**Argonaute Slicing Is Required for Heterochromatic Silencing and Spreading**

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Small interfering RNA (siRNA) guides dimethylation of histone H3 lysine-9 (H3K9me2) via the Argonaute and RNA-dependent RNA polymerase complexes, as well as base-pairing with either RNA or DNA. We show that Argonaute requires the conserved aspartate-aspartate-histidine motif for heterochromatic silencing and for ribonuclease H–like cleavage (slicing) of target messages complementary to siRNA. In the fission yeast Schizosaccharomyces pombe, heterochromatic repeats are transcribed by polymerase II. We show that H3K9me2 spreads into silent reporter genes when they are embedded within these transcripts and that spreading requires read-through transcription, as well as slicing by Argonaute. Thus, siRNA guides histone modification by base-pairing interactions with RNA.

RNA interference (RNAi) results when double-stranded RNA (dsRNA) is processed into siRNA by the ribonuclease (RNase) III–type enzyme known as Dicer. These siRNAs then base-pair with complementary mRNA to target cleavage (and, in some cases, to repress translation). Argonaute proteins facilitate this process by binding the 3′ end of one siRNA strand via the conserved PAZ domain. Target messages complementary to the siRNA are then cleaved by the Argonaute PIWI domain, which is related to RNaseH and contains the highly conserved motif Asp-Asp-His (D-D-H), which is required forendonuclease cleavage (or slicing) (1). Argonaute proteins are required for transcriptional as well as posttranscriptional silencing in Drosophila, Arabidopsis, and Schizosaccharomyces pombe, which has only one Argonaute protein (Ago1) (2–4). Heterochromatic repeats, transposable elements (TEs), and some transgenes are associated with modified chromatin when they are transcriptionally silenced. Histone modification in **S. pombe** depends on Argonaute and RNAi (5) as well as on the Rik1 complex, which contains the histone methyltransferase Ctr4 (6). Two models have been proposed for the role of siRNA in histone modification (7). First, siRNA might interact with DNA, recruiting modified histones via the RNA-induced initiation of transcriptional silencing (RITS) complex, which includes the chromodomain protein Chp1 as well as Ago1 (8). Alternatively, Argonaute might slice heterochromatic transcripts, recruiting RNA-dependent RNA polymerase (RdRP) via free 3′ OH ends, which promote polymerase activity (9). Association with nascent transcripts might then recruit the histone modification apparatus to the chromosome (10).

We tested the ability of fission yeast Ago1 to cleave target messages in a siRNA-dependent fashion. Recombinant glutathione S-transferase (GST)-SpAgo1 fusion proteins were purified from Escherichia coli (Fig. 1A) and incubated with two different 23-nucleotide (nt) RNA oligonucleotides complementary to a target message. When labeled message was added to the reaction, RNA fragments were detected, corresponding in size to products cleaved at each siRNA complementary site (Fig. 1B). These fragments were not observed in control reactions supplemented with EDTA, which chelates Mg2+ and thereby inhibits cleavage. Thus, Ago1 from fission yeast has “slicing” activity and can direct site-specific cleavage of RNA substrates via siRNA.

In order to assess the role of slicing in heterochromatic silencing, we constructed ago1Δ mutants in the D-D-H motif (11–13). Alanine substitutions were introduced at each of the three conserved residues (fig. S1), resulting in much lower catalytic activity in vitro (Fig. 1C). The corresponding mutants in yeast were viable and grew normally but accumulated transcripts from both forward and reverse strands of heterochromatic repeats (Fig. 1D). Run-on transcription assays have previously revealed that the reverse strand is transcribed in wild-type cells, although RNA fails to accumulate, whereas the forward strand is not transcribed in detectable quantities and is silenced at the transcriptional level (3). The reverse-strand transcript [or pre-siRNA (14)] fails to accumulate because it is rapidly converted into siRNA. Thus, our results indicate that both posttranscriptional silencing of (the reverse strand) and transcriptional silencing (of the forward strand) require slicing via the D-D-H motif. In agreement with these results, siRNA from centromeric repeats was undetectable in the slicer mutants (Fig. 1E), as in strains in which the ago1 gene has been deleted (15).

siRNA is derived from dsRNA. Only one strand of the repeats is transcribed in wild-type cells (3), so that generation of dsRNA depends on RdRP activity (9). DsRNA is then cleaved into 23-nt duplexes by Dicer. Ago1 promotes RdRP activity via interactions between the RITS and the RDRC complexes as well as template RNA (9). dsRNA synthesis begins at the 3′ OH end of single-stranded RNA (ssRNA) fragments (9), and such ends are generated by slicing (1). In slicer mutants, reduction in RdRP activity is expected to lead to loss of dsRNA and therefore loss of siRNA (1). Also, sliced transcripts are uncapped, which promotes RdRP activity (16). We therefore tested the association of both RdRP and Ago1 itself with centromeric heterochromatin in slicer mutant strains. We found that association of RdRP with the repeats was reduced (although not abolished) in ago1Δ slicer mutants (Fig. 2A). In contrast, association of slicer-defective Ago1 was slightly enriched (Fig. 2B), indicating that Ago1 catalytic activity, and not just localization to the repeats, is important for silencing. Histone H3 lysine-9 (H3K9me2) quantities were only partially reduced at the centromeric repeats.
themselves, as in ago1– deletions (Fig. 2C). This probably accounts for the retention of slicer-defective Ago1 at the repeats (Fig. 2B), because the RITS complex still binds to H3K9me2, even in the absence of siRNA (6, 8).

The ura4+ reporter genes integrated into the pericentromeric outer repeats of centromere 1 are transcriptionally silenced in wild-type strains via H3K9me2 and Swi6 (17, 18). Pericentromeric ura4+ was strongly derepressed in each of the three slicer mutants: H3K9me2 from the repeat gene was reduced to below the limit of detection, and H3K4me2 was increased fourfold (Fig. 2C). Slicer-defective Ago1 was also substantially lost from ura4+ (Fig. 2B), consistent with the loss of H3K9me2, but most Rdp1 was retained (Fig. 2A). Unlike RITS, whose association with the chromosome depends on H3K9me2, chromatin association of Rdp1 is thought to be dependent on nascent RNA, perhaps accounting for this distinction, although Rdp1 association with ura4+ is variable in replicate experiments (9). In the absence of RNAi, H3K9me2 is retained at pericentromeric repeats by the histone deacetylase Ctt3, as previously reported (19). But H3K9me2 is lost from ura4+ reporter genes integrated into transcribed repeats in ago1– slicer mutants (Fig. 2C), which resemble the RITS mutant chp1– in this respect (6). Thus, spreading of H3K9me2 into neighboring reporter genes depends on slicing.

Several ura4+ reporter genes have been integrated into centromere 1 and differ in the extent to which they are silenced (17, 18). We wondered whether this position effect depended on transcripts from the repeats, and so

Fig. 1. Slicer mutants in S. pombe. (A) 5DS–polycrylamide gel electrophoresis (PAGE) after size exclusion chromatography of GST-SpAgo1. Positions for the molecular weight markers (ink D) are labeled on the left. Bands were identified by mass spectrometric sequencing and are labeled on the right. The asterisk denotes SpAgo1 degradation products. (B) In vitro target RNA cleavage (slicing) assay using 32P-labeled 50-mer target and 23-nt siRNA guides. Target RNA is in red with 32P label as a red circle, siRNAs are in blue, and the cleavage products for each siRNA are shown on the right. Cleavage positions are directed by the siRNA sequences, resulting in two different cleavage products. Cleavage is dependent on Ago1 and magnesium (absence of EDTA). (C) Asp1580 → Ala1580 (DS580A) and Asp1650 → Ala1650 (D650A) mutants have much lower catalytic activity than wild type (WT) by this assay. (D) Centromeric transcripts from the dg and dh repeats, together with regions unaffected by RNAi (act1, ade6, and mat3M), and a ura4+ insertion in the outer repeat of centromere 1 [otr1::ura4+], together with a ura4 minigene (ura4DS/E) at the endogenous locus. (A) Hemagglutinin (HA)-tagged Rdp1. (B) WT and ago15DS80A slicer-defective HA-tagged Ago1. (C) H3K9me2 and H3K4me2.
we sequenced the ends of cDNA clones isolated from dcr1 strains by using the dg centromeric repeat as a probe. Most of these cDNA clones mapped to the 16 dg repeats from centromeres 2 and 3, but one clone matched the dg repeat from centromere 1 (fig. S2). Although it was truncated at the 3′ end, the 5′ end of the clone mapped precisely to the promoter of the reverse transcript identified previously (14). By comparing these sequences with the ura4+ insertion sites, we found that reporter genes inserted downstream of the promoter, within the transcription unit of the dg repeat, were silenced at least fourfold more efficiently than those inserted upstream of the transcription unit (fig. S2), indicating that transcription itself might play a role in spreading.

Read-through transcripts from ura4+ insertions in the dg repeat downstream of the reverse-strand promoter were present in low quantities in wild-type cells but in high quantities in ago1− slicer mutants, indicating they were the targets of slicer activity (Fig. 3, A and B). By using rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR), we could detect full-length cotranscripts in mutant cells (17–18, 20/27 cloned products), but we could not detect either full-length or cleaved transcripts in wild-type cells (Fig. 3C). Cleavage products could be recovered, however, from the exosome mutant rrp6− (Fig. 3C), in which an exonuclease gene had been disrupted. The exosome degrades uncapped and nonpolyadenylated (or cleaved) transcripts (20, 21), such as those cleaved by Ago1 slicer activity on either side of the ura4+ insertion. In rrp6− cells, different cleavage sites were sequenced in the region that matched siRNA (17–18, cloned products), but none were detected in the ura4+ gene itself (Fig. 3D). ura4+ insertions located within the transcribed dg repeat (FY988 and FY939) also gave rise to read-through transcripts in ago1− slicer mutants (fig. S2) and were strongly silenced (17, 18). But read-through transcripts corresponding to ura4+ genes located upstream of the reverse strand promoter (FY496 and FY501) could only just be detected, consistent with reduced silencing in these strains (fig. S2). This reduced silencing is still sensitive to RNAi (3) but, unlike downstream ura4+ insertions silencing, also depends on the histone deacetylase Clr3 (17, 18), which is independent of RNAi (19). Similar read-through transcripts were observed when repeats were fused to reporter genes elsewhere in the genome and were also targets of RNAi (22). Thus, reporter gene silencing depends, at least in part, on slicing of heterochromatic cotranscripts by Ago1 (Fig. 3C).

The siRNA from the ura4+ reporter gene was undetectable (fig. S3), suggesting that interactions between siRNA and DNA are unlikely to account for ura4+ silencing. Instead, recruitment of H3K9me2 and RITS to ura4+ depends on siRNA from the pericentromeric repeats (6, 8), which are required for slicing of read-through transcripts. Sliced nascent transcripts might recruit the silencing apparatus to reporter genes by virtue of cotranscription (Fig. 3E). A heterochromatic role for polymerase (Pol) II in spreading H3K9me2 is reminiscent of its euchromatic role in spreading H3K4me2 (23). In plants, TE insertions bring genes under their control when they integrate within the transcription unit but not when they integrate further away (5, 24). In animals, cotranscription of heterochromatic repeats may also play a role in some forms of position effect variegation, but genes are silenced at much greater distances from heterochromatin and intervening genes can retain activity (25), so that other spreading or antispanning mechanisms are likely to be involved (26), such as those involving Swi6 (27).

References and Notes
Chemical Chaperones Reduce ER Stress and Restore Glucose Homeostasis in a Mouse Model of Type 2 Diabetes

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Endoplasmic reticulum (ER) stress is a key link between obesity, insulin resistance, and type 2 diabetes. Here, we provide evidence that this mechanistic link can be exploited for therapeutic purposes with orally active chemical chaperones. 4-Phenyl butyric acid and taurine-conjugated ursodeoxycholic acid alleviated therapeutic application. Here, we provide evidence that this mechanistic link can be exploited for therapeutic purposes with orally active chemical chaperones. 4-Phenyl butyric acid (PBA), trimethyloxide dihydrate (TMAO), and di-