Slicing and Spreading of Heterochromatic Silencing by RNA Interference

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RNA interference (RNAi) can mediate gene silencing posttranscriptionally by target RNA cleavage, or transcriptionally by chromatin and DNA modification. Argonaute is an essential component of the RNAi machinery that displays endonucleolytic activity guided by bound small RNAs. This slicing activity has recently been shown to be required for gene silencing and spreading of histone modifications characteristic of heterochromatin in *Schizosaccharomyces pombe*. Argonaute proteins with catalytic and nucleic acid binding capacities are found to function in RNAi within both the plant and animal kingdoms. Here we review the requirement of slicing for silencing and spreading in *S. pombe*, plants, and humans.

INTRODUCTION

Heterochromatic Silencing and Position-effect Variegation

Chromosomal material is classically characterized as either euchromatic or heterochromatic, depending on condensation during interphase. Euchromatin has been shown to include regions of active transcription and is characterized by various histone modifications such as histone H3 and H4 acetylation, and histone H3 dimethylation on lysine 4 (K4me2). Heterochromatin, on the other hand, is condensed during interphase and is populated by deacetylated histones enriched in histone H3 dimethylated on K9 and K27 (Richards and Elgin 2002). It was previously thought to be transcriptionally inert.

The chromatin context of a gene can determine its expression, as illustrated by position-effect variegation (PEV) in *Drosophila melanogaster*. (Schotta et al. 2003; Pal-Bhadra et al. 2004; Talbert and Henikoff 2006). PEV is also observed in *S. pombe*, in which a reporter gene that is active in a euchromatic region becomes silenced when located within a region of heterochromatin (Allshire et al. 1994). This implies that the factors which confer transcriptional silence in heterochromatin can spread into transgenes located within its borders that would otherwise be active. In *S. pombe*, there are three main regions of heterochromatin: centromeres, telomeres, and the mating-type locus. Heterochromatin in all eukaryotes is associated with repetitive and transposable elements (TEs) which are often capable of silencing genes when juxtaposed with them (Lippman et al. 2004). Unexpectedly, these elements are a predominant source of small interfering RNA (siRNA) in both plants and fission yeast (Lippman et al. 2004), and RNA interference is required for silencing and heterochromatic modifications associated with PEV in yeast (Volpe et al. 2002), *Arabidopsis* (Zilberman et al. 2003), and *Drosophila* (Pal-Bhadra et al. 2004). Thus, RNAi mediates both transcriptional (TGS) and posttranscriptional (PTGS) gene silencing (Cerutti and Casas-Mollano 2006).

Argonaute, The Catalytic Engine of RNAi

Phylogenetic analysis indicates that the main components of RNAi, namely Dicer, Argonaute, and an RNA-directed RNA polymerase, were present in the last common ancestor of eukaryotes (Cerutti and Casas-Mollano 2006). Argonaute proteins are present in all species that exhibit RNAi and fall into two classes, Argonaute-like and Piwi-like (Cerutti and Casas-Mollano 2006). In Table 1, piwi-like proteins are indicated in bold. Argonaute is a 100-kD basic protein that consists of four domains: the amino-terminal; middle; PIWI, which is responsible for the RNase-H-like catalytic activity; and PAZ, the nucleic-acid-binding domain. The protein forms a positively charged groove between the PIWI domain and the PAZ domain located above (Song et al. 2004). The PAZ domain preferentially binds RNA in a sequence-independent manner by recognizing the 3' ends of single-stranded RNAs (Song et al. 2003). The 5' end of bound siRNA is free to base-pair strongly with complementary target RNA. The slice site is then determined as a distance relative to the 5' end of the siRNA (Hall 2005). The slicing activity of *S. pombe* Ago1 is thus dependent on siRNA for both its catalytic activity and specificity. The PIWI domain contains two aspartates and a histidine that form a "DDH" catalytic motif similar to the "DDE" motif seen in RNase H (Song et al. 2004). Mg²⁺-dependent catalytic activity breaks only one phosphodiester bond, leaving a 3'-OH and a 5' phosphate (Martinez and Tuschl 2004). Argonaute proteins have roles in RNAi, developmental control, stem cell maintenance, and tumorigenesis (Carmell et al. 2002), as well as heterochromatic silencing, but the extent to which catalytic activity is required for each of these is unknown, particularly as Argonaute proteins without the conserved DDH sites exist in many animals (Song et al. 2004). Several recent papers have investigated this possibility in fission yeast, plants, and human cells.

Table 1. Argonaute Proteins of Yeast, Plants, and Humans

<i>S. pombe</i>	<i>A. thaliana</i>	<i>H. sapiens</i>
Ago1*	AGO1*	hAgo1
	AGO2	hAgo2*
	AGO3	hAgo3
	AGO4*	hAgo4
	AGO5	hAgo5
	AGO6	Hiwi
	AGO7/ZIPPY	Hili
	AGO8	
	AGO9	
	PINHEAD/ZWILLE	

(*) Indicates proteins that have been tested and shown to have catalytic activity. Bold type indicates piwi-like proteins.

AGO SLICING ACTIVITY IS REQUIRED FOR SILENCING AND SPREADING IN *S. POMBE*

S. pombe contains only one Argonaute (Table 1) and one Dicer, Dcr1, making it an excellent system in which to study RNAi. Furthermore, heterochromatic regions of the genome are sharply defined and have been sequenced almost to completion (Wood et al. 2002). The *S. pombe* centromeres are composed of a central conserved region, bordered by inner and outer *dg* and *dh* repeats that are heterochromatic in nature (Fig. 1). Reporter genes inserted into these heterochromatic repeats are silenced, while the same genes inserted into a euchromatic region are expressed (Allshire et al. 1994). If Ago1, Dcr1, or the RNA-dependent RNA polymerase (Rdp1) is deleted, silencing of the reporter genes in heterochromatic repeats is no longer maintained (Volpe et al. 2002). Furthermore, RNAi regulates the silencing of centromeric repeats themselves. Levels of H3K4me2 at the centromere increase in *dcr1*, *rdp1*, and *ago1* mutants, while levels of H3K9me2 decrease, although *ago1* maintains higher levels of H3K9me2 than *dcr1* (Volpe et al. 2002). Forward and reverse transcripts from the centromeric repeats accumulate in *dcr1*, *rdp1*, and *ago1* while the mutant of the homolog to the mammalian heterochromatic protein 1 (HP1), *swi6*, only accumulates forward transcripts (Volpe et al. 2002). This suggests that RNAi machinery is responsible for repression of forward strand transcription whereas the reverse strand is always transcribed but is turned over rapidly into siRNA by the RNAi machinery (Volpe et al. 2002).

By making alanine substitutions for each of the conserved aspartates and the histidine required for catalytic

activity, such as *ago1D650A*, the catalytic activity of Ago1, not just its structural contribution or nucleic-acid-binding capacity, has been found to be critical for RNAi-mediated silencing of a reporter gene integrated into the heterochromatic outer repeats of centromere 1 (Fig. 1) (Irvine et al. 2006). Recombinant Ago1 is capable of siRNA-directed endonucleolytic activity in vitro, but each of the mutants is impaired. *ago1D650A* cells do not accumulate detectable siRNA, suggesting that slicing is required for siRNA synthesis (Irvine et al. 2006). Instead, *ago1D650A* cells accumulate both forward and reverse transcripts of heterochromatic repeats (Irvine et al. 2006), which eliminates a possible role for Ago1 in the transcriptional step of pre-siRNA synthesis and rather suggests a role in its processing. It seems counterintuitive, but transcription of the repeats, and processing into siRNA, are necessary for reporter gene silencing. Consistent with this idea, reporter genes inserted upstream of the major heterochromatic repeat promoter were silenced 4 times less effectively than those inserted downstream (H. repeat promoter in Fig. 1), although bidirectional transcription of heterochromatic repeats ensured some level of silencing in both cases (Irvine et al. 2006).

Based on these results, a model for transcriptional silencing in *S. pombe* has been proposed (Fig. 2). Continuous transcription of heterochromatic repeats initiates from a reverse-strand promoter (Volpe et al. 2002). These transcripts are then turned over into siRNA (Reinhart and Bartel 2002). Transcription of the forward strand is silenced by the formation and maintenance of heterochromatin induced by these siRNAs (Volpe et al. 2002). RNase-H-like Ago1 cleaves the reverse-strand transcript, recruiting the RNA-directed RNA polymerase complex (RDRC) for double-stranded RNA (dsRNA) synthesis (Irvine et al. 2006). The RDRC is composed of Rdp1, helicase Hrr1, and oligoadenylate polymerase Cid12 (Motamedi et al. 2004). This complex uses aberrant transcripts, either directly or primed by siRNA, as templates for production of dsRNA (Motamedi et al. 2004). dsRNAs are then processed by Dcr1 into siRNA (Bernstein et al. 2001; Provost et al. 2002). The RNA-induced transcriptional silencing complex (RITS) is composed of Ago1 (bound to siRNA), Tas3, and the chromodomain protein Chp1 (Verdel et al. 2004). RITS associates with chromatin through binding of Chp1 to H3K9me (Partridge et al. 2002). This association, and slicing of transcript by Ago1, somehow recruit the RIK1 complex consisting of Rik1, Dos1/Cir8, Dos2/Cir7, cullin ubiquitin ligase Pcu4, and histone H3K9 methyl-

Figure 1. *S. pombe* centromere 1.

Figure 2. RNAi silencing in *S. pombe*. The symbols used are as follows: (black arrow) reverse promoter, (black lightning bolt) Ago1 endonuclease activity, (black rectangle) reporter transgene, (red hexagon) methyl group on histone H3K9.

transferase Clr4 (Horn et al. 2005; Li et al. 2005; Thon et al. 2005). Clr4 catalyzes the methylation of nearby histone H3K9 (Nakayama et al. 2001). H3K9me2 recruits Swi6, which silences transcription of the forward strand of the repeats as well as the reporter gene (Bannister et al. 2001). Swi6 is required for silencing, and as RNAi continues, H3K9 methylation and Swi6 association spread (Partridge et al. 2000; Martienssen et al. 2005; Irvine et al. 2006).

The targets of Ago1 silencing are determined by the sequence identity between the target (sliced) transcript and that of the bound guide siRNA, rather than with DNA corresponding to the promoter. This is illustrated by the presence of siRNA, and corresponding slice sites, in the repeats but not in the reporter genes embedded within them (Irvine et al. 2006). It is therefore likely that subsequent silencing of the reporter genes is first triggered by slicing and then spread by readthrough transcription by DNA-dependent RNA polymerase II (pol II) into the reporter gene (Fig. 2). Although the mechanism is still unclear, the critical role of pol II in H3K9me2 modification of the reporter genes is revealed by mutants in at least two subunits, Rpb2 and Rpb7 (Sugaya et al. 1998; Djupedal et al. 2005; Kato et al. 2005).

ARGONAUTE4 AND RNA-DIRECTED DNA METHYLATION IN PLANTS

Arabidopsis thaliana has ten Argonaute proteins (Table 1). So far, only AGO1 and AGO4 have been tested for and shown to have catalytic endonuclease activity (Table 1), although all ten have the conserved DDH motif (Baumberger and Baulcombe 2005). AGO1 associates mainly with 21-nucleotide small RNAs whereas AGO4 binds 24-nucleotide small RNAs (Qi et al. 2006), but both are capable of endonuclease activity (Baumberger and Baulcombe 2005; Qi et al. 2005), and both can bind miRNA as well as siRNA (Qi et al. 2006). AGO1 has a major developmental role in vivo in targeting messages via miRNA (Tang et al. 2003; Kidner and Martienssen 2004) but is also required for PTGS and for associated

DNA methylation, which requires the catalytic PIWI domain (Morel et al. 2002). However, most of the small RNAs associated with both AGO1 and AGO4 match to heterochromatic repetitive regions, similar to *S. pombe* (Qi et al. 2006). The siRNA-mediated cleavage of target RNA suggests RNA–RNA recognition as the mechanism of specificity (Qi et al. 2006).

Unlike *S. pombe*, which has no DNA methylation, in plants, RNAi-induced silencing is often associated with DNA methylation. When target transgenes are silenced by homologous regions in viral transgenes, silencing and the associated methylation can spread from the homologous region of the target to other parts of the transgene, both upstream and downstream (Vaistij et al. 2002). This spreading depends on the RNA-dependent RNA polymerase RDR6, but can be subsequently inherited independent of the viral transgene in a fashion that is maintained by the maintenance methyltransferase MET1, a homolog of mammalian DNMT1 (Vaistij et al. 2002). This type of methylation also depends on the Argonaute protein AGO1 (Morel et al. 2002). Thus, although histone modification was not examined in these studies, spreading of methylation resembles the situation in *S. pombe*.

On the other hand, when transgene promoter methylation is induced by homologous hairpin constructs, it is strictly limited to sequences homologous to the hairpin RNA. These sequences can be much shorter than a nucleosome, suggesting a possible RNA–DNA interaction (Matzke and Birchler 2005). In these cases, methylation is partially dependent on AGO4 (Zilberman et al. 2003). Methylation at CpG dinucleotides by MET1 is independent of AGO4, but methylation at non-CpG cytosines by the chromomethyltransferase CMT3 and especially by the DNMT3-related methyltransferases DRM1 and DRM2 depends strongly on AGO4 (Chan et al. 2004).

To investigate the role of slicing in RNA-dependent DNA methylation (RdDM), catalytic mutants of AGO4 have been constructed and used to complement ago4 mutants (Qi et al. 2006). A variety of endogenous methylated sequences were then examined by bisulfite sequencing. The Ac-like transposable element SIMPLE-

HAT has an average of 15.8% non-CpG cytosines methylated in any given molecule. This methylation disappears in *ago4-1* but is almost fully restored (8–11%) when mutants are transformed with both a wild-type and a catalytically inactive form of *AGO4* (Qi et al. 2006). In contrast, higher levels of methylation at the tandem repeat *MEA-ISR* (18.9%), which were also lost in *ago4-1*, could not be restored by catalytically inactive *AGO4* but could be restored by the wild-type form (Qi et al. 2006). Interestingly, levels of siRNA could not be fully restored by mutant *AGO4* either, indicating a requirement for *AGO4* catalytic activity. In the case of *AtMu1*, the results were ambiguous, related perhaps to the presence of a transposed copy in Landsberg *erecta* (Madlung et al. 2005), whose methylation is at least partially dependent on *AGO1* (Lippman et al. 2003).

Non-CpG methylation in epimutants at the *SUPERMAN* (*SUP*) locus is very low with only 21 methylated C out of 374 assayed (less than one per molecule), but has been associated with epigenetic silencing of the locus, which results in a visible phenotype (Chan et al. 2005). This level of methylation is reduced in *ago4-1* to only 17 methylated cytosines out of 765: this difference is low so that restoration by catalytically defective mutants is difficult to assess, although it was noted (Qi et al. 2006). In principle, the *superman* phenotype found in epimutants is a more sensitive assay. *superman* phenotypes are normally observed in “stabilized” strains in which silencing is maintained by a *SUP* transgene and depends on *AGO4*. This stabilization is accompanied by elevated methylation (21% mC) which is partially lost in *ago4-1* (5.5%) (Zilberman et al. 2003). In the absence of the *SUP* transgene, the *superman* phenotype is unstable, but it is even more unstable after propagation in *ago4-1* mutants (only 20–30% phenotypic plants were observed in this background) (Qi et al. 2006). The phenotype was observed at much higher frequencies among the progeny of plants transformed with each of the catalytically inactive transgenes, raising the possibility that catalytically inactive *AGO4* can partially silence *SUP* (Qi et al. 2006).

In cases where *AGO4* catalytic activity is required for DNA methylation (*MEA-ISR*), it is also required for siRNA accumulation, although the correlation with methylation is not absolute. The authors conclude that non-CpG methylation is independent of *AGO4* slicing, although there is clearly a spectrum of methylation levels promoted by catalytic mutants at different loci (Qi et al. 2006). Several explanations can be proposed. First, very low levels of catalytic activity found in the mutants may be sufficient for RdDM in some cases. Second, catalytically inactive *AGO4* might stimulate or stabilize the activity of other Argonaute proteins that retain full activity (such as *AGO1*). Third, non-catalytic *AGO4* may be sufficient for RdDM when it promotes dsRNA synthesis by *RDR2* resulting in siRNA production (Qi et al. 2006). siRNA might then be used to guide a DNA methyltransferase such as *DRM1/2*, in a mechanism completely different from histone modification in fission yeast. In this respect, it would be very interesting to examine catalytic mutants of *AGO4* for histone H3K9 methylation, and for TE silencing, as well as for DNA methylation.

AGO AND TRANSCRIPTIONAL SILENCING IN HUMAN CELLS

Seven Argonaute proteins are encoded in the human genome, of which five are Argonaute-like, but only one of those tested has been shown to be catalytically active as of yet, namely, hAgo2 (Liu et al. 2004). Mutations of the hAgo2 DDH motif eliminate endonuclease activity (Liu et al. 2004; Rivas et al. 2005). However, other Argonaute proteins are implicated in human disease. For example, Wilms' tumors often lack the chromosomal region containing hAgo3, hAgo1, and hAgo4 (Carmell et al. 2002). Also, Hiwi, a homolog to mouse *Piwi*, has been associated with testicular germ cell tumors, seminomas, and ambiguous genitalia (Carmell et al. 2002).

Human cells exhibit siRNA-induced TGS (Morris et al. 2004; Ting et al. 2005). Introduction of a siRNA targeted to the elongation factor 1 alpha (EF1A) promoter region, fused to GFP, results in a reduction of protein expression, as well as a decrease in mRNA transcript levels (Morris et al. 2004). Similar results have been observed with a siRNA targeted to the endogenous gene, containing no GFP fusion (Weinberg et al. 2006). In addition, histone H3K9 methylation increases and spreads to at least 700 bp downstream from the EF1A transcriptional start site (Weinberg et al. 2006).

It has recently been shown that Argonaute proteins are required for TGS in human cells, suggesting a conserved mechanism. siRNA directed against sequences upstream of the transcription start sites of both Huntingtin (HTT) and androgen (AR) receptors expressed in the breast cancer cell line T47D inhibit protein expression and transcript synthesis, establishing RNAi as an inducer of TGS in mammalian cells (Janowski et al. 2006). The ability of HTT-specific siRNA to silence HTT is inhibited by reducing the levels of AGO2 or AGO1, which have both been shown to associate with DNA targeted by siRNA, in T47D cells (Janowski et al. 2006).

The expression level of target genes can alter silencing effectiveness. By comparing two breast cancer cell lines in which the expression level of progesterone receptor (PR) is high in one, T47D, and 33-fold lower in another, MCF-7, it has been determined that siRNA-mediated TGS is dependent on a relatively high level of transcription (Janowski et al. 2006). The authors speculate that high levels of transcription allow access to and recognition of DNA by siRNAs (Janowski et al. 2006). However, similar observations in plants have led some to speculate that high levels of transgene expression exhaust translation cofactors, resulting in a higher concentration of substrate for RNAi (Gazzani et al. 2004). Still others have suggested that a low level of transcription simply does not produce enough aberrant RNAs for rapid and effective initiation of silencing (Melquist and Bender 2003). Whatever the reason, expression level does seem to be a variable in the success of RNAi in any system.

There has been some controversy in human studies concerning the role of siRNA-mediated silencing in histone modification. Few differences were observed in histone H3K4 or K9 modifications at the gene encoding PR when targeted by siRNA, suggesting that H3K9me2 is not required (Janowski et al. 2006). In stark contrast, the pro-

motor region of human immunodeficiency virus-1 coreceptor CCR5 in 293T cells (a human renal epithelial cell line transformed by adenovirus E1A) shows a 14-fold increase of H3K9me2 in response to siRNAs directed to the region (Kim et al. 2006). The H3K9me2 enrichment spread to a region 100–300 bp downstream from the target site at a 7-fold increase (Kim et al. 2006), comparable to the 700 bp spreading mentioned above (Weinberg et al. 2006). AGO1 also binds the CCR5 promoter, and its association also spreads 100–300 bp downstream in response to transfection with siRNA directed against CCR5 promoter (Kim et al. 2006). Knockdown of AGO1 reduces H3K9me2 enrichment and inhibits gene silencing, suggesting that the association of AGO1 with targeted chromosomal DNA acts to recruit histone methyltransferases necessary for siRNA-mediated silencing (Kim et al. 2006). As H3K9me2 enrichment increases, AGO1 association decreases (Kim et al. 2006), suggesting that AGO1 is required for the initial boost of H3K9 methylation. As methylation increases, heterochromatin is formed and silencing occurs stably.

Argonaute Is Involved in Polycomb-mediated Silencing in Animal Cells

Polycomb genes (PcG) are regulatory factors that establish and maintain transcriptional repression by forming multimeric complexes that bind chromosomal regulatory elements (PREs) and modify chromatin structure (Pirrotta 1998; Jacobs and van Lohuizen 2002). They are absent in fission yeast, but are found in animals and in plants. Recently, a link has been described between PcG-mediated silencing in *Drosophila* and RNAi-mediated small RNA synthesis (Grimaud et al. 2006).

In the human Polycomb silencing pathway, the EZH2 histone methyltransferase methylates H3K27, which can be associated with heterochromatin (Kuzmichev et al. 2002). In HeLa cells (an immortal epithelial cell line derived from cervical cancer cells), siRNA directed against the tumor suppressor RASSF1A results in an enrichment of EZH2 and thus H3K27me3 (Kim et al. 2006). The MYT1 promoter, an endogenous target of the Polycomb silencing pathway, shows enrichment not only of EZH2 and H3K27me3, but also of AGO1 (Kim et al. 2006). Similarly, Polycomb component EZH2 was found to be enriched at the CCR5 promoter after transfection of siRNA directed against it, following the same spreading pattern as AGO1 association and H3K9me2 (Kim et al. 2006). These results implicate RNAi and AGO1 with the Polycomb pathway, a process that governs animal development. Although there are no Polycomb proteins in fission yeast, and no H3K27me3 has yet been detected, in *Drosophila*, there is some evidence that Polycomb response elements are transcribed, and that PIWI (an Argonaute homolog) mediates their association in the interphase nucleus, which is thought to be important for silencing (Grimaud et al. 2006). It remains to be seen whether these mechanisms are related.

CONCLUSIONS

The slicing function of Ago1 is necessary for transcriptional silencing and spreading of heterochromatin by RNAi

in *S. pombe* (Irvine et al. 2006). Argonaute proteins are also required for the generation of siRNA, but at least in fission yeast, siRNAs corresponding directly to the silenced promoter are not detectable, and slicing of co-transcripts that read through the silent gene seems to be sufficient. Argonaute proteins are also required for some examples of transcriptional silencing in plants and in mammalian cells. In human cells, silencing is mediated by artificial siRNA, so that a role in siRNA production can be excluded. In cases where histone H3 K9 methylation is induced, spreading of H3K9me2 and AGO1 occurs downstream from the siRNA, reminiscent of the situation in fission yeast.

However, RNAi-mediated silencing through DNA methylation (not found in fission yeast), although dependent on Argonaute, may differ in mechanism. In *Arabidopsis*, the Argonaute protein *AGO4* does not appear to require its slicing function to promote DNA methylation of at least one TE. In humans, AGO1 is required for examples of siRNA-directed DNA methylation but has yet to be demonstrated to have slicing activity. In the *Arabidopsis* study, histone modifications were not examined, but in humans, some examples of siRNA-directed silencing involve histone modification, whereas others involve DNA methylation, consistent with the idea that the two mechanisms are distinct.

In both humans and plants, it is tempting to speculate that DNA methyltransferases might be guided by siRNA themselves, whereas histone modification might be guided by the sliced target (sometimes called “aberrant RNA”). Consistent with this idea, the DNA methyltransferase DNMT3A has been shown to bind artificial siRNA (Weinberg et al. 2006) whereas siRNA have not been found in the Rik1/Cir4 complex in *S. pombe*, which may instead recognize sliced RNA products. The situation is complicated by the fact that DNA methylation can be induced by histone modification and vice versa in both humans and plants (Matzke and Birchler 2005), so that distinguishing these mechanisms may prove challenging.

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