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Two different Argonaute complexes are required for siRNA generation and heterochromatin assembly in fission yeast

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The RNA-induced transcriptional silencing (RITS) complex, containing Ago1, Chp1, Tas3 and centromeric small interfering RNAs (siRNAs), is required for heterochromatic gene silencing at centromeres. Here, we identify a second fission yeast Argonaute complex (Argonaute siRNA chaperone, ARC), which contains, in addition to Ago1, two previously uncharacterized proteins, Arb1 and Arb2, both of which are required for histone H3 Lys9 (H3-K9) methylation, heterochromatin assembly and siRNA generation. Furthermore, whereas siRNAs in the RITS complex are mostly single-stranded, siRNAs associated with ARC are mostly double-stranded, indicating that Arb1 and Arb2 inhibit the release of the siRNA passenger strand from Ago1. Consistent with this observation, purified Arb1 inhibits the slicer activity of Ago1 *in vitro*, and purified catalytically inactive Ago1 contains only double-stranded siRNA. Finally, we show that slicer activity is required for the siRNA-dependent association of Ago1 with chromatin and for the spreading of histone H3-K9 methylation.

RNA interference (RNAi) and related RNA silencing mechanisms regulate gene expression at both the transcriptional and post-transcriptional levels[1–6]. Small RNA molecules, called microRNAs (miRNA) or siRNAs, are central in RNAi as guides that recognize homologous sequences and target them for inactivation. During post-transcriptional gene silencing, siRNAs or miRNAs are loaded onto the RNA-induced silencing complex (RISC), containing a conserved Argonaute protein[7], which binds siRNAs and also directly cleaves target messenger RNA sequences[8,9]. In the fission yeast *Schizosaccharomyces pombe*, repetitive DNA elements that surround centromeres give rise to siRNAs[10], and the formation of heterochromatin at these repeats requires components of the RNAi pathway[11]. Centromeric siRNAs are found in the RITS complex, which also contains the fission yeast Argonaute protein Ago1, the chromodomain protein Chp1, and Tas3, a Gly-Trp (GW)-repeat protein of unknown function[12].

Duplex siRNAs are processed from long double-stranded RNAs by an RNase III–like enzyme called Dicer[13]. However, the mechanisms that generate mature single-stranded siRNAs are poorly understood. In addition to Dicer, siRNA generation requires a number of other proteins or complexes. For example, in fission yeast, siRNA generation also requires Rdp1 (the fission yeast RNA-directed RNA polymerase, RdRP) and Ago1, as well as chromatin factors such as the Clr4 histone H3-K9 methyltransferase[14,15]. Rdp1 is a subunit of the RNA-directed RNA polymerase complex (RDRC), which also contains the Hrr1 helicase and a noncanonical poly(A) polymerase family member, Cid12 (ref. 14). Notably, unlike the situation in plants and *Caenorhabditis elegans*, where RdRP enzymes are required for siRNA amplification[16,17], no siRNAs can be detected in *S. pombe* cells that lack any subunit of the RDRC complex[14]. Furthermore, it is unclear how duplex siRNAs generated by Dcr1 are transferred to the RITS complex and converted into functional siRNAs, which must be single-stranded to base-pair with their nucleic acid targets.

Members of the Argonaute/PIWI family contain two conserved domains: PAZ and PIWI. Whereas the PAZ domain has been shown to be important for siRNA binding[18–20], the PIWI domain of Argonaute is responsible for the endonucleolytic cleavage of a target RNA, referred to as 'slicing'[8,9]. In the minimal RISC complex, containing Ago2, Dicer and TRBP in human (or R2D2 in *Drosophila melanogaster*), Ago2 is initially loaded with double-stranded siRNA[21,22]. As only one strand (the guide strand) is retained in active RISC, the other (the passenger strand) must be removed before a mature complex is formed. *In vitro*, the enzymatic activity of Ago2 is required for the efficient ejection of the passenger strand[23–25]. The passenger strand is cleaved in the same manner as homologous long RNA targets, making it the first target of slicer activity. The requirement of passenger-strand cleavage in RISC maturation is conserved between flies and humans and may be the main mechanism of RISC activation of cleavage-competent Argonaute proteins in these systems. Alternative

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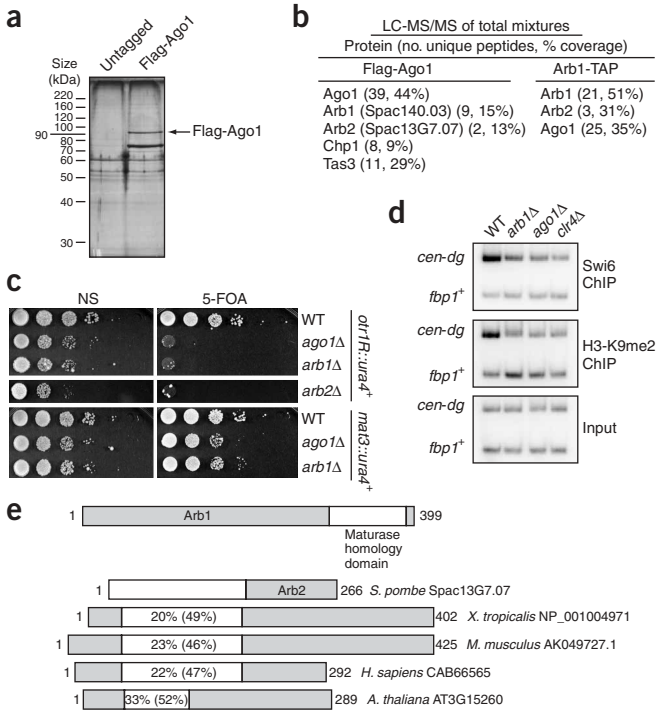

Figure 1 Purification of Flag-Ago1 and identification of Arb1 and Arb2. (**a**) Extracts from a Flag-Ago1 strain and an untagged control strain were purified using anti-Flag agarose, run on a 4%–12% polyacrylamide gel and silver-stained. (**b**) Results of tandem MS sequencing of mixtures of proteins (LC-MS/MS) in each preparation are indicated as the number of unique peptides and percent of total number of amino acid residues covered. (**c**) Silencing of a *ura4*$^+$ reporter inserted at the outermost centromeric repeat (*otr1R::ura4*$^+$) is lost in *arb1*Δ and *arb2*Δ strains to the same extent as *ago1*Δ; *arb1*$^+$, like *ago1*$^+$, is not required for silencing at the *mat* locus. Arb1-TAP is fully functional for centromeric silencing (see **Supplementary Fig. 1**). WT, wild-type; NS, nonselective medium; 5-FOA, 5-fluorotic acid. (**d**) ChIP experiment showing that methylation of histone H3-K9 and localization of Swi6 to the *cen-dg* centromeric repeat are lost in *arb1*Δ cells. (**e**) Schematic diagram of Arb1 and Arb2 sequences, showing region of similarity between Arb1 and organellar maturases, based on BLASTP results (species and accession codes as follows: *S. pombe*, locus; *Xenopus tropicalis*, NCBI Protein; *Mus musculus*, GenBank; *Homo sapiens*, NCBI Protein; *Arabidopsis thaliana*, locus). Percent identity and similarity (in parentheses) are indicated.

mechanisms are likely to be involved in release of the passenger strand from Argonaute proteins that lack cleavage activity[23].

Fission yeast centromeric siRNAs are thought to target the RITS complex to sites of heterochromatin formation through base-pairing interactions between the siRNA and nascent centromeric transcripts[14,26]. This association is then proposed to recruit histone-modifying enzymes as well as the RDRC complex, which may generate more double-stranded RNA using chromatin-associated RNA templates. It has recently been shown that Ago1 has catalytic activity and that this activity is required for silencing of a transgene inserted in the pericentromeric repeats[27]. However, little is known about how RITS is loaded with centromeric siRNA and what role Ago1 itself plays in this process. Here we identify a second Ago1 complex, termed ARC, which contains the previously uncharacterized proteins Arb1 and Arb2. Both Arb1 and Arb2 are required for heterochromatin assembly and siRNA generation. Furthermore, we show that ARC contains mostly duplex rather than single-stranded siRNA. Consistent with the presence of duplex siRNAs in ARC, purified Arb1 inhibits the slicer activity of Ago1 *in vitro*. These observations suggest that Arb proteins regulate the conversion of duplex siRNA to single-stranded siRNA in a process that is required for heterochromatin formation. We provide evidence that catalytically inactive Ago1 cannot associate with centromeric repeats beyond the background level observed in *dcr1*Δ cells, which lack siRNAs. Furthermore, catalytically inactive Ago1 disrupts heterochromatin formation and prevents the spreading of histone H3-K9 methylation into transgene sequences even when a wild-type copy of *ago1*$^+$ is present. This strong dominant-negative phenotype suggests that the ability of siRNAs to base-pair with their target sequences is crucial for the spreading of histone H3-K9 methylation and heterochromatin formation.

RESULTS

A second fission yeast Argonaute complex

To gain further insight into the role of Ago1 in RNAi-mediated heterochromatin assembly, we used a Flag epitope tag to purify the Ago1 protein and any associated factors (**Fig. 1**). Flag-Ago1 was fully functional for RNAi-dependent gene silencing (**Supplementary Fig. 1** online). Mass spectrometry analysis of purified Flag-Ago1 identified several known Ago1-interacting proteins, including the RITS components Chp1 and Tas3 (**Fig. 1b**). In some Flag-Ago1 preparations, we also identified Hrr1, which has previously been shown to copurify with both RITS and the RNA-directed RNA polymerase complex (RDRC)[12,14] (data not shown). Peptides corresponding to the previously uncharacterized SPAC140.03 and SPAC13G7.07 proteins, which we named Argonaute binding protein-1 and Argonaute binding protein-2 (Arb1 and Arb2), were also identified (**Fig. 1b** and **Supplementary Table 1** online). Arb2 was also independently identified by mass spectrometry analysis of Myc-Ago1 immunoprecipitations (T.I. and J.-I.N., unpublished data). Arb1 is conserved throughout fungi, from fission yeast to *Neurospora crassa*, and has a C-terminal domain that resembles those of organellar maturases, which facilitate intron self-splicing (**Fig. 1e** and **Supplementary Fig. 2** online). Arb2 seems to be widely conserved from fission yeast to human but has no obvious functional domains (**Fig. 1e**). To determine whether Arb1, Arb2 and Ago1 form a stable complex, we purified Arb1 using a TAP approach. Mass spectrometry analysis confirmed that Ago1, Arb1 and Arb2 associate together in a complex we have named ARC (**Fig. 1b** and **Fig. 2a**). Notably, no peptides corresponding to the RITS subunits Tas3 or Chp1 were identified in Arb1-TAP preparations (**Fig. 1b**). Coimmunoprecipitation experiments confirmed the Ago1-Arb1 interaction and corroborated the lack of any detectable interaction between other RITS subunits and Arb1 (**Supplementary Fig. 3** online). Strains carrying C-terminally tagged Arb2-TAP fusions were not functional in centromeric silencing assays and were not further pursued (data not shown). A semiquantitative approach based on the relative abundances of tryptic peptide spectra was used to estimate the relative abundances of proteins in the RITS and ARC complexes, which indicated that Ago1 is distributed roughly equally between the two complexes (**Supplementary Table 2** online). Together, these data indicate that fission yeast Ago1 exists in two distinct complexes, RITS and ARC.

Ago1 and other components of the RNAi pathway are required for the assembly of heterochromatin and silencing of reporter genes inserted in the pericentromeric region of chromosomes[11]. To determine whether Arb1 and Arb2 are involved in the same pathway, silencing of a *ura4*$^+$ reporter inserted in the outermost centromeric repeats of chromosome 1 (*otr1R::ura4*$^+$) was assayed in *arb1*Δ and

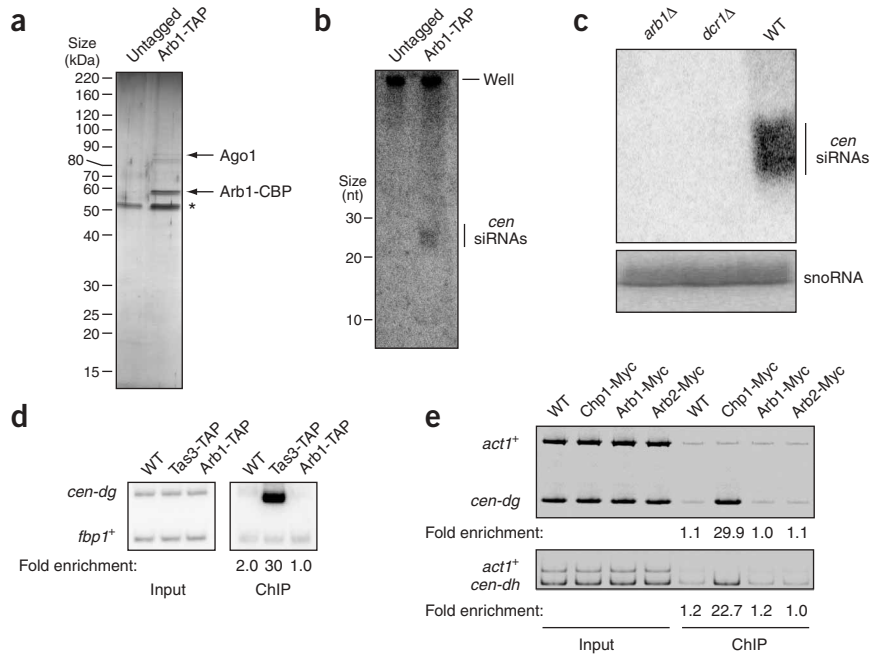

Figure 2 ARC contains siRNAs and is required for siRNA generation but does not localize to centromeric heterochromatin. (**a**) Purification of ARC using Arb1-TAP. Purified material was run on a 4%–12% polyacrylamide gel and silver-stained. Asterisk denotes residual protease used for elution of bound proteins from the IgG-Sepharose column. (**b**) Northern blot showing that siRNAs that associate with ARC hybridize to ^{32}P-labeled probes corresponding to centromeric siRNAs. Nucleic acid was isolated from Arb1-TAP purified as in **a**. (**c**) Northern blot from total RNA preparation showing that *arb1*$^{+}$ is required for the generation of centromeric siRNAs. Blot was probed with oligonucleotides as in **b** and also oligonucleotides that anneal to the loading control snoR69. WT, wild-type. (**d**) ChIP experiment comparing the localization of Arb1-TAP and Tas3-TAP to centromeric DNA. PCR was done using primers that amplify a portion of the *cen-dg* centromeric repeat or the euchromatic *fbp1*$^{+}$ gene. (**e**) Localization of Arb1 and Arb2 to centromeric repeats *cen-dg* and *cen-dh*, compared with the RITS subunit Chp1.

*arb2*Δ cells. The reporter was derepressed in both mutants to the same level as in *ago1*Δ cells (**Fig. 1c**). In addition, noncoding centromeric transcripts originating from the centromeric repeats accumulated in *arb1*Δ cells (**Supplementary Fig. 4** online). Like Ago1, Arb1 and Arb2 are not required for the silencing that occurs at the silent mating type locus *mat3M* (**Fig. 1c** and data not shown), where a redundant pathway is involved in heterochromatin formation[28]. Chromatin immunoprecipitation (ChIP) experiments showed that Arb1, like Ago1 and Clr4, is required for H3-K9 methylation and Swi6 localization to the centromeric repeats (**Fig. 1d**). Thus, like other RNAi components, ARC subunits are required for heterochromatin assembly at centromeric repeats.

ARC is required for siRNA generation

RITS contains centromeric siRNAs thought to target the complex to nascent centromeric transcripts through base-pairing interactions[12,14,15,26]. To determine whether ARC also contains these siRNAs, we performed northern blot analysis on nucleic acid derived from purified Arb1-TAP (**Fig. 2a**,**b**). Using probes that hybridize with cloned centromeric siRNAs[10,12], we consistently observed species of ~25 nucleotides (nt) in Arb1-TAP preparations (**Fig. 2b**). The siRNA signal in Arb1-TAP northern blots was consistently weaker than that observed for RITS preparations (**Fig. 2b**; S.M.B., unpublished data)[12]. This may be due to the substoichiometric presence of Ago1 in most of our Arb1-TAP preparations. Furthermore, Arb1 and Arb2 are required for siRNA generation, as no centromeric siRNAs could be detected in total RNA northern blots from *arb1*Δ and *arb2*Δ cells, as was the case for a *dcr1*Δ control (**Fig. 2c** and T.I., unpublished data).

Subcellular localization of Arb1 and Arb2

Given that centromeric siRNAs are thought to be targeting factors, we next performed ChIP to test whether Arb1 and Arb2 localize to centromeric heterochromatin. Notably, in contrast to Tas3-TAP and Chp1-Myc, we could not detect Arb1-TAP or Arb2-Myc localization to centromeric repeat sequences (**Fig. 2d**,**e**). Moreover, it has previously been shown that Chp1 and Tas3 localize to punctuate nuclear foci that represent sites of heterochromatin[14,29,30].

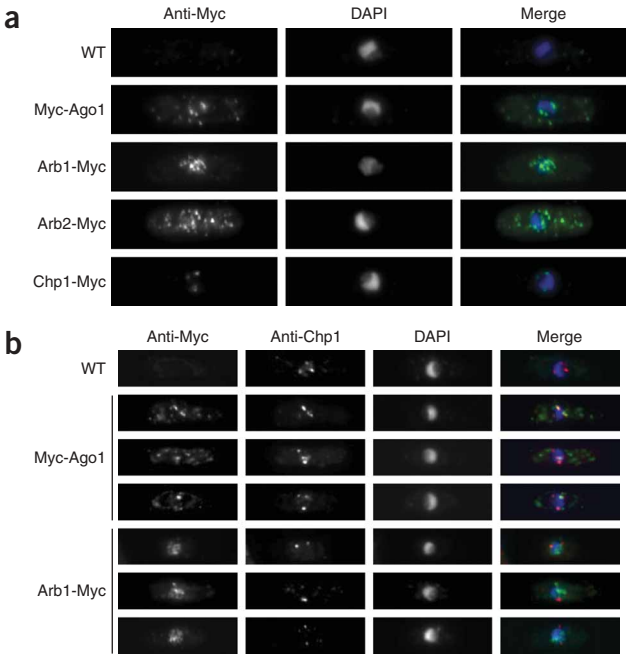

Figure 3 Immunofluorescence localization of ARC subunits. (**a**) Immunofluorescence experiment showing the subcellular localization of Arb1 and Arb2 compared with Ago1 and Chp1. In contrast to Chp1, which localizes to characteristic heterochromatic foci, Myc-Ago1 and Arb2-Myc localize to a large number of foci in both the nucleus and cytoplasm, whereas Arb1 is predominantly nuclear. WT, wild-type. (**b**) Immunofluorescence colocalization experiments showing that some of the nuclear Myc-Ago1 foci overlap with Chp1, but Arb1 and Chp1 stain mostly nonoverlapping regions in the nucleus. All four proteins were expressed from their native promoters and were fully functional in centromeric silencing, except for Arb2-Myc, which had a slight silencing defect.

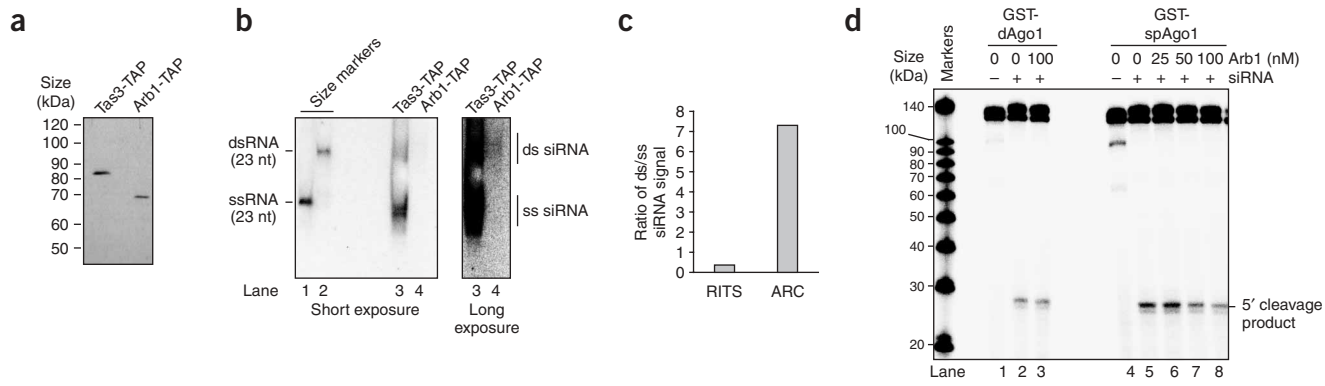

Figure 4 Distribution of single-stranded and duplex centromeric siRNAs in the ARC and RITS complexes and inhibition of Ago1 slicer activity by Arb1. (**a**) Western blot of whole-cell extract from Tas3-TAP and Arb1-TAP strains, showing relative expression of Tas3 and Arb1 from their endogenous promoters. (**b**) Nondenaturing northern blot of purified Tas3-TAP and Arb1-TAP hybridized with centromeric siRNA probes. Lanes 1 and 2, single-stranded (ss) and double-stranded (ds) 5′ end–labeled synthetic RNA, ssRNA and dsRNA, respectively, were used as size markers. (**c**) Quantification of phosphorimaging signals in **b** as described in Methods. (**d**) Purified *Drosophila* GST-Ago1 and *S. pombe* GST-Ago1 (100 nM each) were incubated with a synthetic siRNA. A 5′ end–labeled 130-nt target was added to the reaction that contained sequence complementary to that of the siRNA guide. Position of the 5′ cleavage product is indicated. Purified Flag-Arb1 was added to the reactions at the indicated concentrations. Flag-Arb1 (100 nM, lane 8) usually inhibited the appearance of the 5′ cleavage product by two- to three-fold. See **Supplementary Figure 5** for GST-Ago1 and Flag-Arb1 purifications.

Immunofluorescence experiments with Arb1-Myc and Arb2-Myc showed staining throughout the nucleoplasm as well as in cytoplasmic foci, which were particularly prominent for Arb2-Myc (**Fig. 3a**). In this regard, Arb1-Myc and Arb2-Myc localization resembles that of Myc-Ago1, which is present in both nuclear and cytoplasmic foci (**Fig. 3a**). Furthermore, in contrast to Chp1-Myc, which localized to nuclear foci as is characteristic of heterochromatin proteins, both Arb1-Myc and Myc-Ago1 localized to a larger number of foci, which were not always overlapped by the foci containing Chp1 (**Fig. 3b**). Cytoplasmic Ago1 foci have also been described previously for overexpressed hemagglutinin (HA)-Ago1 and green fluorescent protein (GFP)-Ago1 proteins[31]. The distinct localization patterns of RITS[14,15,30] and ARC (**Fig. 3**) subunits suggest that siRNA biogenesis in *S. pombe* involves nuclear chromatin–associated steps as well as nucleoplasmic and cytoplasmic steps.

ARC contains predominantly duplex siRNA

To understand why siRNAs do not direct the localization of ARC to chromatin, we sought to characterize the siRNAs that reside in ARC. Driven by their endogenous promoters, Tas3 and Arb1 are expressed to similar levels in the cell (**Fig. 4a**). Both RITS and ARC, purified via Tas3-TAP and Arb1-TAP, respectively, were subjected to nucleic acid extraction, nondenaturing PAGE and subsequent northern blot analysis to distinguish single-stranded from double-stranded siRNAs. With this nondenaturing gel system, we were able to resolve a synthetic 23-nt single-stranded RNA from 23-nt RNA duplex (**Fig. 4b**, lanes 1 and 2). RITS was associated with single-stranded siRNAs and a smaller amount of duplex centromeric siRNAs (**Fig. 4b**, lane 3). In contrast, the majority of centromeric siRNAs associated with ARC were double-stranded (**Fig. 4b**, lane 4, dark exposure). Phosphorimaging quantification of the siRNA signals showed that about three-fold more single-stranded siRNA than duplex siRNA was associated with Tas3-TAP (**Fig. 4c**). Although no signal corresponding to single-stranded siRNA was visually detectable with purified Arb1-TAP, phosphorimaging quantification revealed a signal above background in the region where single-stranded siRNAs migrate. This approach showed that at least seven-fold more duplex siRNAs than single-stranded siRNAs were associated with Arb1-TAP (**Fig. 4c**; see Methods). However, it is likely that we underestimate the amount of double-stranded siRNA, as the efficiency of the probe annealing to a siRNA duplex may be low. RITS requires a single-stranded siRNA to target the complex to specific chromosome regions for heterochromatin assembly, but contains both single-stranded and duplex siRNA. Thus, we conclude that the assembly of Ago1 into RITS facilitates the release of the passenger strand, producing mature single-stranded siRNA.

Two possible mechanisms have previously been proposed for the release of the passenger siRNA strand from metazoan Ago2 in the RISC complex. The first involves cleavage of the passenger strand by Ago2 itself, and the second involves a poorly defined slicer-independent release pathway[23,24]. As ARC contains mostly duplex siRNAs, either the slicer activity of Ago1 in ARC is inhibited by Arb1 and/or Arb2, or, alternatively, Arb1 and Arb2 block slicer-independent release of the siRNA passenger strand. To distinguish between these possibilities, we tested whether the *in vitro* slicer activity of Ago1 is inhibited by Arb proteins. A glutathione *S*-transferase–*S. pombe* Ago1 fusion (GST-spAgo1) was expressed in bacteria and purified (**Supplementary Fig. 5** online). As a control, we also purified the *Drosophila* Ago1 (GST-dAgo1, **Supplementary Fig. 5**), which has previously been shown to have *in vitro* slicer activity[25]. Consistent with previous results[25,27,32], both purified proteins cleaved a target RNA after incubation with a synthetic single-stranded siRNA (**Fig. 4d**, lanes 2 and 5), but no cleavage activity was observed in the absence of the siRNA or when a noncomplementary siRNA was used (**Fig. 4d**, lanes 1 and 4, and data not shown). A functional Flag-Arb1 fusion was constructed and purified from yeast (**Supplementary Fig. 5**). The addition of purified Flag-Arb1 inhibited the slicer activity of spAgo1 but not the activity of the heterologous dAgo1 (**Fig. 4d**, compare lanes 2 and 3 with lanes 5–8). We note that even though Flag-Arb1 is overexpressed, preparations of this protein are likely to also contain Arb2 and Ago1, albeit at substoichiometric levels. Nonetheless Arb1, by itself and/or together with Arb2, is a direct inhibitor of the slicer activity of fission yeast Ago1. Moreover, the species-specific nature of the inhibition suggests that it involves an interaction of Arb1 and/or Arb2 with *S. pombe* Ago1 rather than with siRNAs.

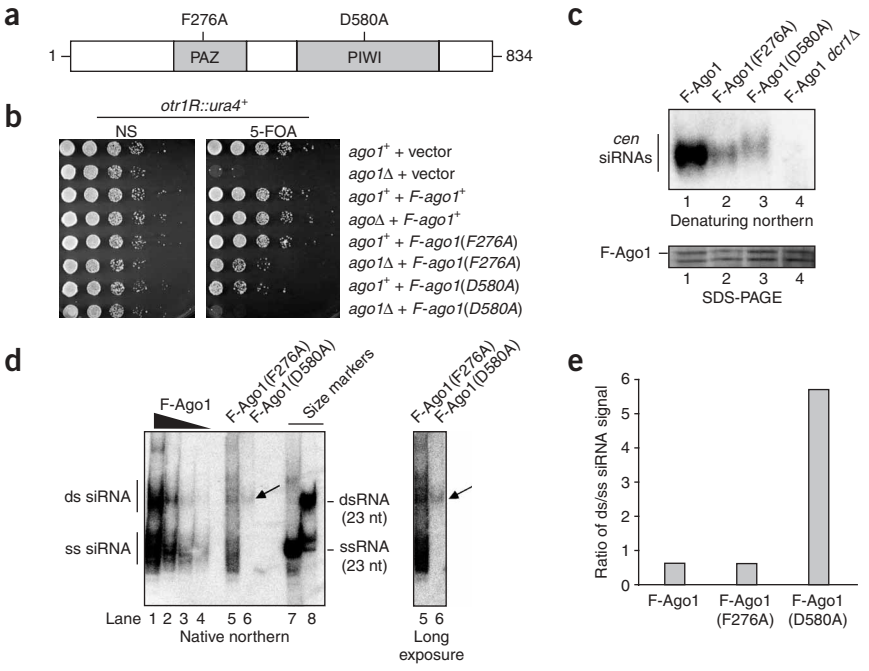

Figure 5 The slicer activity of Ago1 is required for siRNA maturation and heterochromatin assembly. (**a**) Functional domains of Ago1 and the positions of point mutations in the PAZ and PIWI domains. (**b**) Silencing assay with wild-type or mutant Ago1 plasmids transformed into *ago1*$^+$ or *ago1*Δ strain. (**c**) Top, northern blot, probed with oligonucleotides that hybridize to centromeric siRNA from the indicated Ago1 proteins purified from *ago1*$^+$ cells; bottom, silver-stained gel showing the purified Flag-Ago1 (F-Ago1) proteins used for RNA extraction. (**d**) Samples prepared as in **c** but run under nondenaturing conditions to separate double-stranded (ds) from single-stranded (ss) siRNA. Lanes 1–4, two-fold serial dilution of siRNAs associated with wild-type Flag-Ago1. Lanes 5 and 6, siRNAs associated with the indicated mutant Ago1 proteins. (**e**) Quantification of phosphorimaging signals in **d** as described in Methods.

Ago1 slicer activity and siRNA generation

The above experiments indicate that ARC regulates Ago1's slicer activity and the conversion of double-stranded siRNAs to single-stranded siRNAs, which is crucial for RNAi-mediated heterochromatin assembly. Fission yeast Ago1 contains conserved catalytic residues that are required for slicer activity[8,27]. Furthermore, in *Drosophila* Ago2 complexes, slicer activity is required for the ejection of the passenger strand of an siRNA duplex[23,25,33]. To determine whether Ago1 activity is required for siRNA maturation and RNA silencing, we mutated a conserved residue in the putative catalytic site of Ago1 (D580A) and tested the effects on silencing and the conversion of double-stranded siRNA to single-stranded siRNA (**Fig. 5a**). A mutation at a conserved residue important for siRNA binding was also introduced into the PAZ domain (F276A; **Fig. 5a**)[34]. Consistent with previous observations[27], a plasmid carrying *ago1* containing a D580A substitution in the DDH catalytic motif of the PIWI domain did not complement the silencing defect of an *ago1*Δ strain (**Fig. 5b**). Notably, this slicer mutant also acted as a dominant-negative allele, as its overexpression in *ago1*$^+$ cells caused a moderate silencing defect (**Fig. 5b**). To directly assay the slicer requirement for siRNA maturation *in vivo*, we purified Ago1 PAZ and PIWI mutants and performed both denaturing and nondenaturing northern blots on associated small RNAs. Because no siRNAs can be detected in cells with mutations in either RNAi or heterochromatin components[14,15,26,35,36], cells harboring Ago1(D580A), which is defective for centromeric heterochromatin assembly, would be expected to lack siRNAs. We therefore purified Ago1 mutant proteins from cells that also contained wild-type *ago1*$^+$. Both the Ago1(F276A) (PAZ) and Ago1(D580A) (PIWI) mutants were associated with fewer siRNAs than wild-type Ago1, as shown by denaturing northern blots (**Fig. 5c**). As Ago1(D580A) was completely defective for silencing yet was still associated with siRNAs when purified from cells that also contained *ago1*$^+$, we next performed a nondenaturing northern blot to determine whether it contained double- or single-stranded siRNAs. In contrast to wild-type Ago1, Ago1(D580A) contained mostly duplex siRNA *in vivo* (**Fig. 5d**, compare lanes 1–4 with lanes 5 and 6). Quantification of siRNA signals on northern blots showed that the ratios of double- to single-stranded siRNA were ~1:2 in purified wild-type and Ago1(F276A) (**Fig. 5e**), consistent with the data in **Figure 4c**. In marked contrast, the double- to single-stranded siRNA ratio in purified Ago1(D580A) was ~6:1 (**Fig. 5e**), which is similar to the value observed for the ARC complex (**Fig. 4c**). Thus, mutations that disrupt the slicer activity of Ago1 prevent the conversion of duplex siRNA to single-stranded siRNA. Ago1(F276A), on the other hand, reduces the efficiency of siRNA binding but does not affect this siRNA maturation step. These results indicate that the slicer activity of Ago1 is required for the *in vivo* conversion of double-stranded siRNA to single-stranded siRNA and, together with the results in **Figure 3**, suggest that the inhibition of this activity is responsible for the presence of double-stranded siRNA in the ARC complex.

Role of slicer activity of Ago1 in H3-K9 methylation

Having shown that Ago1(D580A) is defective in siRNA maturation, we sought to determine what effect, if any, Ago1 mutations have on heterochromatin assembly and the recruitment of Ago1 itself to centromeric repeats. We used ChIP to examine the localization of wild-type and mutant Flag-Ago1 proteins expressed in both *ago1*$^+$ and *ago1*Δ cells. *dcr1*Δ cells, which lack siRNAs, served as a control for siRNA-independent binding of Ago1 to centromeric repeats[30]. Ago1(D580A) localization to *cen-dg* repeats was similar to that observed in *dcr1*Δ control cells, whereas intermediate amounts of the Ago1(F276A) mutant bound the repeats when it was overexpressed in *ago1*Δ cells (**Fig. 6a**). In *ago1*$^+$ cells, the levels of binding for both mutants were above those observed in *dcr1*Δ cells. These results indicate that the slicer activity of Ago1 is required for its siRNA-dependent localization to centromeric repeats.

We next tested how each one of the above Ago1 mutations affected histone H3-K9 methylation and the recruitment of the Chp1 subunit of the RITS complex. As expected from the lack of siRNAs in Ago1(D580A) mutant cells, H3-K9 methylation and Chp1 binding at *cen-dg* were reduced to the background level observed in *dcr1*Δ cells when Ago1(D580A) was overexpressed in *ago1*Δ cells (**Fig. 6a**). Ago1(F276A), which still contains some siRNAs and has a relatively weak silencing defect, showed nearly wild-type levels of H3-K9 methylation and Chp1 binding in *ago1*Δ cells (**Fig. 6a**). In the presence

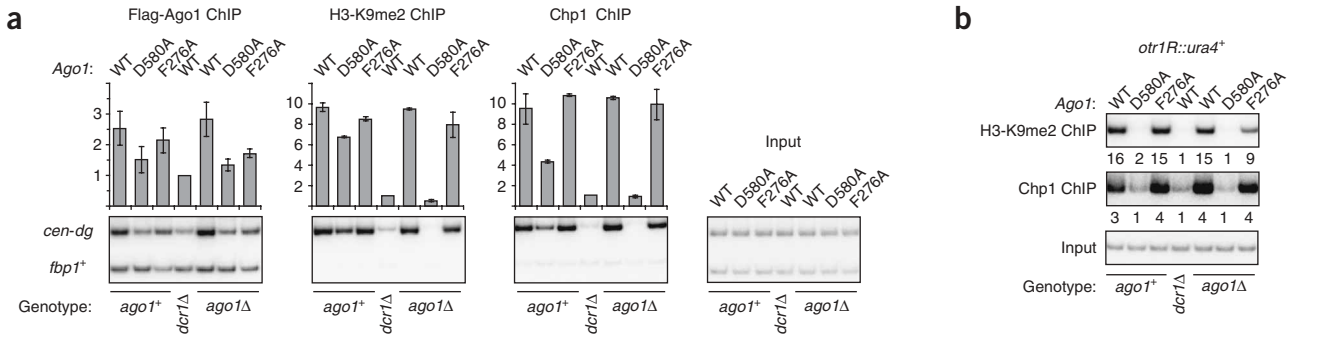

Figure 6 The slicer activity of Ago1 is required for its localization to centromeric repeats and for the spreading of H3-K9 methylation. (**a**) ChIP experiment showing the localization of Chp1 and Flag-Ago1 proteins, as well as H3-K9 methylation at the *cen-dg* repeat in wild-type (WT) or the indicated mutant backgrounds. *dcr1*Δ serves as a control for siRNA-independent background localization. Error bars represent s.d. (**b**) ChIP experiments showing H3-K9 methylation and Chp1 localization at the *otr1R::ura4*$^+$ transgene inserted into the pericentromeric repeat. Error bars represent s.d.

of a wild-type copy of *ago1*$^+$, Ago1(D580A) caused a decrease in both H3-K9 methylation and Chp1 binding at *cen-dg*, but Ago1(F276A) had little or no effect on either H3-K9 methylation or Chp1 localization (**Fig. 6a**). These results indicate that the slicer activity of Ago1 not only is required for the localization of Ago1 itself to *cen* repeats but is also crucial in mediating H3-K9 methylation and RITS recruitment.

Previous studies have shown that the RNAi pathway is essential in the spreading of histone H3-K9 methylation to transgene sequences that are inserted within centromeric repeats[11,12,15,30]. To determine the role of Ago1 in this process, we examined the effect of Ago1 PAZ and PIWI mutations on H3-K9 methylation of and Chp1 localization to a *ura4*$^+$ gene inserted within the outer centromeric repeats (*otr1R::ura4*$^+$). Similar to what we observed for the *cen-dg* repeats (**Fig. 6a**), in cells that carried Ago1(D580A) as their sole source of Ago1, both H3-K9 methylation and Chp1 localization to the centromeric *ura4*$^+$ were reduced to the background levels observed in *dcr1*Δ cells (**Fig. 6b**, *ago1*Δ lanes). The Ago1(F276A) mutant had nearly wild-type levels of H3-K9 methylation and Chp1 localization (**Fig. 6b**, *ago1*Δ lanes). Notably, in contrast to the situation at *cen-dg*, even in cells that carried a wild-type copy of *ago1*$^+$, expression of Ago1(D580A) reduced H3-K9 methylation and Chp1 binding at the centromeric *ura4*$^+$ gene nearly to the background levels observed in *dcr1*Δ cells (**Fig. 6b**, *ago1*$^+$ lanes). These results are consistent with the dominant-negative silencing phenotype of Ago1(D580A) and indicate that the presence of double-stranded siRNA in Ago1(D580A) strongly interferes with the spreading of RITS and histone H3-K9 methylation into transgene sequences.

DISCUSSION

We have identified a novel Argonaute-containing complex with an essential role in heterochromatin assembly at fission yeast centromeres. This complex, which we have named ARC, contains double-stranded siRNAs rather than single-stranded siRNAs and is likely to be a precursor complex involved in siRNA maturation. ARC may act as a receptor that receives siRNAs directly from the Dicer RNase, similar in function to R2D2 and TRBP, which are associated with Dicer and Ago2 in the *Drosophila* and human RISC complexes, respectively[21,22,37]. In *Drosophila*, siRNA duplex generated by Dicer-2 first associates with the Dicer-2–R2D2 heterodimer, which orients the siRNA duplex such that the 5′ end with the lower melting temperature is retained as the guide strand in the mature RISC complex[38]. However, we have not been able to detect an interaction between ARC and Dcr1. Moreover, thermodynamic rules for siRNA strand selection do not seem to operate in *S. pombe*[39]. Furthermore, unlike R2D2 and TRBP, Arb1 and Arb2 have no obvious double-stranded RNA–binding domain. Arb1 shares similarity to organellar maturases, which facilitate the self-splicing of introns that encode them, suggesting that it may contain RNA-binding activity (**Supplementary Fig. 2**). Although Arb2 lacks similarity to previously defined functional domains, it seems to be conserved from fission yeast to human, suggesting that Arb2-like proteins may have a conserved role in regulation of the Argonaute family of proteins.

The binding of Tas3 and Chp1 to Ago1 seems to be mutually exclusive with that of Arb1 and Arb2 to Ago1. Thus, Ago1 may shuttle between its binding partners in the ARC and RITS complexes but can be targeted to chromatin only through base-pairing interactions using single-stranded siRNAs in the RITS complex (**Figs. 6** and **7**). How Ago1 might shuttle between these two complexes remains unknown. Our analysis of endogenous siRNAs associated with Ago1 complexes *in vivo* demonstrates that slicer activity is required for the conversion

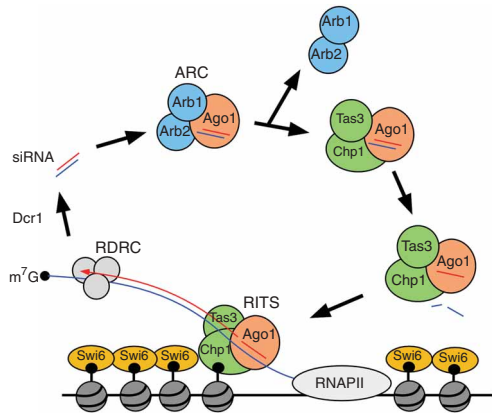

Figure 7 Model for the roles of ARC and RITS complexes. As slicer activity of Ago1 is required for conversion of double-stranded siRNA to single-stranded siRNA, model proposes that Arb1 and Arb2 inhibit slicer activity of Ago1. This explains presence of double-stranded siRNAs in ARC. Arb1 and Arb2, like Ago1 and other RNAi components, are required for siRNA generation and heterochromatin assembly and, in addition to inhibiting slicer, may function in the transfer of duplex siRNA from Dcr1 to Ago1. Black lollipops on nucleosomes (gray circles) represent histone H3-K9 methyl groups to which chromodomain proteins Chp1 and Swi6 bind. See text for details.

of double-stranded siRNA to single-stranded siRNA and thus for the formation of a mature RITS complex. Furthermore, the Ago1(D580A) mutant, which is trapped with siRNA duplex, shows reduced localization to centromeric repeats, further solidifying the importance of Watson-Crick base-pairing interactions for RITS localization to sites of heterochromatin formation. Lastly, expression of a catalytically inactive Argonaute is detrimental to silencing in a wild-type background, as it is likely that mutant Ago1 can titrate away and trap available duplex siRNAs. Two important conclusions can be drawn from this observation and the analysis of mutant cells carrying catalytically inactive Ago1. First, these results suggest that Ago1-mediated cleavage of the siRNA passenger strand is the primary pathway of RITS activation and that the silencing defect of the slicer mutant is due to the inability to eject passenger-strand siRNA, making siRNA-dependent recruitment of the mutant Ago1 to its target impossible. Second, the observation that catalytically inactive Ago1 strongly blocks the spreading of Chp1 and H3-K9 methylation into transgene sequences establishes a role for the base-pairing of siRNAs with target sequences in the heterochromatin spreading mechanism. It remains possible that Ago1 slicer activity is required for events further downstream in heterochromatin-dependent silencing—for example, slicing of nascent centromeric transcripts[27]—but this cannot be concluded yet, because slicer activity is required for the more upstream step of siRNA maturation.

Finally, it is not immediately obvious why slicer activity, a step required for siRNA maturation, is inhibited in ARC. The idea that Arb1 and Arb2 are negative inhibitors of Ago1 function can be ruled out in its simplest form, as both proteins, like other RNAi components, are required for RNAi-mediated heterochromatin assembly. Thus, one possibility is that unchecked Ago1 slicer activity, associated with a precursor complex, inhibits heterochromatin formation. For example, cleavage of nascent centromeric RNA by ARC may prevent the RNA from acting as an assembly platform for the RITS complex, which also associates with chromatin and is thought to recruit chromatin-modifying factors to initiate heterochromatin formation[12,14,26]. The inhibition of slicer activity in ARC would prevent such a potentially destructive association. As RITS also contains duplex siRNA in addition to single-stranded siRNA (**Figs. 4, 5** and **7**), the kinetics of target cleavage in RITS may be slow. We propose that the slow kinetics of the slicer reaction may allow RITS to remain bound to nascent precursor mRNA long enough to recruit chromatin-modifying enzymes and to become tethered to chromatin through the binding of the chromodomain of its Chp1 subunit to methylated H3-K9.

METHODS

Strain construction. *S. pombe* strains used in this study are described in **Supplementary Table 3**. All strains were constructed using a PCR-based gene-targeting method[14,40]. Primer sequences are available on request. Positive transformants were selected for growth on medium containing 100–200 μg ml^{-1} antibiotic (nourseothricin or G418) and confirmed by PCR. The Arb2 CDS, cloned from a complementary DNA library, differs from the published annotation. It is included in **Supplementary Methods** online.

Plasmid construction. A double-stranded DNA oligo encoding the 3×Flag sequence (5′-ATGGATTATAAAGATGACGATGACAAGGATTATAAAGATGACGATGACAAGGATTATAAAGATGACGATGACAAG-3′) flanked by 5′-PacI and 3′-AscI sites was ligated into the PacI and AscI sites of pREP1NT to generate pREP1-3×Flag[41]. The same oligo was also ligated into PacI and AscI of pFA6-natR-P3nmt1 (refs. 14,40) to generate pFA6-natR-P3nmt1-3×Flag, a plasmid used as a template for N-terminal Flag-tagging. The coding sequences of Ago1 and Arb1 were cloned into the AscI and NotI sites of pREP1-3×Flag to generate pREP1-3×Flag-Ago1 and pREP1-3×Flag-Arb1. Mutagenesis was done using the QuikChange kit (Stratagene) according to the manufacturer's instructions.

Protein affinity purification. Tas3-TAP and Arb1-TAP purifications were done as described[12] with the following modifications. Arb1-TAP was fully functional for silencing (**Supplementary Fig. 1**). Rich medium (5 l, containing 5 g l^{-1} yeast extract, 30 g l^{-1} dextrose and 0.225 g l^{-1} each of adenine, leucine, lysine, histidine and uracil) was inoculated with 10–15 ml of a saturated overnight culture and grown to an A_{600} of 2–3. Cells were spun at room temperature and washed once with distilled water. The cell pellet was then resuspended in 0.25 volumes lysis buffer (50 mM HEPES (pH 7.6), 300 mM potassium acetate, 5 mM magnesium acetate, 20 mM β-glycerol phosphate, 1 mM EGTA, 1 mM EDTA, 0.1% (v/v) Nonidet P-40 (NP-40)) containing protease inhibitors. The suspension was then frozen by directly dropping it into liquid nitrogen. Frozen cells were lysed by grinding in a coffee grinder with dry ice for 5 min[12]. After sublimation of dry ice, 1 volume lysis buffer was added, and the extract was spun at 4,000*g* for 10 min. Lysates were then transferred to a 50-ml falcon tube and incubated with 200–400 μl of pre-washed IgG Sepharose 6 Fast Flow beads (Amersham Biosciences) for 2–3 h at 4 °C. The beads and immobilized proteins were harvested by centrifugation at 500*g*, loaded on a Bio-Rad polyprep column and washed three times with 10 ml lysis buffer. Bound protein was eluted with 1 ml tobacco etch virus (TEV) protease cleavage buffer containing 30 units of TEV (Invitrogen) at 4 °C overnight. The TEV eluate was transferred to a 3-ml Bio-Rad polyprep column and bound to 200 μl of calmodulin-Sepharose beads (Amersham biosciences) for 2 h at 4 °C, and the bound protein was eluted as previously described[14]. Purification of Flag-tagged proteins was performed as above, except the extract was incubated with anti–Flag-M2 agarose (Sigma), washed with lysis buffer, then washed with a buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 0.25% (v/v) NP-40, 5% (v/v) glycerol and 0.1 mg ml^{-1} HA dipeptide, and eluted with the same buffer with 0.2 mg ml^{-1} 3×Flag peptide (Sigma).

Silencing assays. Silencing assays were done as described[26].

Chromatin immunoprecipitation. ChIP was done as described[42]. Antibodies used were the following: Swi6 (Abcam no. 14898), dimethylated H3-K9 (Upstate no. 07-212 in **Fig. 1** and Abcam no. ab1220 in **Fig. 6**) and Chp1 (Abcam no. ab18191). For the immunoprecipitation of TAP-tagged proteins, IgG Sepharose 6 Fast Flow beads (Amersham Biosciences) were used.

Immunofluorescence localization. Indirect immunofluorescence staining was conducted using thirteen Myc epitope–tagged strains as described[14].

Affinity purification and protein identification. For the characterization of Ago1- and Abr1-interacting proteins, the Flag or TAP eluates were precipitated with trichloroacetic acid and whole protein mixtures were analyzed after in-solution digestion, by liquid chromatography MS/MS (LC-MS/MS). Peptides were separated across a 50-min gradient ranging from 7% to 30% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid in a microcapillary (125 μm × 18 cm) column packed with C_{18} reverse-phase material (Magic C18AQ, 5 μm particles, 200 Å pore size, Michrom Bioresources) and on-line analyzed on a hybrid linear ion trap–Orbitrap mass spectrometer (LTQ-Orbitrap, Thermo-Electron). For each cycle, one full MS scan acquired on the Orbitrap at high mass resolution was followed by ten MS/MS spectra on the linear ion trap from the ten most abundant ions. MS/MS spectra were searched using Sequest algorithm. Peptide matches were filtered to <0.5% false positives using a target-decoy database strategy. Final lists of Ago1- and Arb1-interacting proteins were obtained by subtracting protein matches that were also found in an untagged control sample. Semiquantification of Ago1 in the two complexes was done indirectly by counting the number of MS/MS spectra for each of the subunits of ARC and RITS in purified Flag-Ago1 preparations, normalized by protein molecular weight[43,44], and assuming stoichiometric amounts for each protein subunit.

RNA analysis. Northern blots used to detect centromeric siRNA were done as described[26] with the following modifications. 20% (v/v) polyacrylamide Tris-borate EDTA gels were run at 4 °C for nondenaturing conditions. 5′ end–labeled 23-nt single-stranded RNA or 23-nt RNA duplex were run as position

markers. To detect RITS- and ARC-loaded siRNAs, RITS and ARC were purified by tandem affinity purification of Tas3-TAP or Arb1-TAP. siRNAs associated with wild-type or mutant Ago1 were purified by Flag purification of cells expressing Flag-Ago1 from a pREP1 plasmid. siRNAs were then recovered by phenol-chloroform extraction and ethanol precipitation. The entire sample was run on a gel, blotted and probed as described[26]. Gel signals were quantified by subtracting background signal intensity from intensities measured for single-stranded and double-stranded siRNA regions. The ratio of double- to single-stranded siRNAs was then calculated.

Recombinant protein expression and purification. *S. pombe* Ago1 was expressed as a GST fusion in *Escherichia coli* BL21 Codon Plus (DE3) RIL (Stratagene) from a pGEX-6P-1 plasmid. *Drosophila* Ago1 was expressed from a pGEX-5X-dAgo1 plasmid[25] (see Acknowledgments). The expression and purification of GST fusions was done as described[25].

***In vitro* target RNA cleavage assay.** *In vitro* target RNA cleavage assay, using *luc130* target RNA complementary to *luc* siRNA, was done as described[25] with the following modifications. *luc130* RNA was 5′ end–labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. To test the effect of Arb1 in slicer reactions, ~100 nM GST-Ago1 and Flag-Arb1 (or two-fold serial dilutions of Flag-Arb1) were incubated together for 1 h at room temperature before addition of 100 nM guide siRNA. After incubation for 1.5 h, the *luc130* target was added and the cleavage reaction was allowed to proceed for 3 h. Reactions were stopped by phenol-chloroform extraction followed by ethanol precipitation and analyzed on denaturing urea acrylamide gels.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

S.M.B., T.I., M.B. and J.V. carried out experiments; J.-I.N., S.P.G. and D.M. supervised research; S.B. and D.M. wrote the paper with input from all other authors.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Hannon, G.J. RNA interference. *Nature* **418**, 244–251 (2002).
2. Meister, G. & Tuschl, T. Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**, 343–349 (2004).
3. Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).
4. Matzke, M.A. & Birchler, J.A. RNAi-mediated pathways in the nucleus. *Nat. Rev. Genet.* **6**, 24–35 (2005).
5. Grewal, S.I. & Moazed, D. Heterochromatin and epigenetic control of gene expression. *Science* **301**, 798–802 (2003).
6. Martienssen, R.A., Zaratiegui, M. & Goto, D.B. RNA interference and heterochromatin in the fission yeast *Schizosaccharomyces pombe*. *Trends Genet.* **21**, 450–456 (2005).
7. Carmell, M.A., Xuan, Z., Zhang, M.Q. & Hannon, G.J. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev.* **16**, 2733–2742 (2002).
8. Song, J.J., Smith, S.K., Hannon, G.J. & Joshua-Tor, L. Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* **305**, 1434–1437 (2004).
9. Liu, J. *et al.* Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**, 1437–1441 (2004).
10. Reinhart, B.J. & Bartel, D.P. Small RNAs correspond to centromere heterochromatic repeats. *Science* **297**, 1831 (2002).
11. Volpe, T.A. *et al.* Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**, 1833–1837 (2002).
12. Verdel, A. *et al.* RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* **303**, 672–676 (2004).
13. Bernstein, E., Caudy, A.A., Hammond, S.M. & Hannon, G.J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366 (2001).
14. Motamedi, M.R. *et al.* Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. *Cell* **119**, 789–802 (2004).
15. Noma, K. *et al.* RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat. Genet.* **36**, 1174–1180 (2004).
16. Sijen, T. *et al.* On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**, 465–476 (2001).
17. Dalmay, T., Hamilton, A., Rudd, S., Angell, S. & Baulcombe, D.C. An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101**, 543–553 (2000).
18. Lingel, A., Simon, B., Izaurralde, E. & Sattler, M. Structure and nucleic-acid binding of the *Drosophila* Argonaute 2 PAZ domain. *Nature* **426**, 465–469 (2003).
19. Song, J.J. *et al.* The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. *Nat. Struct. Biol.* **10**, 1026–1032 (2003).
20. Yan, K.S. *et al.* Structure and conserved RNA binding of the PAZ domain. *Nature* **426**, 468–474 (2003).
21. Liu, Q. *et al.* R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. *Science* **301**, 1921–1925 (2003).
22. Gregory, R.I., Chendrimada, T.P., Cooch, N. & Shiekhattar, R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* **123**, 631–640 (2005).
23. Matranga, C., Tomari, Y., Shin, C., Bartel, D.P. & Zamore, P.D. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**, 607–620 (2005).
24. Rand, T.A., Petersen, S., Du, F. & Wang, X. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* **123**, 621–629 (2005).
25. Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H. & Siomi, M.C. Slicer function of *Drosophila* Argonautes and its involvement in RISC formation. *Genes Dev.* **19**, 2837–2848 (2005).
26. Buhler, M., Verdel, A. & Moazed, D. Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. *Cell* **125**, 873–886 (2006).
27. Irvine, D.V. *et al.* Argonaute slicing is required for heterochromatic silencing and spreading. *Science* **313**, 1134–1137 (2006).
28. Jia, S., Noma, K. & Grewal, S.I. RNAi-independent heterochromatin nucleation by the stress-activated ATF/CREB family proteins. *Science* **304**, 1971–1976 (2004).
29. Petrie, V.J., Wuitschick, J.D., Givens, C.D., Kosinski, A.M. & Partridge, J.F. RNA interference (RNAi)-dependent and RNAi-independent association of the Chp1 chromodomain protein with distinct heterochromatic loci in fission yeast. *Mol. Cell. Biol.* **25**, 2331–2346 (2005).
30. Sadaie, M., Iida, T., Urano, T. & Nakayama, J. A chromodomain protein, Chp1, is required for the establishment of heterochromatin in fission yeast. *EMBO J.* **23**, 3825–3835 (2004).
31. Carmichael, J.B. *et al.* RNA interference effector proteins localize to mobile cytoplasmic puncta in *Schizosaccharomyces pombe*. *Traffic* **7**, 1032–1044 (2006).
32. Rivas, F.V. *et al.* Purified Argonaute2 and an siRNA form recombinant human RISC. *Nat. Struct. Mol. Biol.* **12**, 340–349 (2005).
33. Rand, T.A., Ginalski, K., Grishin, N.V. & Wang, X. Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. *Proc. Natl. Acad. Sci. USA* **101**, 14385–14389 (2004).
34. Ma, J.B., Ye, K. & Patel, D.J. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature* **429**, 318–322 (2004).
35. Hong, E.J., Villen, J., Gerace, E.L., Gygi, S.P. & Moazed, D.A. Cullin E3 ubiquitin ligase complex associates with Rik1 and the Clr4 histone H3–K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. *RNA Biol.* **2**, 106–111 (2005).
36. Iida, T., Kawaguchi, R. & Nakayama, J. Conserved ribonuclease, Eri1, negatively regulates heterochromatin assembly in fission yeast. *Curr. Biol.* **16**, 1459–1464 (2006).
37. Chendrimada, T.P. *et al.* TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**, 740–744 (2005).
38. Tomari, Y., Matranga, C., Haley, B., Martinez, N. & Zamore, P.D. A protein sensor for siRNA asymmetry. *Science* **306**, 1377–1380 (2004).
39. Cam, H.P. *et al.* Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. *Nat. Genet.* **37**, 809–819 (2005).
40. Bahler, J. *et al.* Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast* **14**, 943–951 (1998).
41. Tasto, J.J., Carnahan, R.H., McDonald, W.H. & Gould, K.L. Vectors and gene targeting modules for tandem affinity purification in *Schizosaccharomyces pombe*. *Yeast* **18**, 657–662 (2001).
42. Huang, J. & Moazed, D. Association of the RENT complex with nontranscribed and coding regions of rDNA and a regional requirement for the replication fork block protein Fob1 in rDNA silencing. *Genes Dev.* **17**, 2162–2176 (2003).
43. Liu, H., Sadygov, R.G. & Yates, J.R., III. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* **76**, 4193–4201 (2004).
44. Sanders, S.L., Jennings, J., Canutescu, A., Link, A.J. & Weil, P.A. Proteomics of the eukaryotic transcription machinery: identification of proteins associated with components of yeast TFIID by multidimensional mass spectrometry. *Mol. Cell. Biol.* **22**, 4723–4738 (2002).