while similar amino acids are shaded in grey. Tt = *Tetrahymena thermophila*; Dm = *Drosophila melanogaster*; Mm = *Mus musculus*; At = *Arabidopsis thaliana*. (**B)** Schematic drawings of HEN1 homologues. Identity (I) and similarity (S) of the methyltransferase domains of HEN1 homologues when compared to the founding member of the HEN1 family, At HEN1 (gaps were omitted). Percentages of identical (I) and similar (S) residues are indicated. dsRNA indicates the location of the double-stranded RNA binding domain specific for plant HEN1.

Figure S2. Sequences of synthetic RNAs used for *in vitro* methylation assays

Figure S3. Methylation occurs on single-stranded scnRNA *in vivo*

Small RNAs from Twi1p slicer-dead mutant cells extracted at 4 h post-mixing were combined with synthetic, unmodified RNA of 21 nt and treated with (+) or without (−) periodate oxidation/β-elimination. The reactions were then mixed with synthetic RNA of 32 nt and analyzed by denaturing gel electrophoresis, followed by nucleic acid-specific fluorescent dye staining (left). Densitometric analysis of the fluorescent signal is shown on the right. The gel mobility of scnRNAs from Twi1p slicer-dead mutant cells increased by nearly 1.5 nt after periodate oxidation/β-elimination,

indicating that these scnRNAs bear 2′- and 3′-hydroxyl groups at their terminal nucleotides.

Figure S4. *HEN1* mRNA expression in wild-type (wt) cells

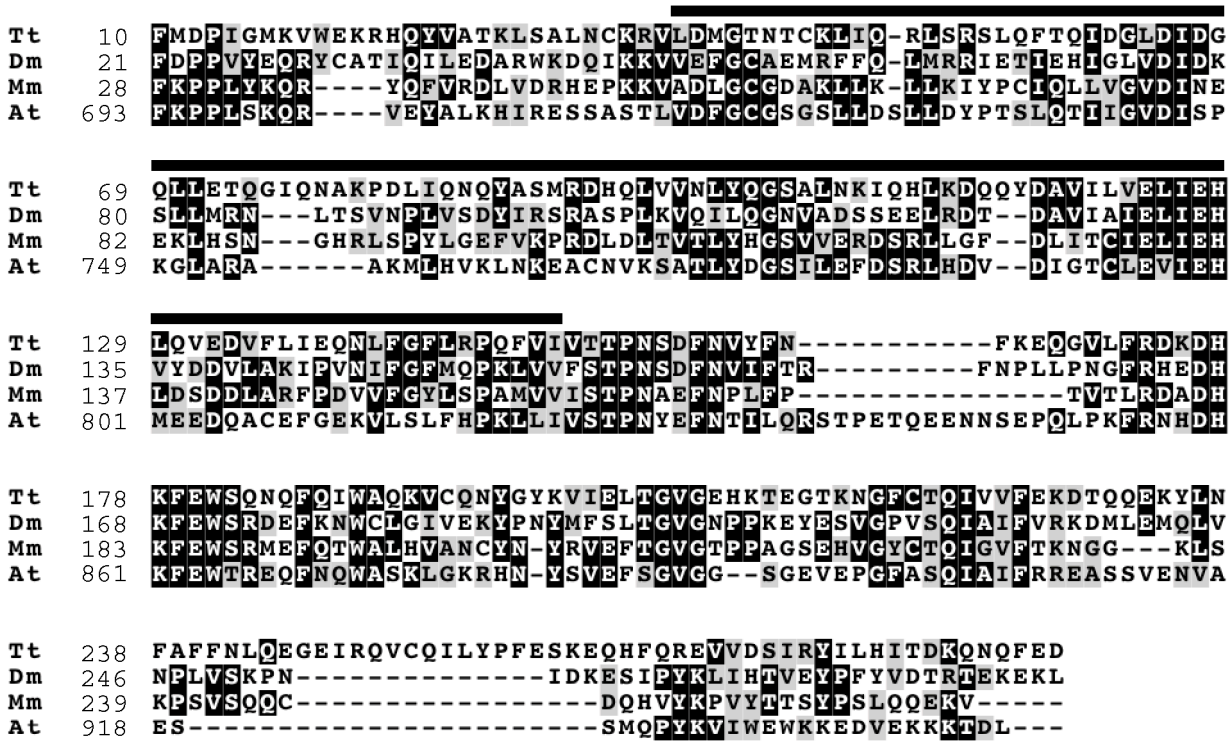
HEN1 mRNA expression in wt cells. Total RNA extracted from log-phase, vegetative, and mating cells was used for RT-PCR.

Figure S5. Construction and analysis of *HEN1* knockout cells

(A) Schematic drawing of the *HEN1* knockout strategy. The entire coding region was replaced with a drug resistance marker. The knockout construct was introduced into the *HEN1* locus by homologous recombination. **(B)** Confirmation of the complete replacement of the endogenous locus. Total DNA isolated from wild-type (wt) and Δ*HEN1* strains was digested with *Bgl*II (b) and analyzed by Southern hybridization using the radiolabelled probe shown in B. **(C)** Δ*HEN1* strains show a defect in viable progeny production. Single mating pairs were placed into drops of medium and were incubated for 60 h at 30 °C. Completion of mating was confirmed by testing the expression of a drug resistance marker that is active only in sexual progeny.

Figure S1

A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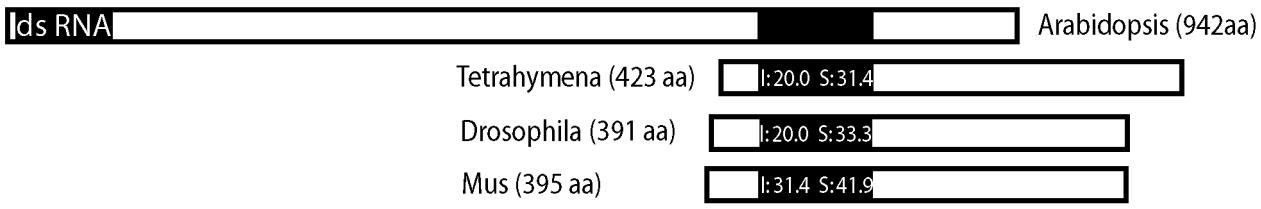
Dm 21 FDPPVYEQRYCATIQILEDARWKDQIKKVVEFGCAEMRFFQ-LMRRIETIEHIGLVDIDK
Mm 28 FKPPLYKQR----YQFVRDLVDRHEPKKVADLGCGDAKLLK-LLKIYPCIQLLVGVDINE
At 693 FKPPLSKQR----VEYALKHIRESSASTLVDFGCGSGSLLDSLLDYPTSLQTIIGVDISP
Tt 69 QLLETQGIQNAKPDLIQNQYASMRDHQLVVNLYQGSALNKIQHLKDQQYDAVILVELIEH
Dm 80 SLLMRN---LTSVNPLVSDYIRSRASPLKVQILQGNVADSSEELRDT--DAVIAIELIEH
Mm 82 EKLHSN---GHRLSPYLGEFVKPRDLDLTVTLYHGSVVERDSRLLGF--DLITCIELIEH
At 749 KGLARA------AKMLHVKLNKEACNVKSATLYDGSILEFDSRLHDV--DIGTCLEVIEH
Tt 129 LQVEDVFLIEQNLFGFLRPQFVIVTTPNSDFNVYFN----------FKEQGVLFRDKDH
Dm 135 VYDDVLAKIPVNIFGFMQPKLVVFSTPNSDFNVIFTR---------FNPLLPNGFRHEDH
Mm 137 LDSDDLARFPDVVFGYLSPAMVVISTPNAEFNPLFP---------TVTLRDADH
At 801 MEEDQACEFGEKVLSLFHPKLLIVSTPNYEFNTILQRSTPETQEENNSEPQLPKFRNHDH
Tt 178 KFEWSQNQFQIWAQKVCQNYGYKVIELTGVGEHKTEGTKNGFCTQIVVFEKDTQQEKYLN
Dm 168 KFEWSRDEFKNWCLGIVEKYPNYMFSLTGVGNPPKEYESVGPVSQIAIFVRKDMLEMQLV
Mm 183 KFEWSRMEFQTWALHVANCYN-YRVEFTGVGTPPAGSEHVGYCTQIGVFTKNGG---KLS
At 861 KFEWTREQFNQWASKLGKRHN-YSVEFSGVGG--SGEVEPGFASQIAIFRREASSVENVA
Tt 238 FAFFNLQEGEIRQVCQILYPFESKEQHFQREVVDSIRYILHITDKQNQFED
Dm 246 NPLVSKPN----------IDKESIPYKLIHTVEYPFYVDTRTEKEKL
Mm 239 KPSVSQQC----------DQHVYKPVYTTSYPSLQQEKV-----
At 918 ES----------SMQPYKVIWEWKKEDVEKKKTDL---

B

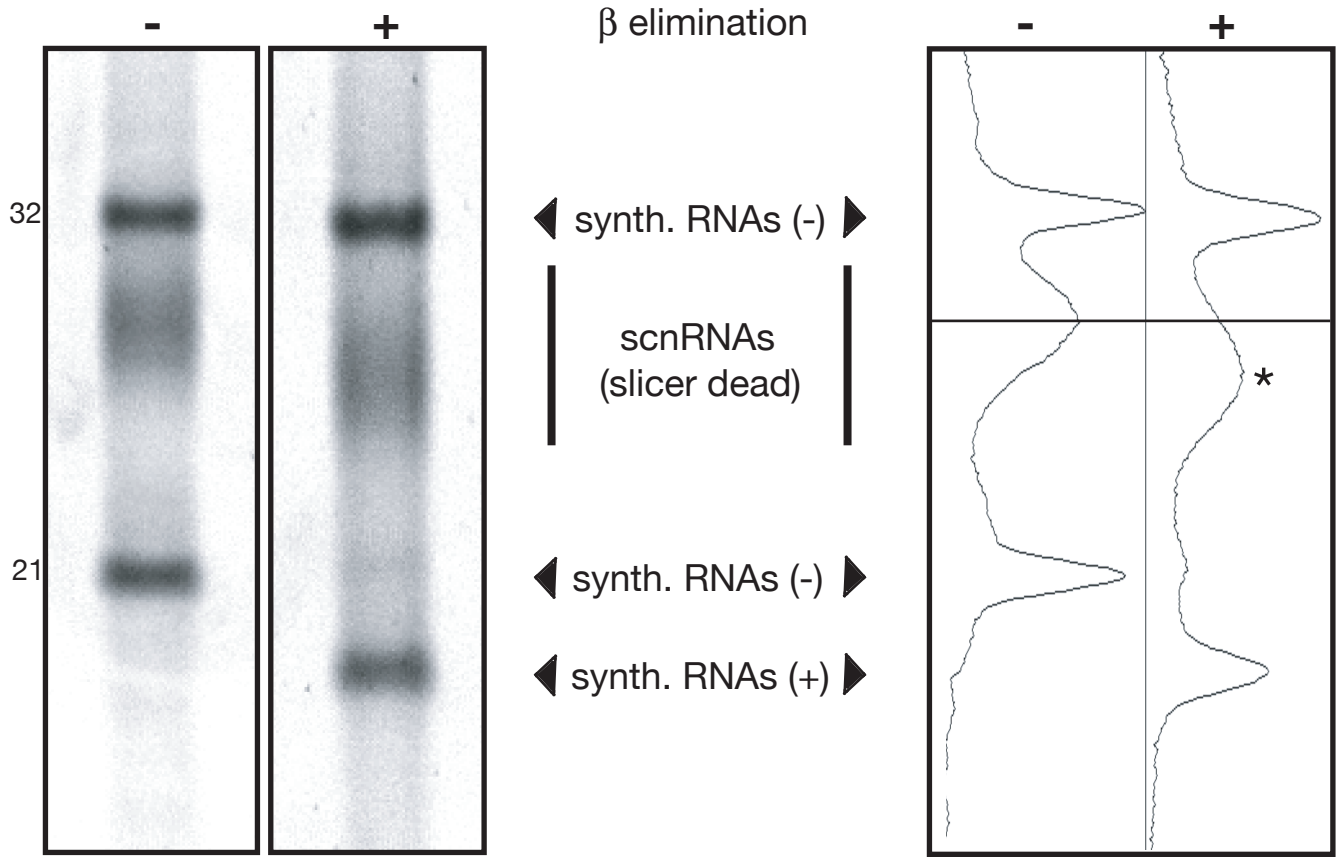

dsRNA
Arabidopsis (942aa)
Tetrahymena (423 aa)
I:20.0 S:31.4
Drosophila (391 aa)
I:20.0 S:33.3
Mus (395 aa)
I:31.4 S:41.9

Supplementary Figure S2

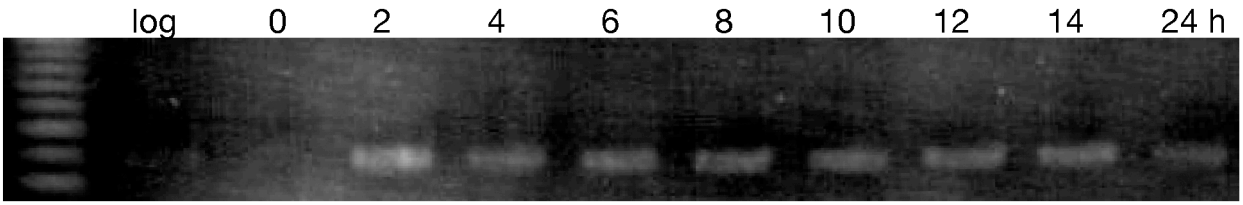
Synthetic RNAs:

I)	29 nt:	UCGAGUCGAGUUGAUCUUAGUUUCUUUUA
II)	29 nt me:	UCGAGUCGAGUUGAUCUUAGUUUCUUUUmA
III)	ds blunt:	UCGAGUCGAGUUGAUCUUAGUUUCUUUUA AGCUCAGCUCAACUAGAAUCAAAGAAAAU
IV)	ds overhang:	UCGAGUCGAGUUGAUCUUAGUUUCUUUUA ACAGCUCAGCUCAACUAGAAUCAAAGAAA
V)	32 nt	UCGAGUCGAGUUGAUCUUUAGUUUCUUUUAGC
	28 nt	UUCGAGUUGAUCUUUAGUUUCUUUUAGC
	24 nt	UGUUGAUCUUUAGUUUCUUUUAGC
	20 nt	UAUCUUUAGUUUCUUUUAGC
	16 nt	UUUAGUUUCUUUUAGC

Supplementary Figure 3

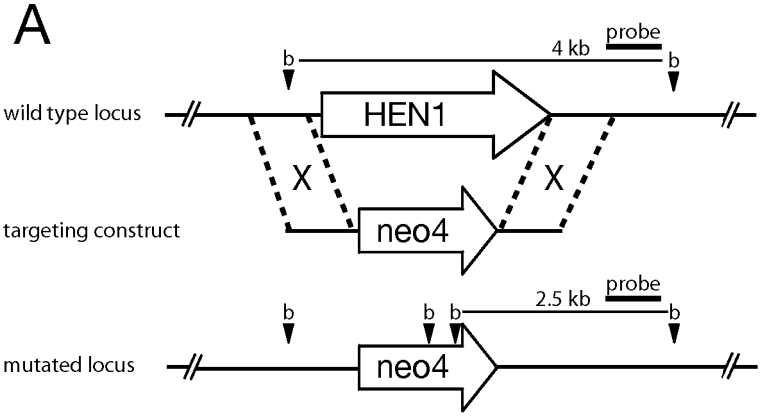


Supplementary Figure 4

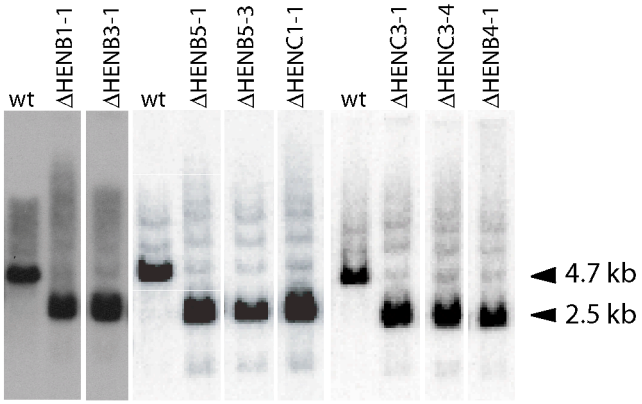


Supplementary Figure 5

A



B



C

Strains mated	Number of pairs examined	Number of pairs that gave rise to viable progeny	
ΔHEN1-B31 x ΔHEN1-C31	101	5	(5.0%)
ΔHEN1-B51 x ΔHEN1-C41	123	1	(0.8%)
ΔHEN1-B53 x ΔHEN1-C34	99	2	(2.0%)
ΔHEN1-B51 x ΔHEN1-C34	103	3	(2.9%)
B2086 x CU428	200	80	(40%)

Cell

The *Tetrahymena* Argonaute-Binding Protein Giw1p Directs a Mature Argonaute-siRNA Complex to the Nucleus

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SUMMARY

Emerging evidence suggests that RNA interference (RNAi)-related processes act both in the cytoplasm and in the nucleus. However, the process by which the RNAi machinery is transported into the nucleus remains poorly understood. The *Tetrahymena* Argonaute protein Twi1p localizes to the nucleus and is crucial for small RNA-directed programmed DNA elimination. In this study, we identify Giw1p, which binds to Twi1p and is required for its nuclear localization. Furthermore, the endoribonuclease (Slicer) activity of Twi1p plays a vital role in the removal of one of the two strands of Twi1p-associated small interfering RNAs (siRNAs), leading to a functionally mature Twi1p-siRNA complex. Slicer activity is also shown to be required for nuclear localization of Twi1p and for its association with Giw1p. These results suggest that Giw1p senses the state of Twi1p-associated siRNAs and selectively transports the mature Twi1p-siRNA complex into the nucleus.

INTRODUCTION

Argonaute family proteins bind to small RNAs (~20–30 nt) and are integral players in all known RNA interference (RNAi)-related gene-regulatory pathways (reviewed in Tolia and Joshua-Tor, 2007). Many Argonaute proteins act in the cytoplasm, where they induce posttranscriptional gene silencing. Recent evidence suggests that Argonaute proteins also act in the nucleus.

In mammals, the Argonaute proteins Ago1 and Ago2 mediate transcriptional silencing (Janowski et al., 2006; Kim et al., 2006) and Ago2 localizes to the nucleus in an Importin 8-dependent manner (Weinmann et al., 2009). Another Argonaute protein, MIWI2, localizes to the nucleus in fetal mouse testes and is required for DNA-methylation-mediated retrotransposon silencing (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). The *Drosophila* Argonaute protein Piwi localizes to nuclei of nurse and follicle cells in the ovary (Cox et al., 2000; Brennecke et al., 2007) and plays a role in transcriptional gene silencing (Pal-Bhadra et al., 2002). In *Arabidopsis*, the nuclear-localizing Argonaute proteins AGO4 and AGO6 are involved in RNA-directed DNA methylation (Li et al., 2006; Pontes et al., 2006; Zheng et al., 2007). In the fission yeast *Schizosaccharomyces pombe*, the Argonaute protein Ago1 is involved in both transcriptional and posttranscriptional gene silencing (Volpe et al., 2002; Sigova et al., 2004) and localizes to both the cytoplasm and the nucleus (Noma et al., 2004).

Two recent studies indicate that nuclear import of some Argonaute proteins is dependent on small RNAs. The *Caenorhabditis elegans* Argonaute protein NRDE-3 needs to associate with a small interfering RNA (siRNA) to localize to the nucleus (Guang et al., 2008). In mice, nuclear localization of MIWI2 requires MILI, which is essential for the production of Piwi-associated (pi) RNAs that bind MIWI2 (Aravin et al., 2008). These studies suggest that some mechanism distinguishes between free Argonaute proteins and those complexed with small RNAs, transporting only the latter into the nucleus. However, little is known about how small RNAs regulate the nuclear localization of Argonaute proteins.

The ciliated protozoan *Tetrahymena thermophila* provides an extreme example of a nuclear-acting Argonaute protein. The Argonaute protein Twi1p plays an essential role in programmed DNA elimination (Mochizuki et al., 2002), which is evolutionarily related to RNAi-directed heterochromatin formation in other eukaryotes (reviewed in Malone and Hannon, 2009). *Tetrahymena* possesses a germline micronucleus and a somatic macronucleus in a single cell. The micronucleus produces both new micronuclei and new macronuclei during sexual reproduction. During macronuclear development, ~6000 different internal eliminated sequences (IESs) are defined by ~28–29 nt siRNAs, termed scan RNAs (scn) RNAs (Mochizuki et al., 2002; Yao et al., 2003; Lee and Collins, 2006), and removed. scnRNAs are processed from bidirectionally transcribed noncoding RNAs (Chalker and Yao, 2001) by the Dicer-like protein Dcl1p in the

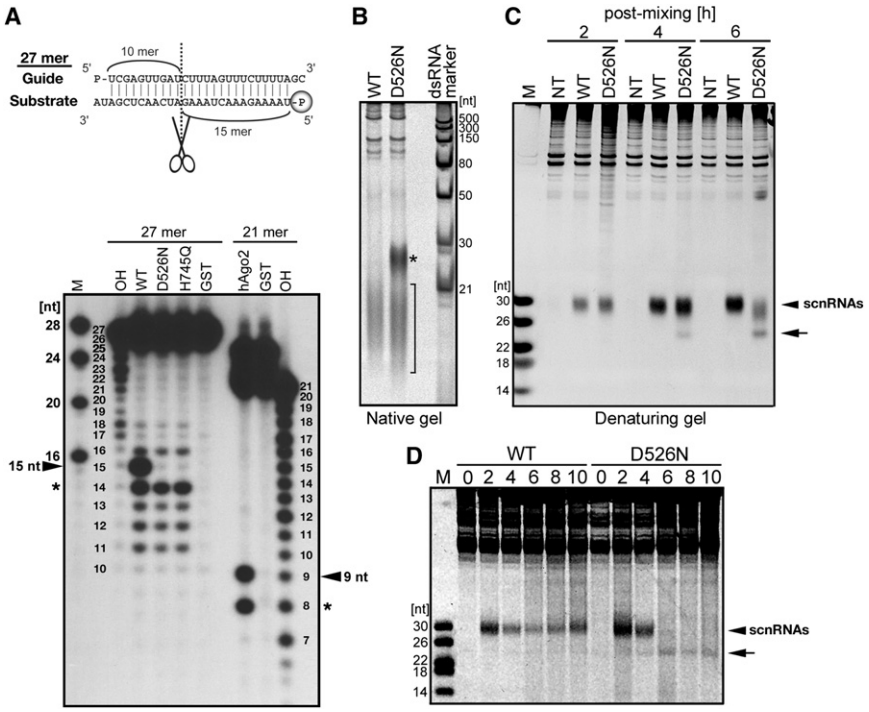

Figure 1. Twi1p Has Slicer Activity that Is Important for Passenger-Strand Removal of scnRNAs

(A) Wild-type (GST-Twi1p-WT) or Slicer-dead (GST-Twi1p-D526N, GST-Twi1p-H725Q) recombinant Twi1p, or GST was incubated with a 27 nt guide-strand RNA and then with 5′-end-labeled substrate RNA. Wild-type, but not Slicer-dead, Twi1p cleaved the substrates between residues base paired to nucleotides 10 and 11 of the guide strands, thus producing 15 nt labeled products. As a control, recombinant human Ago2 (hAgo2) was incubated with 21 nt guide and target RNAs, resulting in 9 nt labeled products. 20 and 24 nt RNA oligos (M) and partially alkaline hydrolysed substrate RNAs (OH) were 5′ end labeled and used as position markers. Asterisks indicate byproducts that have also been detected in other in vitro assays.

(B) scnRNAs coimmunoprecipitated with FLAG-HA-Twi1p-WT (WT) and FLAG-HA-Twi1p-D526N (D526N) at 4 hr postmixing were separated in a native gel and stained by GelRed. Double- and single-stranded scnRNAs are marked with an asterisk and a bracket, respectively.

(C) scnRNAs coimmunoprecipitated with FLAG-HA-Twi1p-WT (WT) or FLAG-HA-Twi1p-D526N (D526N) at 2, 4, and 6 hr postmixing were separated in a denaturing gel and stained by GelRed. Nontagged wild-type strains (NT) were used as a negative control. The position of scnRNAs and ~24 nt RNAs are marked by an arrowhead and an arrow, respectively. M: single-stranded RNA markers.

(D) Total RNA from FLAG-HA-*TWI1*-WT (WT) and FLAG-HA-*TWI1*-D526N (D526N) at 0, 2, 4, 6, 8, and 10 hr postmixing was separated in a denaturing gel and stained by GelRed. The position of scnRNAs and ~24 nt RNAs are marked by an arrowhead and an arrow, respectively.

See also Figure S1.

micronucleus (Malone et al., 2005; Mochizuki and Gorovsky, 2005) and complex with Twi1p in the cytoplasm (Mochizuki and Gorovsky, 2004). The Twi1p-scnRNA complex is then transported into the parental macronucleus, where it has been proposed that IES-specific scnRNAs are enriched through selective degradation of scnRNAs that are complementary to the macronuclear DNA, probably by recognizing nascent transcripts (Mochizuki et al., 2002; Aronica et al., 2008). Finally, Twi1p-scnRNA complexes move into the developing macronucleus, where they induce the formation of heterochromatin, leading to DNA elimination (Mochizuki et al., 2002; Taverna et al., 2002; Liu et al., 2007).

These dynamic changes in the localization of Twi1p are believed to be essential for the conserved small RNA-directed heterochromatin-formation process, providing an attractive model for the study of how Argonaute-small RNA complexes are localized and how their localizations influence their functions in eukaryotes. Here, we report that nuclear localization of Twi1p is regulated by the Twi1p-binding protein Giw1p, which senses the state of siRNAs associated with Twi1p. This mechanism enables *Tetrahymena* to transport only a functionally mature Argonaute-siRNA complex into the nucleus.

RESULTS

Twi1p Has DDH-Motif-Dependent Slicer Activity

Some Argonaute proteins have endoribonuclease (Slicer) activity responsible for cutting RNAs with sequences complementary to those of their small RNA cargos. Slicer activity is provided by the evolutionarily conserved Piwi domain, when it contains a conserved catalytic core composed of an Asp-Asp-His (DDH) motif (reviewed in Tolia and Joshua-Tor, 2007). Comparison of the Piwi domains of the *Tetrahymena* Argonaute protein Twi1p and other Argonaute proteins possessing Slicer activity (Figure S1A, available online; Couvillion et al., 2009) revealed that Twi1p contains a DDH motif (Asp526-Asp596-His745), suggesting that it may have Slicer activity.

Slicer activity of Twi1p was analyzed with the use of recombinant Twi1p expressed in *E. coli* as a GST fusion protein. GST-Twi1p was incubated with 27 nt "guide" RNA to form RISC-like ribonucleoprotein complexes. These complexes were then incubated with a 5′ end radio-labeled 27 nt "substrate" RNA whose 3′ 25 nt were complementary to the guide RNA. As a positive control, recombinant human Ago2 fused to GST (Rivas et al., 2005) was prepared, complexed with 21 nt guide RNA, and incubated with a 5′ end radio-labeled 21 nt substrate RNA whose 3′ 19 nt were complementary to the guide RNA. The cleaved product was observed by denaturing gel electrophoresis followed by autoradiography (Figure 1A). If the GST-Twi1p cleaves the substrate in a manner similar to that of other Argonaute proteins with Slicer activity, which cleave the bond between residues base-paired to nucleotides 10 and 11 of the guide strand (reviewed in Tolia and Joshua-Tor, 2007), the radio-labeled cleavage product should be 15 nt long (see schematic drawing in Figure 1A). Indeed, a 15 nt RNA species was detected in the

GST-Twi1p sample (Figure 1A, 27-mer, WT), but not when GST was used alone (Figure 1A, 27-mer, GST). Similarly, radiolabeled cleavage product indicating cutting between residues base paired to nucleotides 10 and 11 of the guide strand (9 nt) was detected with the use of GST-hAgo2 and 21 nt RNAs (Figure 1A, 21-mer, GST-hAgo2). We conclude that Twi1p possesses Slicer activity.

Cleavage of substrate RNA by GST-Twi1p in our assay was less efficient than that by some other Argonautes in similar assays (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). A pre-steady-state kinetics analysis of substrate RNA cleavage (Förstemann et al., 2007) suggested that only ~0.4% of the recombinant GST-Twi1p was active (Figure S1B). The inefficient substrate cleavage by GST-Twi1p may be caused either by enzymatic inactivity of the majority of GST-Twi1p or by inefficient complex formation between Twi1p-guide-strand scnRNA in vitro.

For determining whether the DDH motif is involved in the Slicer activity of Twi1p, mutants were created in which either the first aspartic acid of the motif was replaced by asparagine (Twi1p-D526N) or the last histidine of the motif was replaced by glutamine (Twi1p-H754Q) (Figure S1A). Twi1p-D526N or Twi1p-H745Q fused to GST was analyzed as described above. As shown in Figure 1A, neither mutated enzyme produced a detectable 15 nt cleavage product, suggesting that the conserved motif is required for the Slicer activity of Twi1p. These mutated Twi1p proteins are referred to as Slicer-dead Twi1p.

Slicer Activity of Twi1p Is Important for scnRNA Passenger-Strand Removal In Vivo

In vivo, Twi1p associates with ~28–29 nt siRNAs, named scnRNAs (Mochizuki and Gorovsky, 2004). Given that scnRNAs are processed from double-stranded noncoding RNA by the Dicer-like protein Dcl1p (Malone et al., 2005; Mochizuki and Gorovsky, 2005), there must be a mechanism responsible for making scnRNAs single stranded. Because the Slicer activities of several Argonaute proteins are involved in the removal of one of the two small RNA strands (passenger strand) in other organisms (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005; Leuschner et al., 2006; Maiti et al., 2007; Steiner et al., 2009), we tested whether the Slicer activity of Twi1p was involved in scnRNA passenger-strand removal in vivo.

We constructed *Tetrahymena* strains whose *TWI1* loci in the polyploid macronucleus were completely replaced by a mutant construct (*FLAG-HA-TWI1-D526N*) encoding Slicer-dead Twi1p-D526N tagged with FLAG-HA (Figures S1C and S1E). *FLAG-HA-TWI1-WT*, expressing wild-type Twi1p tagged with FLAG-HA (Figures S1C and S1D), was also used. Two *FLAG-HA-TWI1-WT* strains of different mating types produced viable sexual progeny (Figure S1M), indicating that the FLAG-HA tag does not disturb the essential function (Mochizuki et al., 2002) of Twi1p. Comparable amounts of FLAG-HA-Twi1p-WT and FLAG-HA-Twi1p-D526N were detected by Western blotting (Figure S1H), indicating that the Slicer activity is not required for the accumulation of Twi1p protein.

scnRNAs coimmunoprecipitated with FLAG-HA-Twi1p-D526N or FLAG-HA-Twi1p-WT from cells at an early stage (4 hr postmixing) of conjugation were separated in native gel and stained with GelRed (Figure 1B). More than half of the FLAG-HA-Twi1p-D526N-associated scnRNAs detected by the staining migrated at positions corresponding to double-stranded ~28 nt RNA (Figure 1B, asterisk). The remaining scnRNAs from FLAG-HA-Twi1p-D526N and all scnRNAs from FLAG-HA-Twi1p-WT migrate as a smear (Figure 1B, bracket). We believe that this smear signal is attributable to the extensive sequence heterogeneity of the scnRNAs, which are believed to be transcribed from the whole micronuclear genome (Mochizuki et al., 2002). Consistent with this, by northern hybridization, two different 28 nt oligo DNA probes complementary to different specific scnRNAs sequences detected distinct bands within the smear region (Figure S1N). Because of their small size and AT richness, a fraction of scnRNAs could dissociate during experimental handling, and this analysis likely underestimates the amount of double-stranded scnRNA associated with FLAG-HA-Twi1p-D526N. In contrast, none of the scnRNA associated with FLAG-HA-Twi1p-WT migrated to the position on the gel corresponding to double-stranded scnRNA (Figure 1B, WT). Denaturing gel analysis of these scnRNAs indicated that similar amounts of scnRNA were associated with FLAG-HA-Twi1p-D526N and FLAG-HA-Twi1p-WT at 2 hr and 4 hr postmixing (Figure 1C). We conclude that the Slicer activity of Twi1p plays an important, possibly essential, role in the scnRNA passenger-strand removal in vivo.

Slicer Activity of Twi1p Is Required for Stable Accumulation of scnRNA

We analyzed the expression of scnRNAs in the absence of the Slicer activity of Twi1p. *FLAG-HA-TWI1-D526N* strains expressed levels of scnRNAs similar to those of *FLAG-HA-TWI1-WT* strains in the early stages of conjugation (Figure 1D, 2–4 hr postmixing). However, in the *FLAG-HA-TWI1-D526N* strains, the amount of scnRNA was greatly reduced at the mid stage of conjugation (Figure 1D, 6 hr) and became undetectable at later stages of conjugation (Figure 1D, 8–10 hr). Thus, Slicer activity of Twi1p is not required for production of scnRNAs, but it is required for their stable accumulation. An exonuclease likely degrades the double-stranded scnRNAs complexed with Slicer-dead Twi1p, as the scnRNAs associated with FLAG-HA-Twi1p-D526N become gradually shorter and less abundant (Figure 1C, 4–6 hr). We previously reported that the RNA methyltransferase Hen1p methylates only single-stranded scnRNAs to protect them from degradation (Kurth and Mochizuki, 2009). The mid-stage disappearance of scnRNAs in Slicer-dead *TWI1* cells could be a result of a lack of methylation of double-stranded scnRNAs.

Approximately 24 nt of RNA bound to FLAG-HA-Twi1p-D526N in the mid stages of conjugation (4–6 hr postmixing) (Figure 1C, arrow) and accumulated (Figure 1D, arrow). Northern blot analysis demonstrated that ~28–29 nt scnRNAs, but not the ~24 nt RNAs, hybridize to a Tlr1-1 oligo DNA probe, which is complementary to a subset of scnRNAs derived from repeated Tlr1 IES elements (Figure S1O). Therefore, the ~24 nt RNAs probably are not degradation products of scnRNAs but are likely constitutively expressed ~23–24 nt siRNAs (Lee and Collins, 2006). These ~23–24 nt siRNAs might misassociate with Twi1p and therefore be stabilized when scnRNAs are reduced.

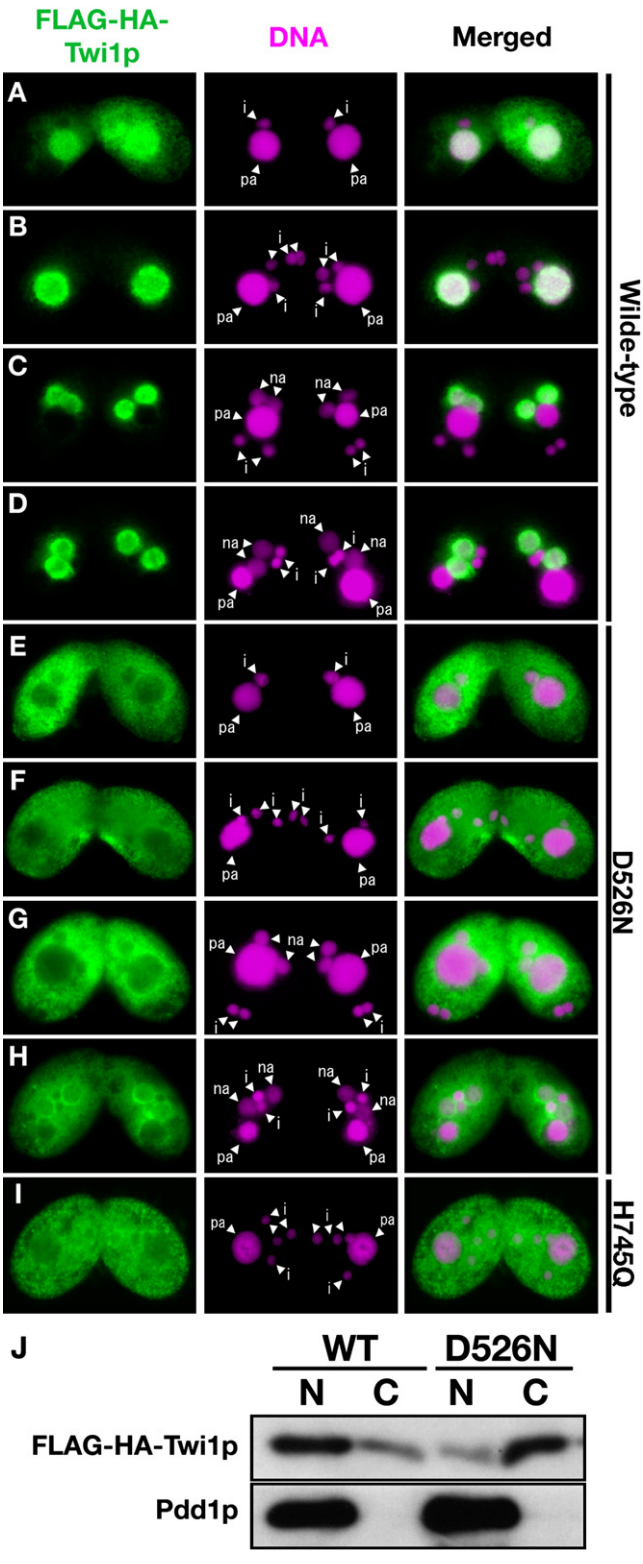

Figure 2. Slicer Activity of Twi1p Is Required for the Macronuclear Localization of Twi1p

(A–I) FLAG-HA-Twi1p-WT (Wild-type, A–D), FLAG-HA-Twi1p-D526N (D526N, E–H) and FLAG-HA-Twi1p-H745Q (H745Q, I) were localized by an anti-HA antibody (green). DNA was stained by DAPI (purple). The micronuclei (i), the parental macronuclei (pa), and the newly developed macronuclei (na) are marked.

(J) The nuclear (N) and the cytoplasmic (C) fractions from FLAG-HA-TWI1-WT (WT) or FLAG-HA-TWI1-D526N (D526N) at 9 hr postmixing (corresponding to the stages shown in D and H) were analyzed by Western blotting. FLAG-HA-Twi1p and Pdd1p were detected by an anti-HA and an anti-Pdd1p antibody, respectively.

See also Figure S2.

Slicer Activity of Twi1p Is Required for Twi1p Nuclear Localization

The localization of FLAG-HA-Twi1p-WT and FLAG-HA-Twi1p-D526N was analyzed by indirect immunofluorescence staining with the use of an anti-HA antibody. FLAG-HA-Twi1p-WT localized to both the cytoplasm and the parental macronucleus during early stages of conjugation (Figure 2A). During mid stages, it localized almost exclusively to the parental macronucleus (Figure 2B). In the later stages of conjugation, FLAG-HA-Twi1p-WT disappeared from the parental macronucleus and appeared in the newly developing macronucleus (Figures 2C and 2D). This localization pattern was indistinguishable from that of nontagged wild-type Twi1p detected with an anti-Twi1p antibody (see below), indicating that the presence of the FLAG-HA tag did not disturb the localization of Twi1p.

In contrast, FLAG-HA-Twi1p-D526N was detected in the cytoplasm throughout conjugation and did not accumulate in the parental macronucleus (Figures 2E–2G). In the late stages of conjugation, FLAG-HA-Twi1p-D526N accumulated at the periphery of the newly developing macronuclei but was still seen mostly in the cytoplasm (Figure 2H). These results were confirmed by analyzing nuclear and cytoplasmic fractions via Western blotting (Figure 2J). Though FLAG-HA-Twi1p-WT was detected mainly in the nuclear fraction, FLAG-HA-Twi1p-D526N was detected mainly in the cytoplasmic fraction. In contrast, the macronuclear protein Pdd1p (Coyne et al., 1999) was detected in the nuclear fraction in both FLAG-HA-*TWI1*-WT and FLAG-HA-*TWI1*-D526N strains. The other Slicer-dead Twi1p mutant (FLAG-HA-Twi1p-H745Q; Figures S1F and S1G) also localized to the cytoplasm (Figure 2I, Figure S2). These results indicate that Slicer activity is required for the Twi1p nuclear localization.

Given that comparable levels of scnRNAs accumulate in both wild-type and Slicer-dead FLAG-HA-*TWI1*-D526N strains at 4 hr postmixing (Figure 1D), at which point wild-type Twi1p was already localized to the parental macronucleus (Figure 2B), the mislocalization of Slicer-dead Twi1p was not likely due to the nucleolytic shortening or reduction of scnRNAs in the mutants; instead, it is probably directly caused by defective passenger-strand removal of scnRNAs.

Two possible mechanisms could explain Slicer-dependent Twi1p nuclear localization. The first suggests that Twi1p complexed with single-stranded scnRNA is anchored in the nucleus through an interaction between scnRNA and nascent macronuclear noncoding transcripts (Aronica et al., 2008). However, this anchoring cannot fully explain the nuclear localization of Twi1p, because *EMA1* KO strains, in which the scnRNA-noncoding RNA interaction is impaired, show normal Twi1p macronuclear localization (Aronica et al., 2008). The second and more likely

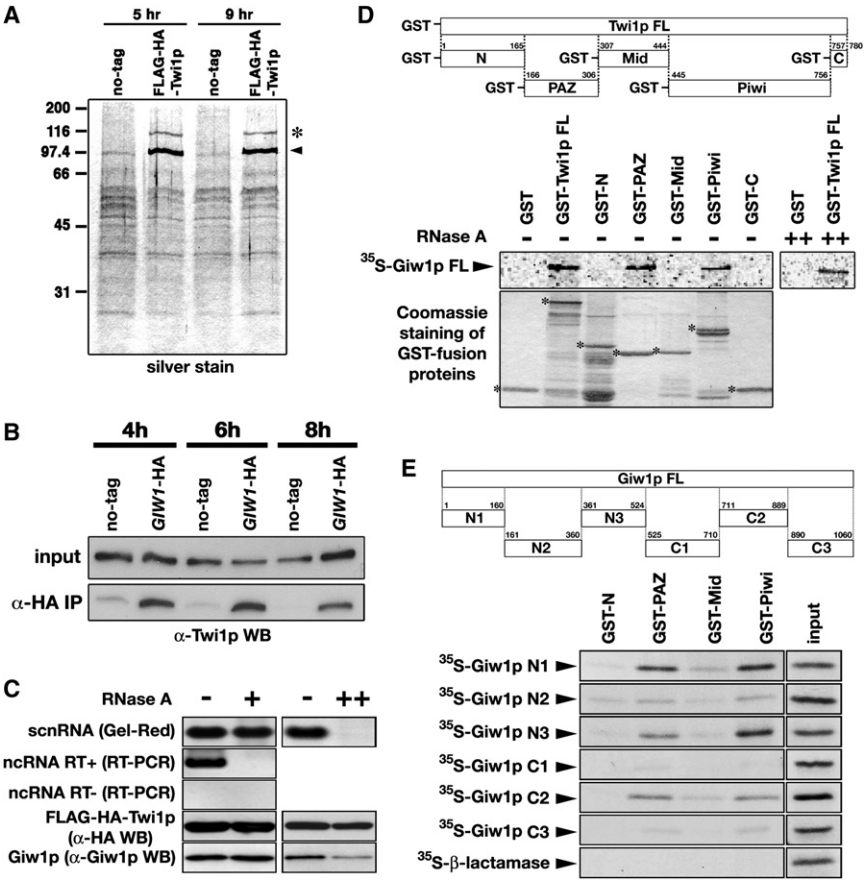

Figure 3. Giw1p Interacts with Twi1p

(A) Proteins copurified with FLAG-HA-Twi1p at 5 and 9 hr postmixing were separated by SDS-PAGE and visualized by silver staining. As a control, wild-type (no-tag) strains were processed in parallel. Positions of FLAG-HA-Twi1p-WT and ~115 kDa protein are marked by an arrowhead and an asterisk, respectively.

(B) Cell lysates were prepared from wild-type (no-tag) or *GIW1-HA* strains at 4, 6, and 8 hr postmixing, and immunoprecipitation was performed with an anti-HA antibody. Twi1p in the cell lysate (input) and immunoprecipitated samples (α-HA IP) were detected by Western blot with the use of an anti-Twi1p antibody.

(C) Cell lysate from FLAG-HA-*TWI1*-WT strains at 4 hr postmixing was incubated with 20 pg/mL (+), 100μg/mL (++), or without (−) RNase A, and FLAG-HA-Twi1p-containing complexes were immunoprecipitated with the use of an anti-FLAG antibody. Coimmunoprecipitated scnRNA was stained by GelRed. Coprecipitated noncoding RNA from the macronuclear R locus was analyzed by RT-PCR with (ncRNA RT+) or without (ncRNA RT−) reverse transcriptase. Precipitated FLAG-HA-Twi1p and Giw1p were detected by Western blotting with the use of anti-FLAG and anti-Giw1p antibodies, respectively.

(D) GST pull-down assays were performed with the use of GST, full-length Twi1p fused to GST (GST-Twi1pFL), or parts of Twi1p (N-terminal, PAZ, mid, Piwi, and C-terminal domain) fused to GST, and ^{35}S-labeled full-length Giw1p (^{35}S-Giw1p FL) with 100 μg/mL (++) or without (−) RNaseA. The precipitated proteins were separated by SDS-PAGE, and ^{35}S-Giw1p FL was detected by phosphorimager. One-fourth of GST and GST-Twi1p recombinant proteins (asterisks) used for the assay were stained by Coomassie blue.

(E) Different segments of ^{35}S-labeled recombinant Giw1p were expressed and pulled down with GST or parts of Twi1p (N-terminal, PAZ, mid, or Piwi domains) fused to GST. The precipitated proteins were separated by SDS-PAGE, and the ^{35}S-labeled Giw1p segments were detected by autoradiography. ^{35}S-labeled β-lactamase was used as a negative control. For comparison of the relative intensity of bands between the experiments, the precipitated proteins were exposed to X-ray films for the same period and processed equally. ^{35}S-labeled Giw1p segments used for the pull-down assay were analyzed in a different gel (input). See also Figure S3.

possibility is that an active macronuclear import mechanism specifically recognizes the complex formed between Twi1p and single-stranded scnRNA.

Giw1p is a Twi1p-Associated Protein

None of the previously identified Twi1p-associated proteins are required for macronuclear localization of Twi1p (Aronica et al., 2008; Bednenko et al., 2009). For the identification of Twi1p-associated proteins involved in the macronuclear localization of Twi1p, FLAG-HA-Twi1p-containing complexes were isolated with lysis and washing conditions that were milder (see Experimental Procedures) than those used in the previous studies. Immunoprecipitated samples from cells at the mid (5 hr postmixing) and the late (9 hr) stages of conjugation were separated by SDS-PAGE and analyzed by silver staining (Figure 3A). In addition to a band corresponding to FLAG-HA-Twi1p, a previously unidentified ~115 kDa protein was detected in *FLAG-HA-TWI1* strains but not in nontagged, wild-type strains. In this study, the three previously identified Twi1p-associated proteins (Ema1p [211 kDa], CnjBp [200 kDa], and Wag1p [123 kDa]) were undetectable by silver staining, although Ema1p and Wag1p were weakly detectable by Western blotting (Figure S3A), most likely due to the milder lysis conditions employed. The milder lysis procedure used here solubilizes mainly cytoplasmic components, including the 115kDa protein (see below), whereas all three previously identified Twi1p-associated proteins localize mainly to nuclei (Aronica et al., 2008; Bednenko et al., 2009) and require harsher lysis conditions to be observed.

We identified the 115 kDa protein by mass spectrometry (Figure S3B) and named it Giw1p (gentleman-in-waiting). The molecular weight of Giw1p as predicted from the *GIW1* mRNA sequence (GenBank XM_001029843) is 125 kDa. Giw1p shows no obvious similarity with any previously identified protein from any organism.

The interaction between Twi1p and Giw1p was confirmed by coimmunoprecipitation with the use of *GIW1*-HA strains in which all of the macronuclear *GIW1* loci were replaced by a *GIW1*-HA construct encoding C-terminal HA-tagged Giw1p (Figures S1I and S1J). *GIW1*-HA can replace essential function (see below) of *GIW1* in the production of sexual progeny (Figure S1M),

indicating that Giw1p-HA was functional and retained normal Giw1p physical interactions with other molecules. Two *GIW1*-HA or two nontagged strains were crossed, Giw1p-HA-containing complexes were immunoprecipitated with an anti-HA antibody, and the precipitated proteins were analyzed by Western blot with the use of an anti-Twi1p antibody. As shown in Figure 3B, a substantially higher amount of Twi1p was precipitated from the *GIW1*-HA strains than from the nontagged strains at all developmental stages tested, confirming that Twi1p and Giw1p are found in the same complex. Silver staining of these precipitated proteins detected only two specific proteins with the sizes of Giw1p-HA and Twi1p (Figure S3C), suggesting that Giw1p may complex only with Twi1p.

Giw1p Directly Binds to Twi1p

Because Twi1p associates with long noncoding RNAs (ncRNA) (Aronica et al., 2008), we determined whether the interaction between Twi1p and Giw1p was mediated by ncRNA. Lysates from *FLAG-HA-TWI1-WT* cells at 4 hr postmixing were incubated with 20 pg/mL of RNase A to degrade ncRNAs, and the Twi1p-Giw1p interaction was analyzed by immunoprecipitation with the use of an anti-FLAG antibody. The amount of Giw1p coimmunoprecipitated with FLAG-HA-Twi1p was comparable with (+) and without (−) RNaseA treatment (Figure 3C, Giw1p), whereas ncRNA was undetectable by RT-PCR in the immunoprecipitated sample from the RNase-treated lysate (Figure 3C, ncRNA RT+). These data suggest that the interaction between Twi1p and Giw1p is not mediated by long ncRNAs. This conclusion is further supported by the fact that Giw1p was coimmunoprecipitated with Twi1p from *EMA1* KO strains (Figure S3D), in which the Twi1p-ncRNA interaction is impaired (Aronica et al., 2008).

In the conditions described above, the amount of scnRNAs was unchanged after RNase A (20 pg/mL) treatment (+ in Figure 3C). However, scnRNAs were eliminated when we treated the lysate with a much higher concentration (100 μg/mL) of RNase A (++ in Figure 3C). Even in this condition, a significant, albeit reduced, amount (~60%) of Giw1p was coprecipitated with FLAG-HA-Twi1p (Figure 3C). This result suggests that Giw1p can interact with Twi1p in the absence of scnRNA in cell lysate. This conclusion is further supported by a GST pull-down assay using recombinant Twi1p expressed in *E. coli* and in vitro translated Giw1p. Giw1p was coprecipitated with full-length Twi1p fused with GST but not with GST alone (Figure 3D). Treatment with 100 μg/mL RNase A did not affect precipitation of Giw1p with GST-Twi1p (Figure 3D), suggesting that contaminating RNA does not mediate interaction of these two proteins. We conclude that Giw1p and Twi1p interact directly without RNA.

Twi1p shares conserved PAZ and Piwi domains with other Argonaute proteins (Mochizuki et al., 2002). To determine the domain(s) of Twi1p that interacts with Giw1p, we performed GST pull-down assays, using Giw1p and N-terminal, PAZ, Mid, Piwi, or C-terminal domains of Twi1p, each fused with GST. Giw1p coprecipitated with the PAZ and the Piwi domains but not with other domains of Twi1p (Figure 3D), indicating that Twi1p directly interacts with Giw1p through its PAZ and Piwi domains.

To determine which parts of Giw1p mediate the interaction with these domains, we divided Giw1p into six segments (Figure 3E), all of which we examined for binding with N-terminal, PAZ, Mid, or Piwi domains of Twi1p. Three of the six segments of Giw1p (N1, N3, and C2) were efficiently coprecipitated with PAZ and Piwi domains of Twi1p but were less efficiently coprecipitated with N-terminal and Mid domains (Figure 3E). Small amounts of the other three segments (N2, C1, and C3) were also coprecipitated with PAZ and Piwi domains, whereas a part of β-lactamase, which was used as a negative control, was not (Figure 3E), suggesting that these Giw1p segments also have binding activity, albeit weak, to PAZ and Piwi domains of Twi1p. Interaction between the N3 fragment of Giw1p and PAZ and Piwi domains of Twi1p was further confirmed by a reverse GST pull-down assay using GST-tagged Giw1p-N3 and His-tagged PAZ and Piwi domains (Figure S3E). His-PAZ and His-Piwi were coprecipitated with GST-Giw1p-N3 but not with GST alone. These results indicate that Giw1p has several different sites that have the ability to bind PAZ and Piwi domains of Twi1p and could bridge these domains.

Giw1p Is Specifically Expressed during Conjugation and Localizes to both the Cytoplasm and the Nuclei

Like *TWI1* mRNA expression (Mochizuki et al., 2002), *GIW1* mRNA expression occurs exclusively during early conjugation stages (2–4 hr postmixing) but was not detected in exponentially growing or starved vegetative cells (Figure 4A).

For study of the expression and localization of Giw1p, two *GIW1*-HA strains were crossed, and Giw1p-HA was detected with the use of an anti-HA antibody. Giw1p-HA was specifically detected during conjugation by Western blotting (Figure 4A). Indirect immunofluorescent staining showed that Giw1p-HA was localized to both the cytoplasm and the nuclei throughout conjugation (Figures 4B–4F).

Giw1p Is Required for Twi1p Nuclear Localization

To elucidate the function of Giw1p, we constructed *GIW1* knockout (KO) strains. All copies of the *GIW1* gene in the polyploid macronucleus were replaced by genes in which the entire coding sequence had been replaced by a drug-resistance marker (see Figures S1K and S1L).

Two wild-type or two *GIW1* KO strains were mated, and the localization of Twi1p was analyzed by indirect immunofluorescence staining with the use of an anti-Twi1p antibody. In wild-type cells, Twi1p was detected mainly in parental (Figures 5A and 5B) or newly developing (Figure 5C) macronuclei, whereas in *GIW1* KO cells, Twi1p localized to the cytoplasm throughout conjugation (Figures 5D–5F). These data indicate that Giw1p is required for nuclear localization of Twi1p.

We also analyzed the localization of Ema1p, Pdd1p, and Wag1p, which show localization patterns similar to those of Twi1p in wild-type cells (Figure S4). All of these proteins localize to macronuclei in *GIW1* KO cells (Figure S4), indicating that Giw1p is not a general nuclear transporter but is dedicated to Twi1p or to a limited set of proteins.

Giw1p Is Dispensable for Loading and Passenger-Strand Removal of scnRNAs

Given that Slicer-dead *TWI1* and *GIW1* KO strains showed a similar nuclear Twi1p localization defect, Giw1p could have a

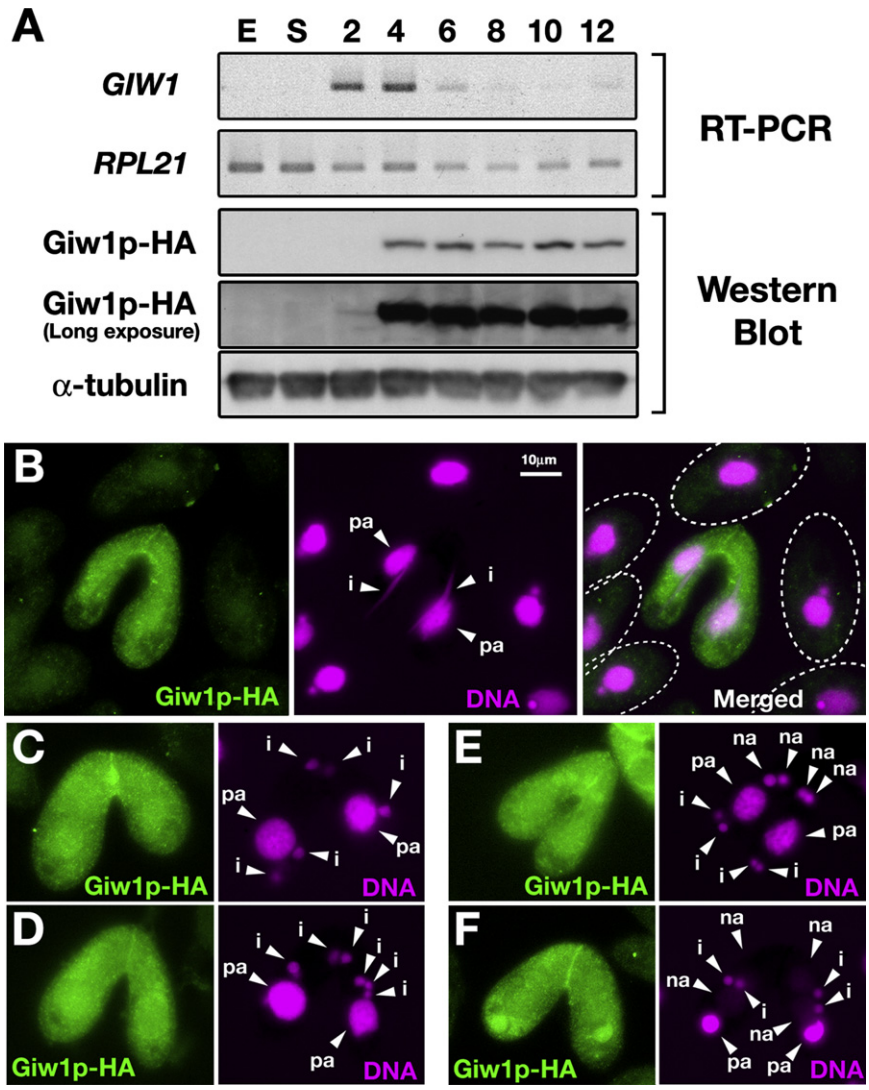

Figure 4. Giw1p Is Expressed Exclusively during Conjugation and Localizes to Both the Cytoplasm and the Nuclei

(A) Expression of *GIW1* mRNA and Giw1p-HA was analyzed by RT-PCR and Western blot, respectively. Total RNA of wild-type cells or total protein of *GIW1*-HA strains was extracted from exponentially growing vegetative (E), starved (S), or conjugating (2, 4, 6, 8, 10, and 12 hr postmixing) cells. Constitutively expressed *RPL21* mRNA and α-tubulin protein were also analyzed. Giw1p-HA and α-tubulin were detected by an anti-HA and an anti-α-tubulin antibody, respectively.

(B–F) *GIW1*-HA strains at early (B: meiotic prophase, C: meiosis), mid (D: pronuclear exchange), or late (E: macronuclear anlagen, F: nuclear alignment) stages of conjugation were fixed, and Giw1p-HA was localized by an anti-HA antibody (green). DNA was stained with DAPI (purple). In (B), nonmating cells (dotted lines) were included to show background staining. Micronuclei (i), parental macronuclei (pa), and new macronuclei (na) are marked.

function in passenger-strand removal of the Twi1p-associated scnRNAs. To assess this possibility, we immunoprecipitated Twi1p from wild-type and *GIW1* KO strains by using an anti-Twi1p antibody, and we analyzed Twi1p-associated scnRNA. Denaturing gel analysis indicated that similar amounts of scnRNA were associated with Twi1p in the presence and absence of Giw1p (Figure 6A). Native gel analysis detected little double-stranded scnRNA in the absence of *GIW1* (Figure 6B). We therefore conclude that Giw1p is not required for the production, loading, or passenger-strand removal of scnRNAs and that Giw1p most likely acts downstream of scnRNA passenger-strand removal in the pathway of Twi1p macronuclear import.

Presence of Double-Stranded scnRNA Inhibits Giw1p-Twi1p Interaction

For understanding of the relationship between the slicing of the scnRNA passenger strand and the action of Giw1p, two wild-type *FLAG-HA-TWI1-WT* strains or two Slicer-dead *FLAG-HA-TWI1-D526N* strains were mated, and FLAG-HA-Twi1p-WT or FLAG-HA-Twi1p-D526N were immunoprecipitated with the use of an anti-FLAG antibody. The coimmunoprecipitation of Giw1p was analyzed by Western blot with the use of an anti-Giw1p antibody. As shown in Figure 6C, Giw1p was coimmunoprecipitated with FLAG-HA-Twi1p-WT (WT), whereas no detectable Giw1p was precipitated with FLAG-HA-Twi1p-D526N (D526N). Similar results were obtained with the use of the other Slicer-dead mutant FLAG-HA-TWI1-H745Q (Figure S5). These results indicate that the Slicer activity of Twi1p has an essential role in the Twi1p-Giw1p interaction in vivo. Because Giw1p is required for the macronuclear localization of Twi1p (Figure 5), the lack of interaction between the Slicer-dead Twi1p mutants and Giw1p explains why Twi1p macronuclear localization is inhibited in the Slicer-dead *TWI1* strains. Because Slicer activity of Twi1p is important for the passenger-strand removal of scnRNAs, the inability of Slicer-dead Twi1p mutants to interact with Giw1p in vivo is likely caused by the association of double-stranded scnRNAs with these mutants.

To test this hypothesis, we analyzed the effect of double-stranded scnRNAs on Twi1p-Giw1p interaction in vitro (Figure 6D). Recombinant GST alone (lane 1) or wild-type Twi1p fused to GST (GST-Twi1p) (lanes 2–4) was first incubated with (lanes 3 and 4) or without (lanes 1 and 2) a 28 nt guide RNA, then with (lane 4) or without (lanes 1–3) a phosphorothioate-modified noncleavable 28 nt target (passenger) RNA. Then, GST-pull down assays were performed with the radiolabeled Giw1p. The amount of Giw1p coprecipitated with GST-Twi1p was greatly reduced when GST-Twi1p was preincubated with a guide and a noncleavable target RNA (lane 4), but not

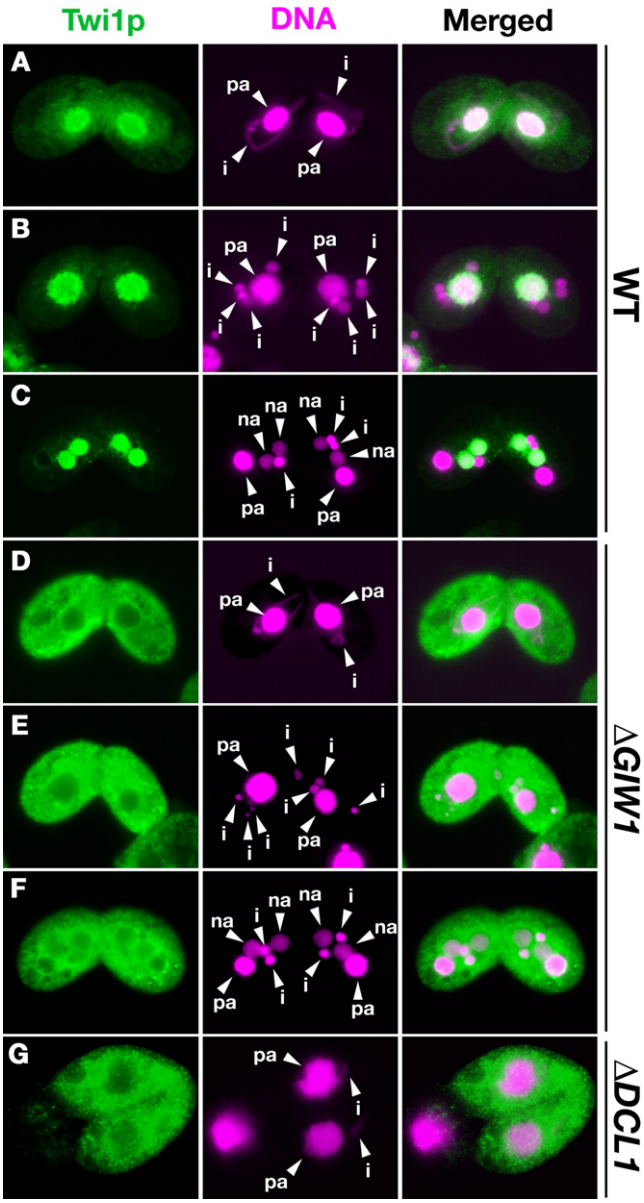

Figure 5. Twi1p Is Mislocalized to the Cytoplasm in the Absence of *GIW1*

Localization of Twi1p in wild-type (WT), *GIW1* KO (Δ*GIW1*), and *DCL1* KO (*ΔDCL1*) strains at early (meiotic prophase; A, D, and G), mid (postzygotic mitosis; B and E), and late (nuclear alignment; C and F) stages of conjugation was analyzed with the use of an anti-Twi1p antibody (green). DNA was stained by DAPI (purple). Micronuclei (i), parental macronuclei (pa), and new macronuclei (na) are marked with arrowheads.

See also Figure S4.

with a guide RNA alone (lane 3), indicating that the presence of double-stranded scnRNAs in Twi1p inhibits the Giw1p-Twi1p interaction. These results suggest that Giw1p can sense the state of the scnRNA complexed with Twi1p and binds to both unloaded Twi1p and Twi1p that is associated with single-stranded scnRNA.

Dicer-like Protein Is Required for Nuclear Localization of Twi1p

Given that Giw1p can bind to Twi1p without scnRNA in a cell lysate (Figure 3C) and in vitro (Figure 3D), it seems reasonable to expect that unloaded Twi1p could localize in the macronucleus. However, Twi1p was localized to the cytoplasm in the *DCL1* KO cells (Figure 5G), in which no detectable scnRNAs are produced (Malone et al., 2005; Mochizuki and Gorovsky, 2005). One possibility is that loaded single-stranded scnRNA may be required for binding to the nuclear import machinery. Alternatively, given that a significant proportion of Twi1p that enters into the macronucleus in a wild-type cell is predicted to be released from scnRNA by the selective degradation of scnRNAs complementary to the macronuclear DNA (Mochizuki and Gorovsky, 2004), there may be a mechanism that exports unloaded Twi1p to the cytoplasm, where Twi1p could load a new scnRNA cargo, thereby preventing accumulation of unloaded Twi1p in the nucleus.

Twi1p-Slicer and Giw1p Are Required for DNA Elimination

In both the Slicer-dead *TWI1* and *GIW1* KO strains, Twi1p is not localized to the developing macronucleus (Figures 2G and 2H, Figure 5F), where the Twi1p-scnRNA complexes are required for DNA elimination. We studied DNA elimination at four different loci by single-progeny PCR (Figure 7A) and found that their eliminations were indeed inhibited in the progeny of Slicer-dead *FLAG-HA-TWI1-D526N* strains (Figure 7B).

Because most of the *GIW1* KO cells are blocked midconjugation (see below), it was difficult to study their DNA elimination by PCR. Instead, we used fluorescence in situ hybridization (FISH) to analyze DNA elimination of the Tlr1 and the REP IES elements, which are moderately repeated in the micronuclear genome (Wuitschick et al., 2002; Fillingham et al., 2004). Both elements remained present in the new macronucleus of most of the progeny of *GIW1* KO, as well as of *FLAG-HA-TWI1-D526N* strains at 36 hr postmixing, but were completely removed in the progeny of wild-type cells (Figure 7C). Therefore, the absence of Giw1p inhibits DNA elimination of these IES elements.

Like all other known mutants showing defective DNA elimination, Slicer-dead *TWI1* and *GIW1* KO strains did not produce viable mating progeny (Figure S1M). In addition, *GIW1* KO strains showed developmental arrest, and ~70% of cells aborted mating midconjugation (Figure S6). This phenotype was not observed in *TWI1* KO strains (Mochizuki and Gorovsky, 2004) and Slicer-dead *TWI1* mutants (data not shown). Given that scnRNAs are believed to be derived from genic as well as nongenic sequences, they potentially target many different mRNAs for degradation if they are not properly regulated. Thus, the pleiotropic defects in *GIW1* KO cells could be due to the presence of mature Twi1p-scnRNA complexes in the cytoplasm. Alternatively, because Giw1p binds to the PAZ and Piwi domains of Twi1p (Figures 3D and 3E), Giw1p might block Twi1p-associated scnRNAs from binding to mRNAs (or other RNAs) or directly inhibit Slicer activity of Twi1p.

DISCUSSION

In this study, we have shown that the *Tetrahymena* Argonaute protein Twi1p has Slicer activity and that this activity is essential

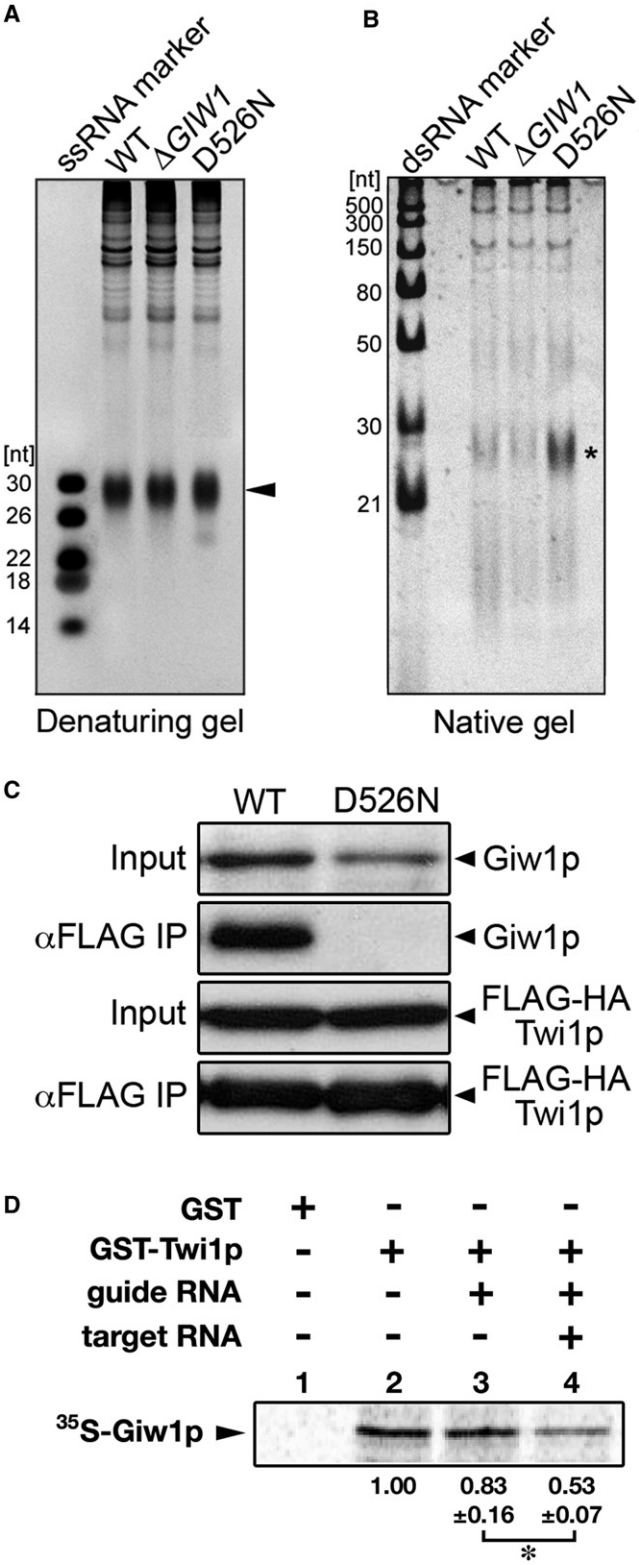

Figure 6. Giw1p Binds to Twi1p Complexed with Single-Stranded scnRNA

(A and B) Twi1p-containing complexes from wild-type (WT), *GIW1* knockout (Δ*GIW1*), and *FLAG-HA-TWI1-D526N* (D526N) strains at 4 hr postmixing were immunoprecipitated with the use of an anti-Twi1p antibody. Coprecipitated RNA was separated in a denaturing (A) or in a native (B) gel and stained by GelRed. Single-stranded (ss) or double-stranded (ds) RNA markers were included. scnRNA in the denaturing gel is marked with an arrowhead. Double-stranded scnRNA in the native gel is marked with an asterisk.

(C) Cell lysate (input) was prepared from FLAG-HA-TWI1-WT (WT) or FLAG-HA-TWI1-D526N (D526N) strains at 4 hr postmixing, and FLAG-HA-Twi1p-containing complexes were immunoprecipitated with the use of an anti-FLAG antibody (α-FLAG IP). Giw1p and FLAG-HA-Twi1p were detected by Western blot with the use of anti-Giw1p and anti-FLAG antibodies, respectively.

(D) GST pull-down assays were performed with the use of GST (lane 1) or GST-Twi1p (lanes 2–4), and ^{35}S-labeled Giw1p (^{35}S-Giw1p). In the experiment shown in lanes 3 and 4, GST-Twi1p was first incubated with 28 nt guide-strand RNA, then with (lane 4) or without (lane 3) 28 nt noncleavable target RNA that was complementary to the guide-strand RNA prior to the incubation with ^{35}S-Giw1p. Precipitated proteins were separated by SDS-PAGE, and ^{35}S-Giw1p was detected by phosphorimager. Relative amounts of ^{35}S-Giw1p detected (average ± standard deviation from four experiments) are shown at the bottom. Asterisk (*) indicates p = 0.014.

See also Figure S5.

for its macronuclear localization. We have also identified a Twi1p-associated protein, Giw1p, which is required for macronuclear localization of Twi1p. Giw1p binds to wild-type Twi1p, but not to Slicer-dead Twi1p, in vivo. These results indicate that Slicer-dependent passenger-strand removal of scnRNAs is a prerequisite for the Twi1p-Giw1p interaction, which, in turn, is essential for the macronuclear localization of Twi1p. Thus, Giw1p serves as a gatekeeper that allows only mature Twi1p-scnRNA complexes to enter macronuclei. Currently, the detailed mechanism by which Giw1p functions is not clear. The simplest hypothesis is that Giw1p might be an adaptor protein that connects the Twi1p-scnRNA complex to nuclear import machinery.

Given that the nuclear localization of some Argonaute proteins is dependent on the presence of their small RNA cargos in nematodes (Guang et al., 2008) and in mice (Aravin et al., 2008), small RNA-dependent nuclear localization of Argonaute proteins is probably widespread among eukaryotes. This study reveals yet another layer of the regulatory mechanisms for the nuclear localization of small RNA-Argonaute complexes: the requirement for passenger-strand removal for the nuclear import of a small RNA-Argonaute complex. This mechanism might have evolved to provide Argonaute proteins enough time to release aberrant RNAs and to find correct RNAs before they are imported into the nucleus. Alternatively, proteins that block or modulate the activity of mature small RNA-Argonaute complexes during their transport might have evolved first and then may have later acquired a direct role in the nuclear import process. Because it is not yet known whether maturation of Argonaute-small RNA complexes is required for nuclear transport of Argonaute proteins in other eukaryotes, the localization of Slicer-dead Argonautes will be of interest to study in other systems.

The conformation of a bacterial Argonaute protein changes according to the state of nucleic acids with which the protein is complexed, such that the space between the PAZ and Piwi domains is wider when it is associated with both guide and substrate strands than when it is associated with only a guide

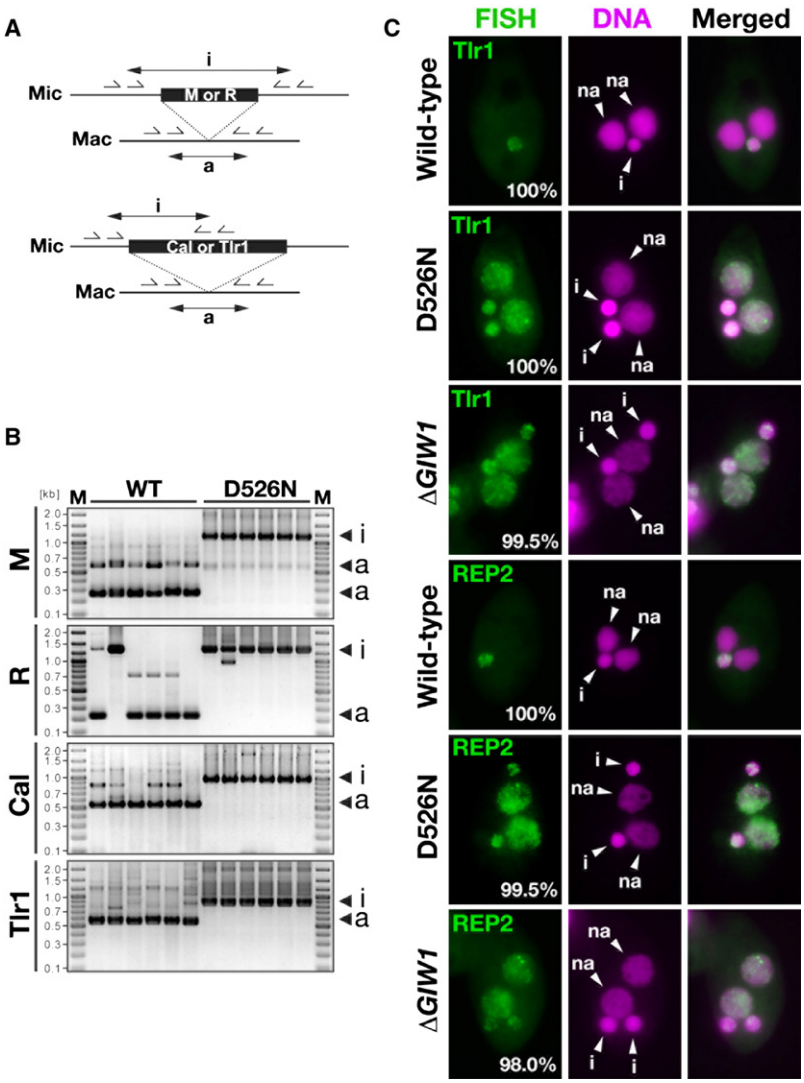

Figure 7. DNA Elimination Is Inhibited in the Progeny of Slicer-Dead *TWI1* and *GIW1* KO Cells

(A and B) DNA-elimination assays by PCR. (A) Horizontal lines, filled boxes, and arrows indicate macronuclear-destined sequences, eliminated DNAs (IESs), and primers for nested PCR, respectively. (B) Single exconjugants (sexual progeny) from FLAG-HA-*TWI1*-WT (WT) and FLAG-HA-*TWI1*-D526N (D526N) were assayed. The sizes of the unprocessed (micronuclear form) and the processed (macronuclear form) products are marked with "i" and "a," respectively.

(C) Exconjugants of wild-type (WT), Slicer-dead FLAG-HA-TWI1-D526N (D526N), and *GIW1* KO (Δ*GIW1*) at 36 hr post mixing were used for detecting Tlr1- and REP-IES elements by FISH (green). DNA was stained with DAPI (purple). The micronuclei (i) and the new macronuclei (na) are marked. The percentage of total exconjugants that showed phenotypes represented in the pictures is given (n = 200).

See also Figure S6.

strand (Wang et al., 2008). Because Giw1p binds to both the PAZ and the Piwi domains of Twi1p (Figure 3D), we propose that scnRNA passenger-strand removal also alters the distance between the PAZ and Piwi domains, allowing binding of Giw1p. Given that Giw1p is the only currently known protein that can detect the state of small RNAs (double or single stranded) associated with Argonaute proteins, identification of functional homologs of Giw1p in other eukaryotes could aid in understanding how conformational changes of Argonaute proteins affect their functions. Because Giw1p shows no obvious similarity with any previously identified proteins, determination of its crystal structure should prove valuable in identifying such homologs and in elucidating mechanisms of this process.

EXPERIMENTAL PROCEDURES

General Methods and Oligonucleotides

Tetrahymena strains, culture conditions, DNA-elimination assay, progeny-viability assay, and oligonucleotide used are described in the Extended Experimental Procedures.

Production of Recombinant Proteins

GST- or His-tagged Twi1p, hAgo1, and Giw1p were expressed in *E. coli*. ^{35}S-labeled full-length and partial Giw1p were synthesized by an in vitro translation system. See the Extended Experimental Procedures for the detailed procedures.

Slicer Assay

Approximately 3 pmol of the recombinant GST or GST fusion proteins were preincubated with 3 pmol of 27-mer or 21-mer guide RNAs (27-mer for Twi1p and 21-mer for hAgo2) in 30 μl of 1× cleavage buffer [30 mM HEPES (pH 7.4), 40 mM KOAc, 5 mM $Mg(OAc)_2$, 5 mM DTT] containing 1 μg BSA, 0.5 μg yeast RNA (Ambion), and 40 U RNasin (Promega) for 90 min at 25°C (for Twi1p) or 37°C (for hAgo2). 27-mer (for Twi1p) or 21-mer (for hAgo2) ^{32}P-labeled Target RNA was added and incubated for 90 min at 25°C (for Twi1p) or 37°C (for hAgo2). RNA was extracted with phenol-chloroform followed by ethanol precipitation, separated in a 20% denaturing polyacrylamide gel, and analyzed by autoradiography.

Antibody Production

Rabbit anti-Giw1p and anti-Wag1p antibodies were produced with the use of synthetic peptides. See the Extended Experimental Procedures for the detailed procedures.

Coimmunoprecipitation

Cells (2×10^6 in total) were lysed by sonication in 1 ml lysis buffer A [20 mM Tris pH 7.5, 100mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Tween 20, 0.1 mM PMSF, 1× complete protease inhibitor cocktail (Roche), and 0.4 U/ml RNasin (Promega)]. For Giw1p-HA immunoprecipitation, lysis buffer B (buffer A without Tween 20) was used. FLAG-HA-Twi1p, Giw1p-HA, or Twi1p complex was immunoprecipitated with the use of anti-FLAG (M2, Sigma), anti-HA (HA-7, Sigma) or anti-Twi1p (Aronica et al., 2008) antibody, respectively. FLAG-HA-Twi1p complexes were eluted in 0.3 mg/ml 3× FLAG peptide (Sigma). RNA in the eluate was extracted by TRIzol. Giw1p-HA and Twi1p complexes were eluted by boiling the gels in SDS-PAGE sample buffer or by incubating gels in the TRIzol. RNAs were separated in 15% denaturing gels or on 18% native polyacrylamide gels and were detected either directly by GelRed (Gentauer) or by northern blot (Aronica et al., 2008) probed with 5′-end-radiolabeled

oligo DNAs (M-28nt, Tlr1-28nt, or Tlr1-1). ncRNA was analyzed by RT-PCR (Aronica et al., 2008). FLAG-HA-Twi1p, Giw1p, and Twi1p were detected by Western blot with the use of anti-FLAG, anti-Giw1p, and anti-Twi1p antibodies, respectively.

Immunofluorescence Staining

Cells were fixed and processed as described previously (Loidl and Scherthan, 2004). See the Extended Experimental Procedures for the detailed procedures.

Nuclear-Cytoplasmic Fractionation

A pellet of 2×10^6 cells was gently resuspended in the ice-cold 1 ml lysis buffer [10 mM Tris pH 7.5, 5 mM $MgCl_2$, 10 mM KCl, 0.05% Triton X-100, 1× complete protease inhibitor cocktail (Roche)] and immediately centrifuged at 3,000 rpm for 5 min at 4°C. The supernatant was mixed with an equal volume of 2x SDS-PAGE sample buffer. The pellet was resuspended with the lysis buffer to make final volume 1 ml and was mixed with an equal volume of 2x SDS-PAGE sample buffer.

Identification of Giw1p

FLAG-HA-*TWI1* strains at 5 or 9 hr postmixing were lysed with a Dounce homogenizer in lysis buffer B, proteins were immunoprecipitated with anti-HA (HA-7) agarose, and eluted with 0.2 mg/mL HA peptide (Sigma). The eluate was separated by SDS-PAGE and visualized by silver or Coomassie Blue staining. The Coomassie Blue-stained ~115 kDa band was analyzed as described (Bowman et al., 2005).

GST-Pull Down Assay

For the experiment shown in Figures 3D and 3E, GST, GST-Twi1p, or GST-Giw1p-N3 (~1 μg) was incubated with 20 μl glutathione sepharose 4B resin (GE Healthcare) in GST pull-down buffer (GPB) [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% BSA] for 30 min at 4°C. For the experiment shown in Figure 6D, GST or GST-Twi1p (~2 μg) in PBS with 5 mM $Mg(OAc)_2$, 5 mM DTT, and ribolock RNase inhibitor (Fermentas) were incubated with or without 4.4 nmol 28 nt guide RNA for 90 min at 26°C. Then, 17–22 nmol of 28 nt noncleavable target RNA, 24 nt or 28 nt nontarget RNA (both provided similar results), or water was added and the reaction was incubated for 90 min at 26°C. A total of 20 μl glutathione sepharose 4B resin in GPB was added, and the reactions were incubated for 30 min at 4°C. The beads were washed with GPB and incubated with ^{35}S-labeled full-length or partial Giw1p recombinant protein (1.2 to 2 μl reaction of in vitro translation) or with His-tagged PAZ, mid, or Piwi domain of Twi1p (~0.4 μg) in GPB for 90–120 min at 4°C. The beads were washed with GPB and boiled in SDS-PAGE sample buffer, and the elutions were separated by SDS-PAGE. ^{35}S-labeled proteins were detected by phosphorimager (GE Healthcare) or by autoradiography. His-tagged proteins were detected by Western blot with the use of an anti-His antibody (QIAGEN).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cell.2010.02.010.

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Cell

EXTENDED EXPERIMENTAL PROCEDURES

Oligo DNAs

Sequences of oligo DNAs (primers) used in this study are listed below.

TWI1-D526N-EcRc-FW: CCATGGTGGTGGGCATGAATGTGTTCCATAACACCCCGGGCAAG
TWI1-H745Q-EcRc-FW: CGAGCGCGGTGCGTTATGCACAGACCCTGAGCAACTTTGTGGGCG
TWI1-RHm-FW-Xho: GGCTCGAGAAGATATGTCTCCTAATGATGC
TWI1-RHm-RV-Spe/Bam: GCGGATCCACTAGTTGATTCTTAAAGCTAATCG
RHD-D526N-FW: GGAATGAATGTTTTCCACAATACTCCTGG
RHD-D526N-RV: GTGGAAAACATTCATTCCTACCACCATTGTTGG
TWI1-H745Q-FW: CTCCATCTGCAGTTAGGTATGCATAAACCCTTTCCAACTTTGTAGGAGACAG
p115KO-FW-Xho: GGCTCGAGGATCTCAGCGAGCAGCTC
p115KO-RV-Bam: GCGGATCCGATCATAACAGGATCTTTGGCTGG
p115Tag-FW-Xho: GGCTCGAGAAAGAAGAAACTTAGTCAG
p115Tag-RV-Bam: GCGGATCCTGACTTATGAAAATAATAAAGTCGC
p115Tag-C-term-FW: GCTAGCGTCGACAGATCTAATTTTTTTTTGTGTAATTATATTAAA
p115Tag-C-term-RV: AGATCTGTCGACGCTAGCGCTTTGTAATCTCTAAAGAC
p115Tag-3′mid-FW: GTCTCTAATGATATCCTATCAAAGTATTGATTAG
p115-Tag-3′mid-RV: CTTTGATAGGATATCATTAGAGACTAATTTTTTTGTCAAG
HAsense: CTAGCTATCCTTATGATGTTCCTGATTATGCTT
HAanti: GATCAAGCATAATCAGGAACATCATAAGGATAG
REP2FW: TTGATGACTTAGATGACATTGATGAC
REP2RV: ACATTTCCAGCAGAATTGTCCAGC
M-28nt: TCACAGGTAGATCTGATAAATTCTAACA
Tlr1-28nt: GCTATTTATTCATCACTTTCTTAAGTCA
Tlr1-1: TCCAAATCATTTAATTATTCAGCTATTTATTCATCACTTTCTTAAGTCAT

Oligo RNAs

27-mer guide: 5′-P-UCGAGUUGAUCUUUAGUUUCUUUUAGC-3′
27-mer target: 5′-^{32}P-UAAAAGAAACUAAAGAUCAACUCGAUA-3′
21-mer guide: 5′-P-UCGAAGUAUUCCGCGUACGUG-3′
21-mer target: 5′-^{32}P-CGUACGCGGAAUACUUCGAAA-3′
28 nt guide RNA: 5′-P-UACUGUAGUUAUGUGUCGAUUGUCCAUA-3′
28 nt non-cleavable target RNA: 5′-P-UGGACAAUCGACACAU*AACUACAGUAAC-3′, * = phosphorothioate-modified
24 nt non-target RNA:5′-UGUUGAUCUUUAGUUUCUUUUAGC-3′
28 nt non-target RNA: 5′-P-UUCGAGUUGAUCUUUAGUUUCUUUUAGC-3′:

Synthetic Genes

Nucleotide sequences of synthetic genes for expressing recombinant Giw1p and Twi1p are:
>GIW1Ec

ATGCAGTCATTCGTAAACCTGATCCAGCTGGAAGAAAACTATAATCTGTCTCTGCTGTCATATAGTAACTCAATTCGTCAATA
CCTGCTGGATAACAAAGTGCATGAGGATAAGTATATCCAACTGATGAAAGACATTAAAGATCTGTTTAGTTCCCCAA
CCTTCCTGATGCCAAACTTCTCGGATAAACGTAAGCAATTTTTCTCTCAGCTGTGTCTGATTGCCTTTGATTTTGCAAATAATCA
GGTGACGGAGCAGCAGAGCCTGTCTGAGTTCCGTAAAAAATTTAAATTTCCATTTGTGATCATCGCCATCATTTATACCAA
GCTGCTGGAAATCAAAGAGGCTCTGCCAGAAGATTTTCTGACCCTGGTGGACTGCCACTTTAAAAACAAAGGCCCACAATCGA
TCTTCGCCAGTCTGACCCTGATCTCTCTGTTTCTGAATGAAGTCGATCAGTATGAGATTGATTGTGACCACATCGAATCTTCCAA
TACGCTGAGTTCATGTAGCGAAGCAGCGGAATCGAACATTCCAAAGAAAGTCAACATCACGGTTAAGACCGTGCAGAA
TTTCTTTGATAAACAGGGCCTGCAGATGATCGCCTTTCCACTGAACGTTATCAAAAATATCCACTGCGTCGCCCATGATCAA
CAATCGATTATCCAGCTGCTGCTGAAGATTGTGGTTATTATCATCAAAAACATGAGCAATAAAATCAAACAGGAGTTCTTCTA
CTTCCATCAGAAAGATCTGAACGTGATCCTGTCATGTTTCATCGGTTTCCTGCAGCAAAATAATCAGGCTCGTGAAGACCGTGA
TGTCGCACTGGCCAATATCTTTGAGTTTTGCGAGCTGCTGGCAAAGTCAACATCCCCGGCCCAGCTGTACGTAGCATTTAA
GGACTTTCTGGTCTATATTTCTCAAAACTTCAGCTTCGCTAATGACTCGATCGAATATTTTGATTTCAACAGTAAATGCGTGGA
TTTTCTGGTTAGCATTACTCCATGCTTCATCCAGCAGTCCGAAAACCTGTCAGCCTATCCACTGATTATCCAGCTGCTGAAACA

GTCGGCCAAAATTAATCTGGTGAACCTGGAAGATCAGATTGGCTGCCTGCACATTTGGTCACACTATTCAGACCAGTTCA
TTCAAGCCTATGATAATAACAATAACAGCATTAAGGCAGAAACCATTAACAATCTGGCTATCCTGAATAGTACATTCATCGAA
TCGCTGCAATTTGATATTGAACTGCTGTTTAACCTGCAGATTAAGGATCATGACAGTGAAGACGTCAACCAATTTGTCGATCA
GCTGAGCCGTCTGGTCATCCATTCGAACCTGATTGATAATCAGCTGTTCGTTGAGGTGATCAAATCTCTGTCCCTGCTGTACCA
GAAAAATATGCTGCTGTACTATACCTTCCAAAAATGCCTGATGAAACAGTATCTGAATAGCAAGGATACGCTGTCACAGATTTA
TCAGGTCTTCGGTAAAAGTAATGATTTTCTGCAAATTACGGAGCTGCTGTTCGAATCCTTTTCCCAGGCTCAGAAGCA
GTTCGCAAATCTGGACCTGAATACGATCGCGGCTATTGAAAAATTTACACAATTTATGTCGCAAATTGGTGCAAAACTGTTCAA
TACCCAAATCAGCCACCTGGAACAAAGTCAAATTCTGGGCATCTTCCAAAAGTACATTCTGAACCCAATTAAAATGTTTAA
TCTGAACATCTTCTCCCAGCTGCCTGAAAATTTTATCCAGCTGAATGAAGTGCAGTTCTCGCAGGTATGCACCATGATGGA
GTCTGTACATTATTTCATCTTTAGCGACAACAAATCTAATAGCGATCAGGTTTTCGAAACCGAGGTCGACGTTTCCATGATTAA
GAAAATTCTGCTGTTTAACCAGTCTGTCAATCAGGACATGGCCTACTGGATTAACCCACAGCTGAAAAATGAACGTAAGAAAA
TCAAGCAGTTTTACCAGAGTACCATTTATTGGGTCATCTTTGCAAGTATTAACCTGGAAAATGCAGCCAATATCTTCGAAAGCTA
TTTCTTCCAAAAAGAATTTAACATCTGGGTCCTGAACTCCACGATCAAAAAACTGAAAGGACGTATCCTGCCTAATA
CCTTCCTGCTGTACCTGTGTTACTATCTGAAATCTCTGACAGACTCCGACTCTTTTTCGAAAATCGTGCAAAAAAGCGAATA
CGAAGAAAAATTTCGTAAGCTGATGGTTAATATCAGTAAGAGTGTTCCTAGTGAAATTTATGTCGACTATTCTAAGATTCAGTA
CTATCTGCAACTGTATATCAACGAAGAGTGTCAGATGTTTCGTTTTCAGATTCTGAGCGATCAGACTGTTAGCCTGCTGA
CCCTGGAAAATCGTTTCTCTCACGTGTATAAGGTTTTCATTATCCTGGAAGACCTGTTTACTATTGTACAGCGTTTTCCTAA
GTTCTATTATGAGAATACGTTCATGGGACTGAAGCGTAATTTCTTTCTGAATCGCGTGTTCTGGATTATTGTGAAGTGCTTCA
CGATTGTGCCAAAACAGGAGCTGCTGGAGAACTCACAAATCTATAAGACCATTAGTCTGTACCTGAACCTGGCCA
TTCGTTTCAAAATGGACTTTCTGGAGAAAGAGGAAACCCAAAGCGAAAATAGCCACTATTTTAATTTTATTCTGGAAATCCTGGA
TCATATGTATTTCACGAATAAAAAGATCGTGCTGGATTTCTGTCAAAAGATCCTGAACTATTTCGATCAGGATTGTTACAATCA
GAATATCATTCAGCTGGCCCAGTCTAGTCAAACGTTTCAGAATATCACTCTGCAGCTGTTTACCAATATTTGGGAACAAA
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GTTCCAATAGTCAAATTATCTATTCGACGATTATTGAAAATCTGGTTATCAAAGAAATCGATGGTGTGAAGGTGCTGCAGAA
TGTTTTTCTGAAATCAATTATCAAAGATCTGCAGATCTGGGCTAAATCGATCTGTCCTCAACGTCTGCAGTCGTAA

>TWI1Ec

GGATCCATGAGCAACAAAGGCCTGGTGCAGAACAATCCGCGTCTGCGTTTTCTGTGCAACTATTACCAGGTGATGCTGCACAA
CGAGAAAATCTACAGCTATCAGTTTAGCGTGGAAGGCATGGAAGCGGCGGAAGTGATGAAACGTACCGGCGAAATTCTGAAA
GCGTGCATTAACAAACTGCGTGAAGTGTTCACCAACAAATTTCTGTTTCAGCACAGCGTGCTGTATAGCCCGGTGCAGCTGCA
GGCGAACCAGATGACCGAAATTGGCACCGTGGATACCAAAACCGTGAGCCTGAAACTGCTGGGCGAAGCGAACAACGAA
CGTGAAGTGCAGAACATTTTTGGCCGTCTGATTAAAACCGTGTTCGCGAAAATCAAACTGAACAAACTGGGCCGCAAATA
TTTTGATCCGGCGAGCCGTAAAGAATATAACGAATATGATCTGGCCGTGTTTAGCGGCTATGAAAGCAGCCTGGAAAAATATA
GCTTCAACCAGAAACTGCTGAACATTGATAGCTGCTTTAAAGTGCAGCGCACCAGCAATCTGCTGCAAGCGCTGCAACA
GGTGAAAGATCGTAACGATCTGAGCGGCTTTGTGGGCAACACCGTGATTACCAGCTATAACGGCAAATTTCATCGCATTGAAA
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GCGTATAACATCGGCAACATTGATCCGAACCAGCCGCTGGTGAAATGCGTGGAGCTGAAAGGCAAGACCAAAACCCCGTTCA
CCTATTATCTGATTCCGAGCCTGTGCCAGGTGACCGGCCTGACCGATCAGCAGCGTAACGATTTCAACCTGATGAAGAAA
CTGGCCGAAGTGACCAAACCGAAAGCGGGCGAACGTATTGCGCAGGCGTCTAAATTTATTCAGCGCATCAAACAGGA
TGCGGATAAAACCCTGAAAGATTGGAGCGTGGAAATTTGCGAAAAACCGCAGACCATCGATTATAAACAGCTGGA
TGCGGGCAACATGGAAATGCAGCAGGGCAAGAAGAAAATTAACGTGGCGACCGGCAACCTGGATCGTGATACCCAGACCCA
GATGTTTGAACAGCCGAGCCTGAACATGTGGGCGATTATTTATAGCGATCGTGATCAGAAAACCTTTGATAGCCTGCTGCGCA
CCTTTCAGGAATGCCTGAAATTCTATAACTATCCGTGCAAACCGCCGAACTGCATTAGCGTGCAGAGCCGTGGCGCGCAGGA
TTGGATTAACGCGCTGAAAAACGCGCCGGATAACGTGCAGATGGCGGTGTTTCTGCTGCCGGGTAAGAAGAAAGCGGGCA
TGTATTATGATGAAATCAAACGCTACTTCACCAACGTGAAACCGATTCCGACCCAGGTGATTCTGGCCAGCACCGCGATGAAA
GATAAAGGCCTGCGTAGCGTGGTGAACAAACTGCTGATGCAGATTTGCGCGAAAACCGGCGGTGTGCCGTGGGTGATGGA
TAACCTGCCGTTTCAGAACCTGCCGACCATGGTGGTGGGCATGGATGTGTTTCATAACACCCCGGGCAAGAAAGAAAGCA
TCTTTGGCTTTGTGAGCACCGTGGATCGTAACTTCAGCAAATATTACAGCCATAGCCATGTGCTGCCGACCGGCCAGGAAA
TTACCCCGTTTCTGCAGCAGGTCTATGAACAGGCGCTGAAAGAATTCAAAGATAGCAACGGCGTGTATCCGCAGCAGATTA
TTATTTTTCGTGATGGCGTGGGCGAAGGCCAGTTTAACGCGGTGAAAGATATTGAAATGCCGCAGCTGAAACAGGCGTGCCA
GAAAATTAACGGCTGCGAAAACATCAAATTCACCCTGATTATCGTGAACAAGAAAGTGGGCGCGAAATTCTATCAGAGCCA
GGATGGTAACGTGGGCAGCGCGGAAAATCCGCCGCAGGGCGCGCTGATTGAAGATCGTGTGACCAAAGGCGTGAACGA
TTTCTTTATCGTGAGCCAGAAAACCAACCAGGGCACCGCGAGCCCGACCCATTATACCATCATCTACAACGATATGATTGA
TGAAGCGCTGCAGAAAGATAACAGCCCGCAGTATCGTGAATTTAAACGTGATCTGCAGGTGCTGGCCTTTAAA
CTGTGCTTCCTGTATTATAACTGGACCGGCGCGATTAAAACCCCGAGCGCGGTGCGTTATGCGCATACCCTGAGCAA

CTTTGTGGGCGATCGTTATAATCCGCGCAAAAACGATGATACCCTGATTCAGGCGCATCCGAAATATGATAAATTCCGCA
GCCTGTATTTCATCTGACTCGAG

Production of Recombinant Proteins

To produce recombinant Giw1p and Twi1p, DNAs encoding Giw1p and Twi1p that had been optimized for codon usage in *E. coli* were synthesized (GenScript Corporation, Piscataway, USA and ATG:biosynthetics, Merzhausen, Germany, respectively). The sequences of the synthesized DNAs are shown in the "Synthetic genes" section above. Synthesized full-length *TWI1* DNA or DNA encoding the N-terminal (1 aa −165 aa), PAZ (166 aa-306 aa), mid (307 aa-444 aa), Piwi (445-756 aa) and C-terminal (757 aa −780 aa) domains were cloned into pGEX4T-1 (Amersham Biosciences). To generate pGEX-TWI1-D526N and pGEX-TWI1-H745Q, mutations were introduced into pGEX-TWI1, which contained the full length *TWI1* in pGEX4T-1, using the GeneEdior in vitro Mutagenesis System (Promega), and DNA oligos TWI1-D526N-EcRc-FW or TWI1-H745Q-EcRc-FW. Sequences of the oligo DNAs (primers) used in this study are listed in "Oligo DNAs" section above. To produce His-tagged PAZ, mid and Piwi-domains, these domains were cloned in to pET-28a(+) (Novagen). All of the GST or His fusion proteins above and GST alone were expressed in *E. coli* strain BL21(DE3). The bacteria strain expressing human Ago2 (BL21-RIPL cells with pGEX4T1-Ago2 and pET28a-HSP90) is a gift from Leemor Joshua-Tor (Cold Spring Harbor Laboratory) (Rivas et al., 2005). After cultivation at 37°C to an A_{600} of ~0.8, cells were incubated with 0.5 mM IPTG for 8 hr at 16°C for GST-fusion proteins or for overnight at 18°C for His-tagged proteins. Cells were lysed in 500 mM NaCl, 80 mM Tris-HCl (pH 8.0), 0.5% Triton X-100, 0.2 mM PMSF and 1× complete proteinase inhibitor cocktail (Roche). The cell lysate was incubated with glutathione sepharose 4B (Amersham Biosciences) for GST-fusion proteins or with Ni-NTA superflow (QIAGEN) for His-tagged proteins. at 4°C. After washing with 500 mM NaCl, 80 mM Tris-HCl (pH 8.0) and 0.1% Triton X-100, the GST fusion proteins or GST were eluted in 160 mM reduced glutathione, 500 mM NaCl and 80 mM Tris-HCl (pH 8.0), and subsequently the buffer was replaced with PBS by dialysis. His-tagged proteins were eluted in 50 mM Tris pH 8.0, 5 mM MgCl2, 100 mM KCl, 5% glycerol, 250 mM imidazole, and subsequently the buffer was replaced with the elution buffer without imidazole by dialysis. Radio labeled full-length (1060 aa) and parts (shown in Figure 3E) of Giw1p recombinant proteins were synthesized using PCR-amplified *GIW1* synthetic DNA, ^{35}S-methionine and PURExpress In Vitro Protein Synthesis Kit (New England Biolab).

Strains and Culture Conditions

Wild-type B2086, CU427 and CU428 strains of *Tetrahymena thermophila* were from Dr. P. J. Bruns (Cornell University). FLAG-HA-*TWI1, EMA1* KO and *DCL1* KO strains were described previously (Mochizuki and Gorovsky, 2004, 2005; Aronica et al., 2008). Other strains are described below. Cells were grown in SPP medium (Gorovsky et al., 1975), containing 2% proteose peptone, at 30°C. For conjugation, log phase cells (~3-5 × 10^5/ml) of two different mating types were washed, starved (12~24 hr) and mixed in 10 mM Tris (pH 7.5) at 30°C.

Slicer-Dead Tetrahymena *TWI1* Mutants

The FLAG-HA-*TWI1*-WT construct was produced by inserting a NsiI-SpeI fragment of genomic DNA, amplified by PCR using the primers TWI1-RHm-FW-Xho and TWI1-RHm-RV-Spe/Bam, into the NsiI-SpeI site of the FLAG-HA-TWI1 construct (Aronica et al., 2008). To make FLAG-HA-*TWI1*-D526N, the D526N mutation was introduced by overlapping PCR (1st PCR "A" with TWI1-RHm-FW-Xho and RHD-D526N-RV as primers and genomic DNA as template; 1st PCR "B" with RHD-D526N-RV and TWI1-RHm-RV-Spe/Bam as primers and genomic DNA as template; 2nd PCR with TWI1-RHm-FW-Xho and TWI1-RHm-RV-Spe/Bam as primers and 1st PCR products A and B as templates). The PCR product was digested with NsiI and SpeI and inserted into the NsiI-SpeI site of the FLAG-HA-TWI1 construct (Aronica et al., 2008). FLAG-HA-*TWI1*-H745Q was produced by introducing the H745Q mutation in the FLAG-HA-*TWI1*-WT construct using the GeneEdior in vitro Mutagenesis System (Promega) and TWI1-H745Q-FW. For the constructs, see Figures S2A and S2B. To produce FLAG-HA-*TWI1*-D526N strains, CU427 and CU428 were transformed with the construct as described (Cassidy-Hanley et al., 1997). FLAG-HA-*TWI1*-WT and FLAG-HA-*TWI1*-H745Q constructs were introduced into *TWI1* KO homozygote homokaryon strains cΔ*TWI1*-5-3 and cΔ*TWI1*-65-3, which were paromomycin sensitive, due to elimination of the *neo3* cassette in their macronuclei. The endogenous Mac *TWI1* loci or *TWI1* KO loci were completely replaced with the FLAG-HA-*TWI1*-WT, FLAG-HA-*TWI1*-D526N or FLAG-HA-*TWI1*-H745Q constructs by stepwise selection in increasing concentrations of paromomycin sulfate (pm, Sigma) in the presence of 1 μg/mL $CdCl_2$. Complete replacement was confirmed by Southern hybridization (Figures S1C, S1D, and S1F).

Antibodies

The monoclonal anti-alpha-tubulin antibody 12G10 was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. To generate the anti-Giw1p antibody, a rabbit was immunized with a peptide (SDNKSNSDQVFETEVC) corresponding to amino acids 589-603 (underlined) of Giw1p. To make anti-Wag1p antibody, a rabbit was immunized with a peptide (CTKRPSKDPRLQEKKQT) corresponding to amino acids 790-805 (underlined) of Wag1p (DDBJ/EMBL/GenBank ACJ39431).

Immunofluorescence Staining

Cells were fixed and processed as described (Loidl and Scherthan, 2004) with 1:250-500 dilutions of primary antibodies [anti-HA (16B12, Covance), anti-FLAG; anti-Twi1p, anti-Ema1p (Aronica et al., 2008), anti-Pdd1p (Abcam); anti-Wag1p (see below), followed by incubation in 1:500-5,000 diluted Alexa488 conjugated anti-rabbit or anti-mouse IgG (Invitrogen). The samples were stained with 10 ng/ml DAPI and observed by fluorescence microscopy.

Construction of *GIW1*-HA Strains

To make the *GIW1*-HA construct (Figure S1H), an HA coding sequence was inserted just before the stop codon of *GIW1* by overlapping PCR. The C-terminal segment of *GIW1*, followed by NheI-SalI-BglII sequences, was amplified with p115Tag-FW-Xho and p115Tag-C-term-RV (Product-A). NheI-SalI-BglII sequences, followed by the stop codon, the 3′-UTR and part of the 3′-flanking sequences, were amplified by overlapping PCR first using p115Tag-C-term-FW + p115-Tag-3′mid-RV (Product B) or p115Tag-3′mid-FW + p115Tag-RV-Bam (Product-C), then using Product B, Product C and p115Tag-C-term-FW + p115Tag-RV-Bam (Product-D). Product B and Product C had an overlapping sequence that produced an EcoRV site. Next, Products A and D were joined by overlapping PCR with p115Tag-FW-Xho and p115Tag-RV-Bam. This product was cloned into the pBlueScript SK(+) vector using the XhoI and BamHI sites. Next, two DNA oligos, HAsense and HAanti, were annealed to produce HA-coding DNA, which was inserted into the NheI and BglII sites of the plasmid. Finally, the *neo3* cassette was introduced into the EcoRV sites in the 3′-flanking sequence. The construct was excised out of the vector backbone using BamHI and XhoI. CU427 and CU428 were transformed with the construct, and the endogenous Mac *GIW1* loci were completely replaced by phenotypic assortment and selection in increasing concentrations of pm as described above. Complete replacement was confirmed by Southern hybridization (Figure S1I).

Construction of *GIW1* KO Strains

To make the disruption construct (Figure S1J), a genomic region including the *GIW1* gene was amplified by PCR with primers p115KO-FW-Xho and p115KO-RV-Bam, inserted into the BamHI and XhoI sites of the pBlueScript SK(+) vector and the internal XbaI fragment was replaced by the *neo3* cassette. The construct was excised from the vector backbone using BamHI and XhoI and introduced into the Macs of B2086 and CU428 cells by biolistic transformation. The endogenous wild-type Mac *GIW1* loci were completely replaced with the disrupted loci by phenotypic assortment as described above. Complete replacement was confirmed by Southern hybridization (Figure S1K).

DNA-Elimination and Progeny-Viability Assays

DNA-elimination and progeny-viability assays were performed as described (Aronica et al., 2008). The DNA-elimination assay by FISH was performed as described (Loidl and Scherthan, 2004). FISH probes were produced by nick translation using pMBR 4C1, pMBR 2 and Tlr IntB plasmid DNA (Wuitschick et al., 2002) for Tlr1-elements, or using a part (~5.6 kb) of REP2-2 genomic DNA (Fillingham et al., 2004) PCR amplified with primers REP2FW and REP2RV (see the Extended Experimental Procedures for sequences) for REP-elements.

SUPPLEMENTAL REFERENCES

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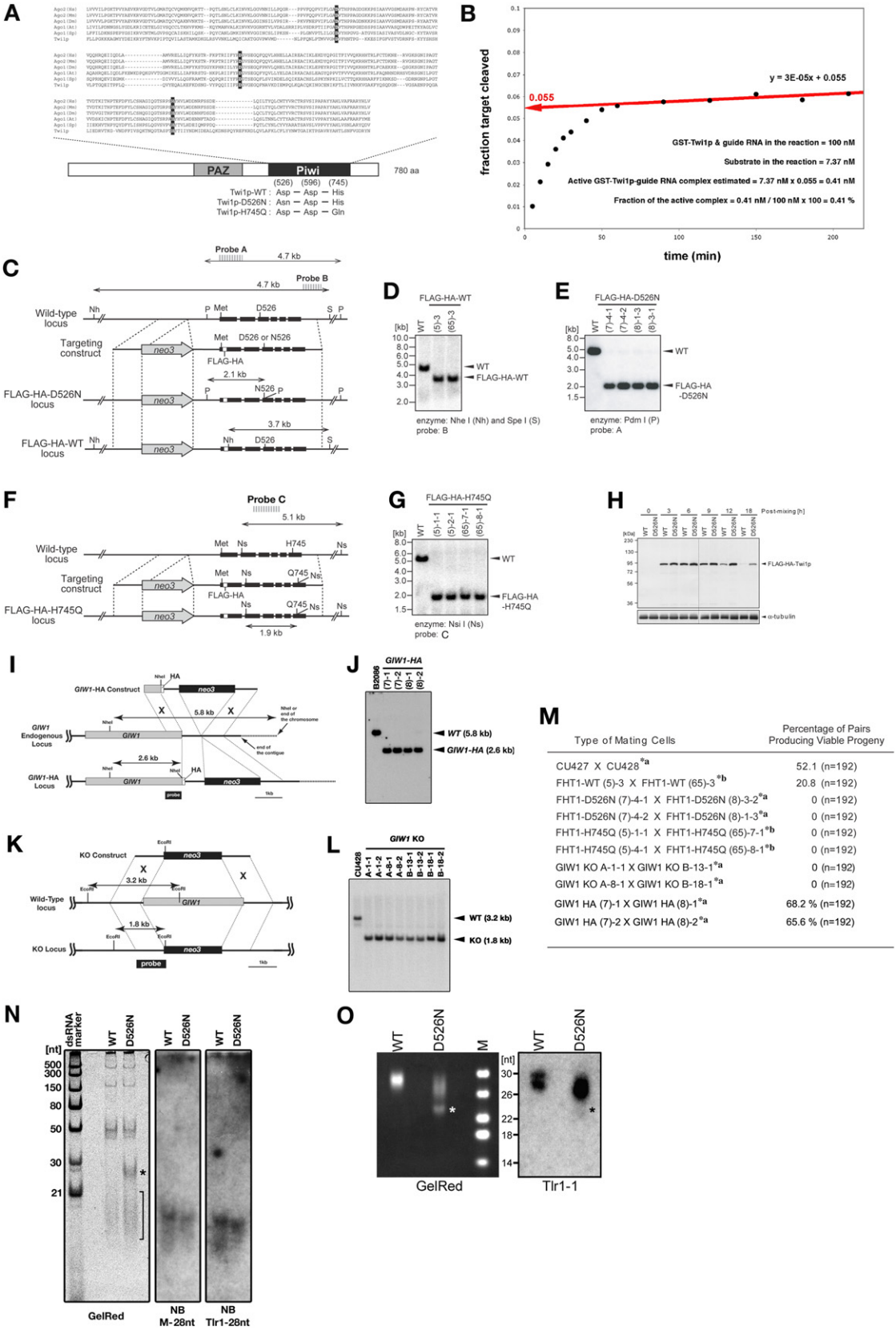

M

Type of Mating Cells	Percentage of Pairs Producing Viable Progeny
CU427 X CU428 *a	52.1 (n=192)
FHT1-WT (5)-3 X FHT1-WT (65)-3 *b	20.8 (n=192)
FHT1-D526N (7)-4-1 X FHT1-D526N (8)-3-2 *a	0 (n=192)
FHT1-D526N (7)-4-2 X FHT1-D526N (8)-1-3 *a	0 (n=192)
FHT1-H745Q (5)-1-1 X FHT1-H745Q (65)-7-1 *b	0 (n=192)
FHT1-H745Q (5)-4-1 X FHT1-H745Q (65)-8-1 *b	0 (n=192)
GIW1 KO A-1-1 X GIW1 KO B-13-1 *a	0 (n=192)
GIW1 KO A-8-1 X GIW1 KO B-18-1 *a	0 (n=192)
GIW1 HA (7)-1 X GIW1 HA (8)-1 *a	68.2 % (n=192)
GIW1 HA (7)-2 X GIW1 HA (8)-2 *a	65.6 % (n=192)

Figure S1. Construction and Analyses of Strains, Related to Figure 1

(A) Slicer-dead *TWI1* mutant strains. Comparison of the Piwi domains of Slicer Active Argonaute Proteins and Twi1p. The Piwi domains of Argonaute proteins known to have Slicer activity were compared with the Piwi domain of Twi1p. Twi1p has the conserved DDH motif (box shaded), which is the catalytic core of Slicer activity. The Slicer-dead mutations (D526N and H745Q) used in this study are shown at the bottom. At: *Arabidopsis thaliana*, Dm: *Drosophila melanogaster*, Hs: *Homo sapiens*, Mm: *Mus musculus*, Sp: *Schizosaccharomyces pombe*.

(B) Cleavage kinetics by recombinant Twi1p. Cleavage by GST-Twi1p was biphasic, suggesting that product release is the rate-determining step. The active GST-Twi1p-guide-strand scnRNA complex estimated by burst analysis (7.37 nM X 0.0545, red arrow) account for ~0.4% of total GST-Twi1p proteins included in the reaction.

(C) Diagrams of the wild-type *TWI1,* FLAG-HA-*TWI1*-WT and FLAG-HA-*TWI1*-D526N loci.

(D and E) Southern blot demonstrating the complete replacement of endogenous *TWI1* genes by FLAG-HA-*TWI1*-WT (D) and FLAG-HA-*TWI1*-D526N (E) constructs. Total DNA isolated from the indicated strain was digested with NheI and SpeI (D) or PdmI (E) and hybridized with the probe shown in (C). Positions of the DNA fragments from wild-type (WT), FLAG-HA-*TWI1*-WT (FLAG-HA-WT) and FLAG-HA-*TWI1*-D526N (FLAG-HA-D526N) are marked with arrowheads.

(F) Diagrams of the wild-type *TWI1,* and FLAG-HA-*TWI1*-H745Q loci.

(G) Southern blots demonstrating the complete replacement of endogenous *TWI1* genes by FLAG-HA-*TWI1*-H745Q constructs. Total DNA isolated from the indicated strain was digested with NsiI and hybridized with the probe shown in (F). Positions of the DNA fragments from wild-type (WT) and FLAG-HA-*TWI1*-H745Q (FLAG-HA-H745Q) are marked with arrowheads.

(H) Expression of FLAG-HA-Twi1p-WT (WT) and FLAG-HA-Twi1p-D526N (D526N) during conjugation was analyzed by Western blot using an anti-HA antibody (top). Expression of α-tubulin was analyzed as a loading control (bottom).

(I) Diagrams of the *GIW1*-HA target construct, wild-type *GIW1* and *GIW1*-HA loci.

(J) Southern blot demonstrating the complete replacement of endogenous *GIW1* genes by the *GIW1*-HA construct. The dotted lines in the diagram indicate the region not found in the *Tetrahymena* genome database. Total DNA isolated from wild-type (B2086) and different *GIW1*-HA strains was digested with NheI and hybridized with the probe shown in the diagram. Positions of the DNA fragments from the wild-type (WT) and *GIW1*-HA (HA) loci are marked with arrowheads.

(K) Diagrams of the *GIW1* KO target construct, wild-type *GIW1* and *GIW1* KO loci.

(L) Southern blot demonstrating the complete replacement of endogenous *GIW1* genes by the KO construct (bottom). Total DNA isolated from wild-type (CU428) and different *GIW1* KO strains was digested with EcoRI and hybridized with the probe shown in the diagram. The positions of the DNA fragments from the wild-type (WT) and *GIW1* KO (KO) loci are marked with arrowheads.

(M) Progeny-viability tests. At 6~8 hr post-mixing, single mating pairs of the indicated strains were placed into drops of SPP medium and incubated for ~60 hr at 30°C. Completion of conjugation was confirmed (*a) by testing for expression of the 6-methypurine resistance gene, which is specific for the newly developed macronucleus, or (*b) PCR analysis for loss of the FLAG-HA-TWI1 locus, which is specific for the parental macronucleus.

(N) Single-stranded scnRNAs migrate as smear in a native gel. scnRNAs coimmunoprecipitated with FLAG-HA-TWI1-WT (WT) and FLAG-HA-TWI1-D526N (D526N) at 4 hr post-mixing were analyzed by native gel electrophoresis and were visualized with GelRed (a nucleic acid-specific fluorescent dye) (left). Double-stranded RNA markers were included (leftmost lane). Double- and single-stranded scnRNAs are marked with an asterisk and a bracket, respectively. These separated RNAs were then analyzed by Northern blots (NB) probed with a 28 nt oligo DNA complementary to either M-IES (M-28nt, middle) or Tlr1-IES (Tlr1-28nt, right). Single-stranded scnRNAs complementary to each oligo moved slightly differently. Double-stranded scnRNAs were not visible on the Northern blots probably because the probes did not hybridize well to non-denatured double-stranded RNAs.

(O) ~24 nt RNAs bind to Slicer-dead Twi1p do not hybridize with a Tlr1 IES sequence. RNAs coimmunoprecipitated with FLAG-HA-Twi1p-WT (WT) or FLAG-HA-Twi1p-D526N (D526N) at 6 hr post-mixing were separated in a denaturing gel, visualized using GelRed (left), transferred to a nylon membrane, and hybridized with Tlr1-1 probe (Aronica et al., 2008) which was complementary to Tlr1 IES sequence and thus could detect a subpopulation of scnRNAs (right). The positions of ~24 nt RNA are marked by asterisks.

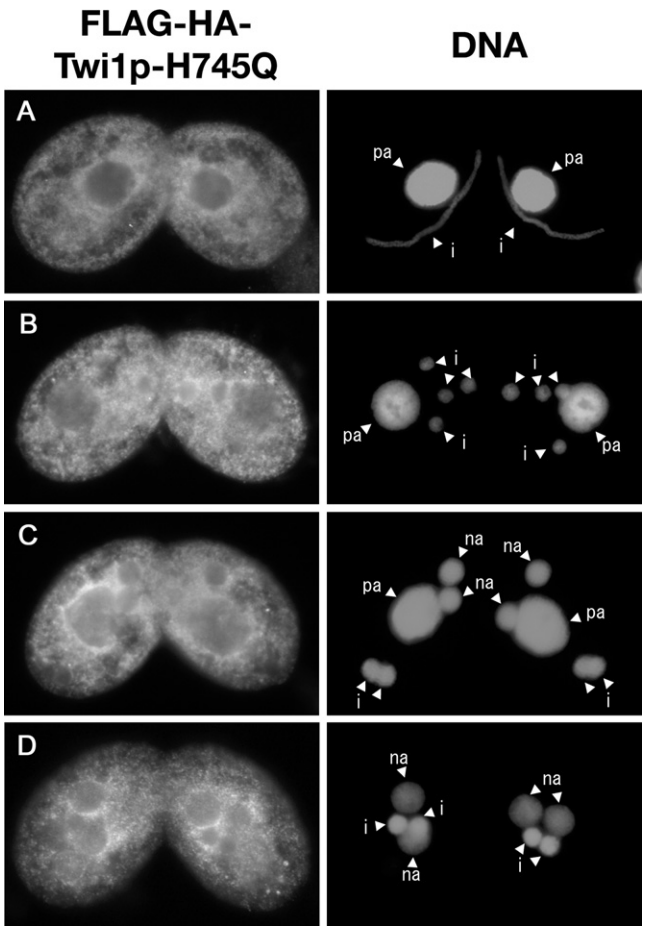

Figure S2. FLAG-HA-Twi1p-H745Q Is Mislocalized in the Cytoplasm, Related to Figure 2

Localization of FLAG-HA-Twi1p-H745Q at meiotic prophase (A), second meiosis (B), macronuclear anlagen (C) and nuclear alignment (D) stages was analyzed by immunofluorescence staining using an anti-HA antibody (left). DNA was counterstained with DAPI (right). The micronuclei (i), the parental macronuclei (pa) and the newly developed macronuclei (na) are marked with arrowheads.

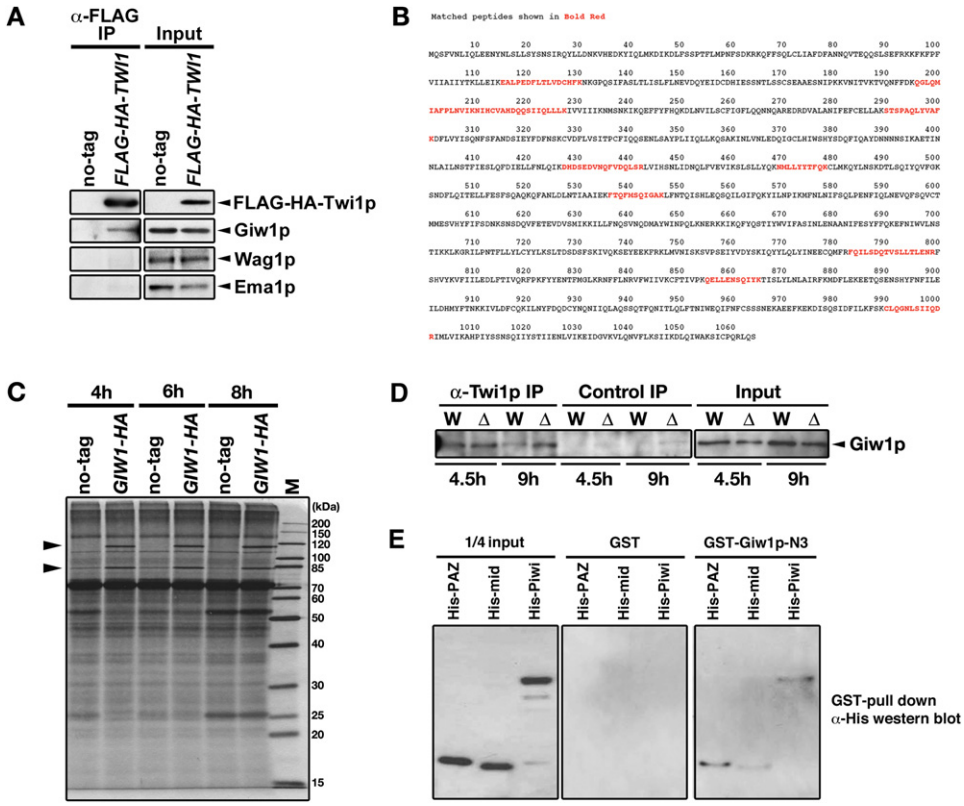

Figure S3. Identification and Analyses of the Twi1p-Binding Protein p115 (Giw1p), Related to Figure 3

(A) Small amounts of Ema1p and Wag1p were coimmunoprecipitated with Twi1p. Two FLAG-HA-TWI1-WT strains were mated and the FLAG-HA-Twi1p containing protein complex was immunoprecipitated with an anti-FLAG antibody at 5 hr post mixing. As a negative control, two wild-type (no-tag) strains were analyzed similarly. The coimmunoprecipitated proteins were separated by SDS-PAGE and analyzed by Western blotting using an anti-HA antibody (to detect FLAG-HA-Twi1p), an anti-Ema1p antibody, an anti-Giw1p antibody or an anti-Wag1p antibody.

(B) Identification of Giw1p. Predicted amino acid sequence encoded by cDNA (DDBJ/EMBL/GenBank XM_001029843) encoding Giw1p is shown. Peptides identified by tandem mass spectrometry sequencing of p115 are highlighted in red.

(C) Analysis of proteins coimmunoprecipitated with Giw1p-HA. Two GIW1-HA strains were mated and Giw1p-HA containing protein complex was immunoprecipitated with an anti-HA antibody at 2, 4 and 6 hr post mixing. As a negative control, two wild-type (no-tag) strains were processed in parallel. The proteins were separated by SDS-PAGE and analyzed by silver-staining. Protein molecular markers (M) were included. The positions of two bands specifically detected in GIW1-HA strains, which correspond to the sizes of Twi1p (90 kDa) and Giw1p-HA (124 kDa), are marked with arrowheads.

(D) EMA1 is not required for Giw1p-Twi1p interaction. Twi1p was immunoprecipitated using an anti-Twi1p antibody from wild-type (W) or EMA1 KO (Δ) strains at 4.5 hr and 9 hr post mixing and coimmunoprecipitated Giw1p was analyzed by Western blot using an anti-Giw1p antibody. As a negative control, a normal rabbit serum was used for immunoprecipitation (Control IP). 1/12.5 of cell lysates used for the immunoprecipitations were also analyzed by Western blot using the anti-Giw1p antibody (Input).

(E) N3 fragment of Giw1p interact with PAZ and Piwi domain of Twi1p. GST-tagged Giw1p-N3 segment (see Figure 3E), GST alone, and His-tagged PAZ, mid and Piwi domain of Twi1p (see Figure 3D) were expressed in and purified from *E. coli*. GST pull-down assays were performed by incubating GST or GST-Giw1p-N3 with His-tagged PAZ, mid or Piwi domain of Twi1p. The precipitated proteins were separated by SDS-PAGE and His-tagged PAZ, mid and Piwi domain of Twi1p were detected by Western blotting using an anti-His-tag antibody. 1/4 of His-tagged proteins used were also analyzed similarly (input).

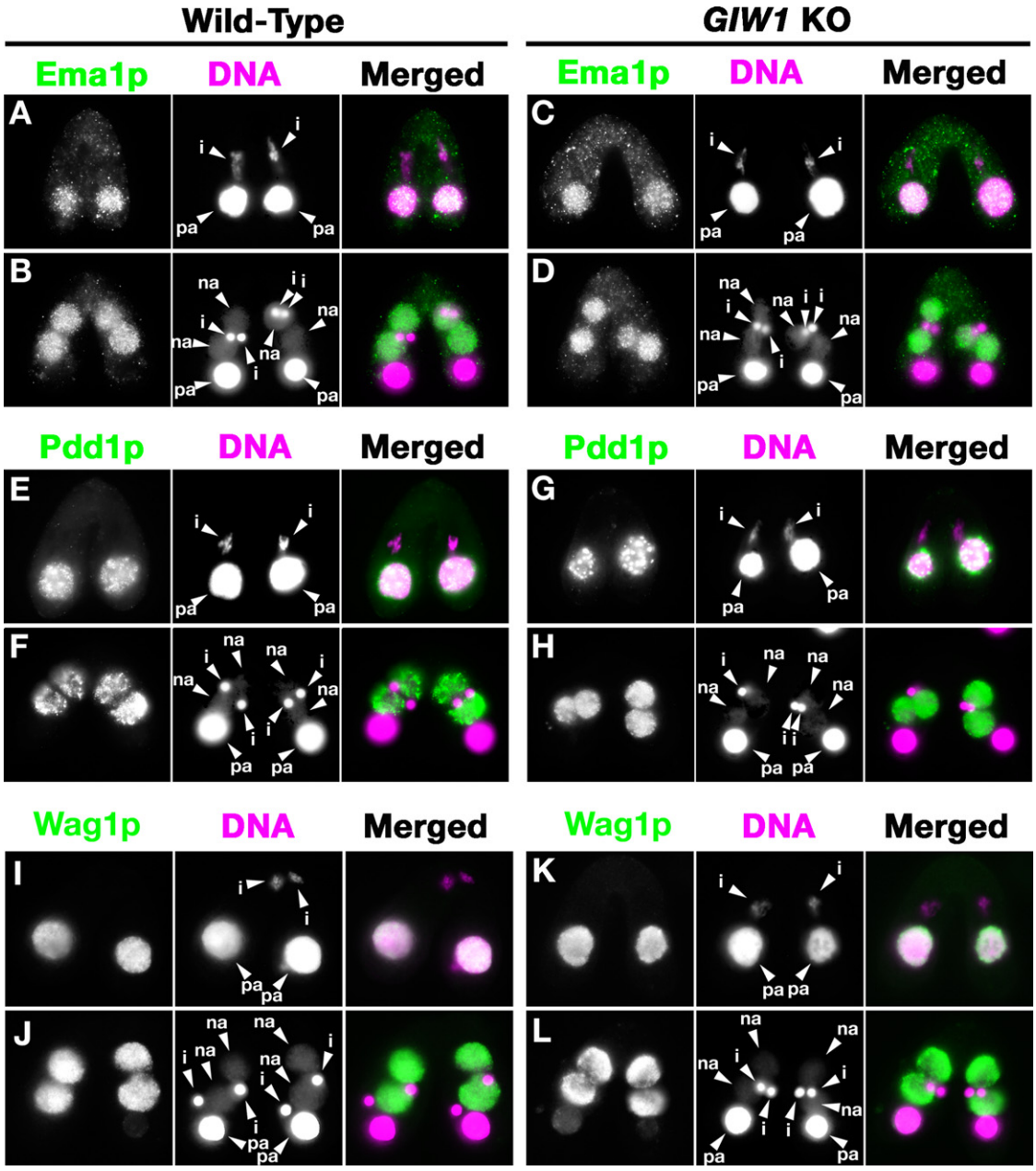

Figure S4. Localization of Ema1p, Pdd1p and Wag1p in *GIW1* KO Cells, Related to Figure 5

Localization of Ema1p (A-D), Pdd1p (E-H) and Wag1p (I-L) in wild-type (left) and *GIW1* KO (right) cells at meiotic prophase (an early conjugation stage; A, C, E. G, I, K) and nuclear alignment stage (a late conjugation stage; B, D, F, H, J, L) were analyzed by immunofluorescence staining using anti-Ema1p, anti-Pdd1p or anti-Wag1 antibodies, respectively (green). DNA was counterstained with DAPI (purple). The micronuclei (i), the parental macronuclei (pa) and the newly developed macronuclei (na) are marked with arrowheads.

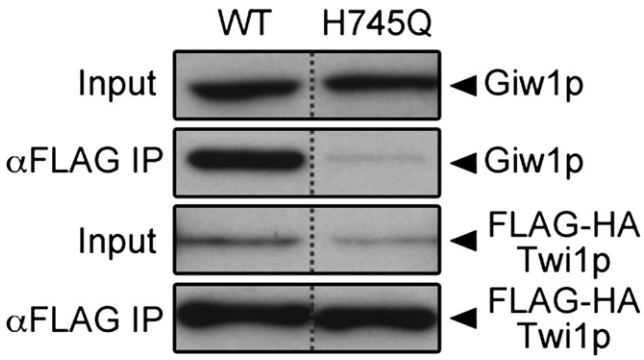

Figure S5. Slicer-Dead Twi1p-H745Q Does Not Interact with Giw1p In Vivo, Related to Figure 6

Cell lysate (input) was prepared from FLAG-HA-TWI1-WT (WT) or FLAG-HA-TWI1-H745Q (H745Q) strains at 4 hr post-mixing and FLAG-HA-Twi1p containing complexes were immunoprecipitated using an anti-FLAG antibody (α-FLAG IP). Giw1p and FLAG-HA-Twi1p were detected by Western blot using anti-Giw1p and anti-FLAG antibodies, respectively.

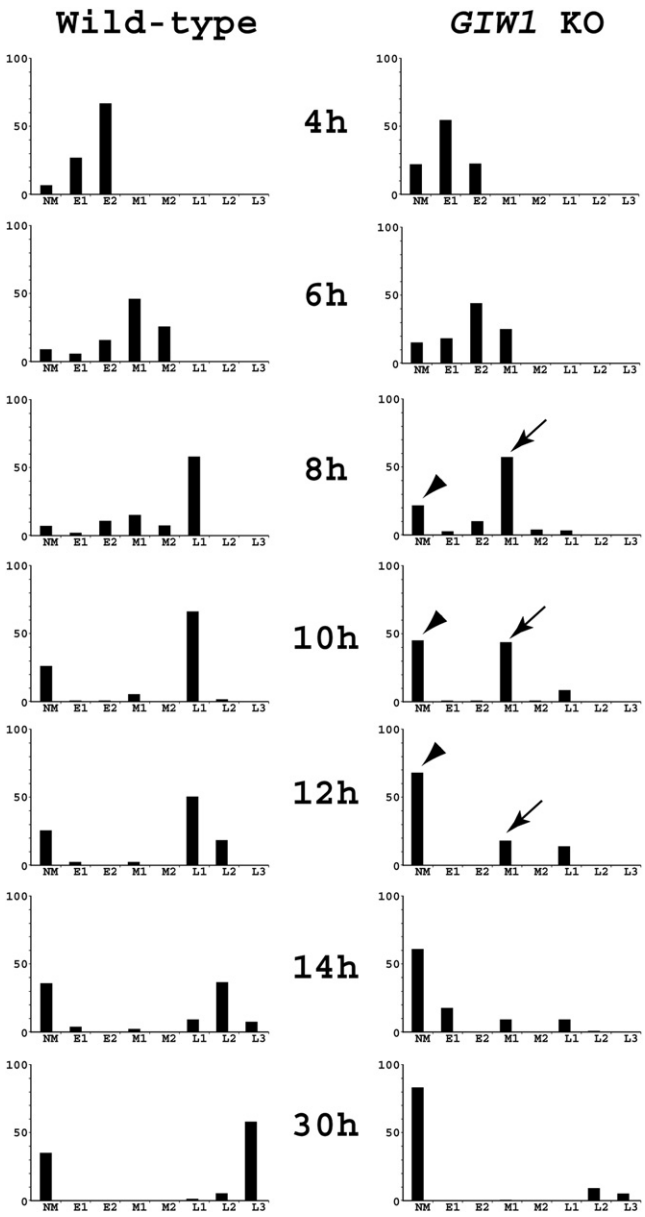

Figure S6. Developmental Profile of *GIW1* KO Cells, Related to Figure 7

Conjugation stages of the wild-type and *GIW1* KO strains were observed by DAPI staining. The stages we categorized were: NM, single unmated cells; E1, Pre-Meiosis; E2, Meiosis; M1, Prezygotic; M2, Postzygotic; L1, Mac Development; L2, Pair Separation (2Mics); L3, Mic Elimination. See Figure S1 of Aronica et al. (2008) for the developmental stages. The majority of the *GIW1* KO cells were arrested at the M1 stage (arrows) and aborted the conjugation process (returned to single cells; NM, arrowheads).

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Point of View

Non-coding RNA: A bridge between small RNA and DNA

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Abbreviations: IES, internal eliminated sequences; scnRNA, scan RNA

Key words: non-coding RNA, small RNA, DNA elimination, Tetrahymena, Argonaute

Small RNAs are not only involved in post-transcriptional gene silencing in the cytoplasm, but also modulate activities of the nuclear genome. Although silencing of mRNAs involves RNA-RNA base-pairing, it remains unclear whether the RNAi machinery responsible for chromatin or DNA modification interacts with DNA or RNA. The identification of an RNA helicase required for chromatin-Argonaute protein interaction as well as the presence of Argonaute-associated long non-coding transcripts in Tetrahymena suggest the presence of RNA-RNA interactions prior to small RNA-induced heterochromatin formation in ciliates.

RNA interference (RNAi) was first described as a post-transcriptional gene silencing mechanism where double-stranded RNA triggers the destruction of complementary mRNAs.[1] In this pathway, Argonaute proteins and small non-coding RNAs form effector complexes that target homologous sequences via base-pairing interactions. It is now recognized that these Argonaute-small RNA complexes are not only involved in a variety of post-transcriptional silencing pathways, but are also able to induce transcriptional gene silencing at the chromatin level.[2] This includes heterochromatin formation in a variety of eukaryotes,[3] as well as RNA-directed DNA methylation in plants.[4] Although the complexes responsible for this transcriptional gene silencing have been well studied, the mechanism how the RNAi machinery interacts sequence-specifically with the genome to establish chromatin or DNA modifications remains unclear. Small RNAs might interact directly with DNA, or alternatively might interact indirectly with nascent transcripts cis-anchored at the locus to be silenced.[5, 6] Studies of heterochromatin formation in *S. pombe* support the model of small RNA-RNA interactions,[7] whereas RNA-targeted DNA methylation in plants seems to involve direct RNA-DNA interactions.[8] We and others have studied the recognition of genomic sequences by small RNAs in ciliates,[9, 10] which show small RNA-induced heterochromatin formation followed by DNA elimination.

In Tetrahymena *thermophila*, small RNA-mediated heterochromatin formation is an essential step in sexual reproduction.[11] Tetrahymena, like most ciliated protozoa, contains two distinct nuclei in each cell: the somatic nucleus (macronucleus) contributes to gene expression, and the germline nucleus (micronucleus) performs reproductive functions. Although both nuclei are descendants of the same zygotic nucleus (Fig 1A), they differ in their DNA: the micronucleus contains the entire DNA sequence, whereas the macronucleus contains a truncated version. Since micronuclei generate new micronuclei and macronuclei during sexual conjugation, targeted DNA elimination is necessary to shape the genome of the new macronuclei (Fig 1B). The approximately 6000 removed sequences (internal eliminated sequences, IES) mainly consist of repeated sequences that contain transposon-like elements and other repeats often categorized as junk DNA.[11] IES are precisely eliminated and believed to be labeled for removal by RNAi-directed heterochromatin marks.[12, 13] This targeted DNA elimination involves the concerted action of three nuclei (micronucleus, parental macronucleus, and new macronucleus) and includes the comparison of their genomes using small RNAs.

Soon after the beginning of sexual conjugation, long double-stranded RNAs are produced by bidirectional transcription of the micronuclear genome (Fig. 2, step 1)[14] and serve as substrates for the Dicer-like protein Dcl1p; they are processed into ~28-29 nt small RNAs (Fig 2, step 2).[15, 16] These so-called scnRNAs move to the cytoplasm, where they complex with the Argonaute protein Twi1p (Fig 2, step 3). Since scnRNAs are derived from promiscuous transcription of the micronuclear genome, they are not specific for the IES sequences they are supposed to target for elimination. Therefore, scnRNAs need to be selected for IES specificity.

We have proposed the scan RNA model,[17] in which scnRNAs are compared to the parental macronuclear genome and are enriched for sequences homologous to IES (scanning process) (Fig 2, step 4). Indeed, it has been observed that Twi1p translocates to the parental macronucleus and that the portion of scnRNAs homologous to IES sequences gradually increases during conjugation. The scanning mechanism remains unknown. It is clear, however, that scnRNAs must base-pair either with DNA or RNA in the parental

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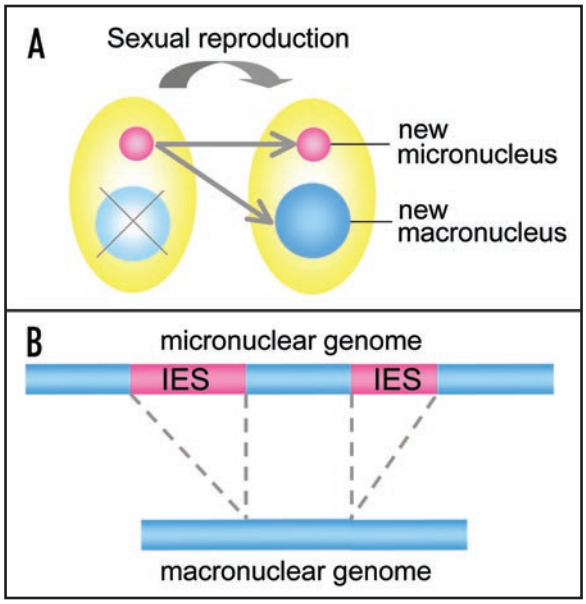

Figure 1. Development of macro- and micro-nuclei during sexual reproduction in Tetrahymena. (A) Ciliates have two distinct nuclei in each cell. During sexual reproduction, a zygotic nucleus derived from parental micronuclei gives rise to a new micronucleus and a new macronucleus; the parental macronucleus is then destroyed. (B) DNA elimination. Internal eliminated sequences (IES, pink) are precisely removed during the development of the new macronucleus. Macronucleus-destined sequences (blue) are re-ligated.

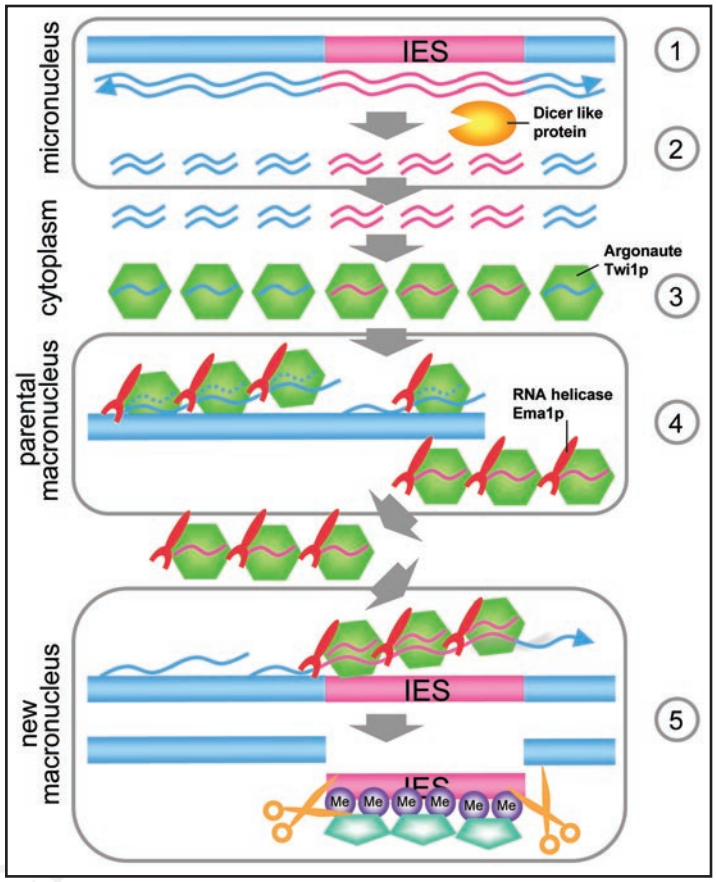

Figure 2. The scan RNA model. Consecutively occurring events are shown from top to bottom. Step 1: production of double stranded RNA (thin lines) by bidirectional transcription of genomic DNA (thick line); step 2: production of scnRNAs by dicer-like protein Dcl1p (yellow); step 3: association of scnRNAs with Twi1p (green) in the cytoplasm; step 4: scanning in the parental macronucleus. The RNA helicase Ema1p (red) is required for the association of Twi1p with non-coding RNA; step 5: heterochromatin formation and IES elimination in the developing new macronucleus (histone methylation: purple; chromodomain proteins: dark green; hypothetical excisase: orange).

macronucleus in order to be sorted. The selected scnRNAs specific for IES sequences then move into the newly developed macronucleus, where they induce heterochromatin formation on the IES prior to their elimination (Fig 2 step 5). Also in the developing new macronucleus, scnRNA-Twi1p complexes must interact with nucleic acids in order to target the correct sequences for elimination.

Our recent study in Tetrahymena[9] suggested that nascent non-coding transcripts mediate the interaction between chromatin and Twi1p-scnRNA complexes in the parental as well as in the developing macronuclei. First, we identified the putative RNA helicase Ema1p as a binding partner of Twi1p. *EMA1* knock-out strains have defects in DNA elimination, and comparisons of chromatin spreads from wild-type and *EMA1* knock-out strains demonstrate that this helicase is necessary for the interaction between Twi1p and chromatin. Since the DExH box RNA helicase Ema1p has potential homologs in diverse organisms, ranging from Paramecium to humans, its role might be conserved. RNA helicases modulate the structure of RNA, and the identification of a putative RNA helicase as an essential factor for the chromatin-Argonaute interaction provided the first hint that scnRNAs might base-pair with RNA transcripts instead of DNA. Indeed, analysis of RNA expression revealed that long non-coding RNAs are not only transcribed from the micronucleus early in conjugation, when they are necessary for the production of scnRNAs, but also from both the parental and newly developing macronuclei at later stages of conjugation. We also demonstrated that these non-coding RNAs from parental and new macronuclei co-immunoprecipitated with Twi1p, and that this interaction was *EMA1*-dependent. This result suggests that Twi1p-scnRNA complexes interact with parental macronuclear transcripts in the middle stages of conjugation (when the scanning is supposed to take place) as well as with the new macronuclear transcripts later in conjugation (when the IES sequences are heterochromatized).

More direct evidence supporting the necessity of the parental macronuclear transcripts for proper IES elimination is provided by studies of another ciliate, *Paramecium tetraurelia*. Lepére et al.[10] used a dsRNA feeding technique to degrade non-coding transcripts in the parental macronucleus. It was found that specific down-regulation blocked the scanning process in the targeted regions and induced ectopic DNA elimination. It was thus proposed that non-coding transcripts from the parental macronucleus serve as molecular sponges that sequester scnRNAs homologous to sequences in the parental macronucleus. It was also suggested that scnRNAs that could not find homologous transcripts would then move to the developing macronucleus to induce DNA elimination. A similar sequestering mechanism has been described in plants, where the activity of miRNAs is regulated by the expression of RNAs that mimic their targets and thereby reduce the amount of available miRNA.[18] The sequestration scanning model does not account for

the observed loss of Tetrahymena scnRNAs during conjugation,[9, 19] although these two concepts are not mutually exclusive.

While the association of scnRNAs with transcripts might explain the mechanism of the scanning process, it is more difficult to envision how small RNAs could specifically target IES removal by associating with non-coding RNA. The estimated 6000 internal eliminated sequences, ranging in size from hundreds to thousands of base pairs, do not seem to share a common sequence motif necessary for their removal. Nevertheless, DNA elimination occurs precisely and reproducibly, and only minor variations have been observed (less than 10 bp for the elimination boundaries).[20] While it is recognized that histone H3 methylation is required to tag sequences for DNA elimination,[12, 13] it is unclear how the precision of IES removal is achieved. It is unlikely that mere changes in histone modifications or placement are sufficient since these occur in the context of nucleosomes, which contain ~150 bp DNA. Future research might identify additional proteins necessary for IES excision and thus might provide a mechanism for this DNA editing.

Another area of interest for future research is the production of macronuclear non-coding RNAs. These non-polyadenylated bidirectional transcripts vary in size and have been shown to have heterogenous 5'- and 3'-termini.[14] However, it is not known which RNA polymerase is responsible for transcription or how this seemingly promiscuous transcription is initiated. Efforts to identify the RNA polymerase and/or transcription factors required for production of macronuclear non-coding RNAs are ongoing.

Recent studies of non-coding RNAs have revealed that their complexity is far greater than expected,[21] and it is not surprising that their functions remain mostly unknown. The number of such transcripts that are actually intentionally transcribed and the number of those that represent transcriptional by-products with no cellular effects remain unclear. The genome-wide production of non-coding RNAs in Tetrahymena is developmentally regulated and controls three different steps of DNA elimination. In the micronucleus, non-coding RNAs are used for production of small RNAs; in the parental macronucleus, they are involved in scanning, and in the developing new macronucleus, they induce heterochromatin formation followed by DNA elimination. Tetrahymena is an ideal model to study these non-coding RNAs both biochemically and genetically. Sexual reproduction, which leads to DNA elimination, can easily be synchronously induced in several billions of Tetrahymena cells, and genetic manipulation and gene targeting methods have been established for this organism. Future research on this organism should thus provide further insights into the biogenesis and functions of non-coding RNAs in eukaryotes.

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