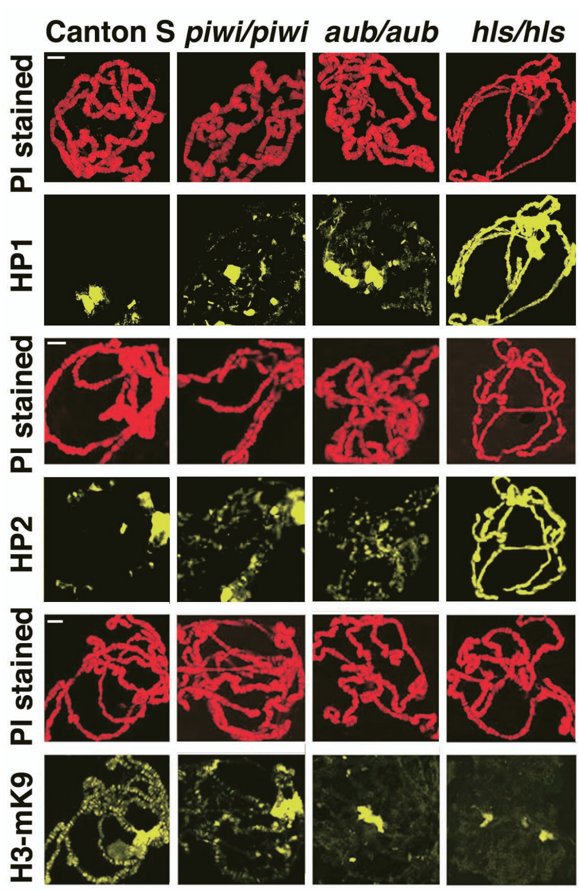

Fig. 4. Mutations in components of the RNAi system result in a loss of histone H3-mK9, and a delocalization of heterochromatin proteins HP1 and HP2. Polytene chromosomes (prepared as in Fig. 3) were treated with rabbit polyclonal primary antibodies specific to HP1, HP2, or histone H3-mK9, as specified, and with antibodies against the female specific protein, Sex-lethal, used to distinguish mutant from wild-type chromosomes. Antibodies were applied to mixtures of Canton S wild type with $piwi^{1}/piwi^{2}$, $aub^{QC42}/\Delta P\text{-}3a$, or hls^{E1}/hls^{E616} glands; $piwi^{1}/piwi^{1}$, hls^{E1}/hls^{DE8}, and $hls^{E1}/hls^{\Delta 125}$ showed similar results. In the supporting online material, adjacent nuclei on the same slide, but of different genotype, are presented for each comparison (figs. S2 to S4). The level of H3 methylated at Lys^{9} is progressively reduced, both at heterochromatic and euchromatic sites, in the *piwi/piwi*, *aub/aub*, and *hls/hls* lines, with a progressive delocalization of HP1 and HP2. Scale bar, 10 μm.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/303/5658/669/DC1
Materials and Methods
Figs. S1 to S5
References and Notes

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RNAi-Mediated Targeting of Heterochromatin by the RITS Complex

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RNA interference (RNAi) is a widespread silencing mechanism that acts at both the posttranscriptional and transcriptional levels. Here, we describe the purification of an RNAi effector complex termed RITS (RNA-induced initiation of transcriptional gene silencing) that is required for heterochromatin assembly in fission yeast. The RITS complex contains Ago1 (the fission yeast *Argonaute* homolog), Chp1 (a heterochromatin-associated chromodomain protein), and Tas3 (a novel protein). In addition, the complex contains small RNAs that require the Dicer ribonuclease for their production. These small RNAs are homologous to centromeric repeats and are required for the localization of RITS to heterochromatic domains. The results suggest a mechanism for the role of the RNAi machinery and small RNAs in targeting of heterochromatin complexes and epigenetic gene silencing at specific chromosomal loci.

The fission yeast *Schizosaccharomyces pombe* contains large stretches of heterochromatin that are associated with telomeres, repetitive DNA elements surrounding centromeres, and with the silent mating-type loci (*1*). Assembly of heterochromatin at these loci involves an orchestrated array of chromatin modifications that lead to the recruitment of two chromodomain histone-binding proteins Swi6, a homolog of the *Drosophila* and mammalian HP1 proteins, and Chp1 (*2*, *3*). The RNAi pathway has also been implicated in regulation at the DNA and chromatin level in *Arabidopsis* (*4*–*6*), *Drosophila* (*7*), and *Tetrahymena* (*8*), and in heterochromatin assembly in *S. pombe* (*9*, *10*).

RNAi silencing is triggered by double-stranded RNA (dsRNA), which is cleaved by the ribonuclease III (RNase III)–like enzyme Dicer to generate small RNA molecules of ~22 nucleotides (nt) (*11*–*13*). These small interfering RNAs (siRNAs), load onto an effector complex called RISC (RNA-induced silencing complex) that contains an Argo-

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naute/PIWI family protein and targets cognate mRNAs for inactivation (*12–15*).

Factors involved in the RNAi pathway in other organisms are required for heterochromatin formation in *S. pombe*. Deletion of any of these factors, such as Dicer (*dcr1*$^{+}$), Argonaute (*ago1*$^{+}$), and RNA-dependent RNA polymerase (*rdp1*$^{+}$), disrupts heterochromatin assembly (*9*, *10*). In support of a role for RNAi in heterochromatin assembly, both DNA strands of the *S. pombe* centromeric repeats are transcribed (*9*), and siRNAs have been identified that match the *S. pombe* centromeric repeats (*16*). Moreover, recent experiments suggest that artificial generation of dsRNA from a hairpin construct can silence homologous sequences by heterochromatin formation in an RNAi-dependent manner

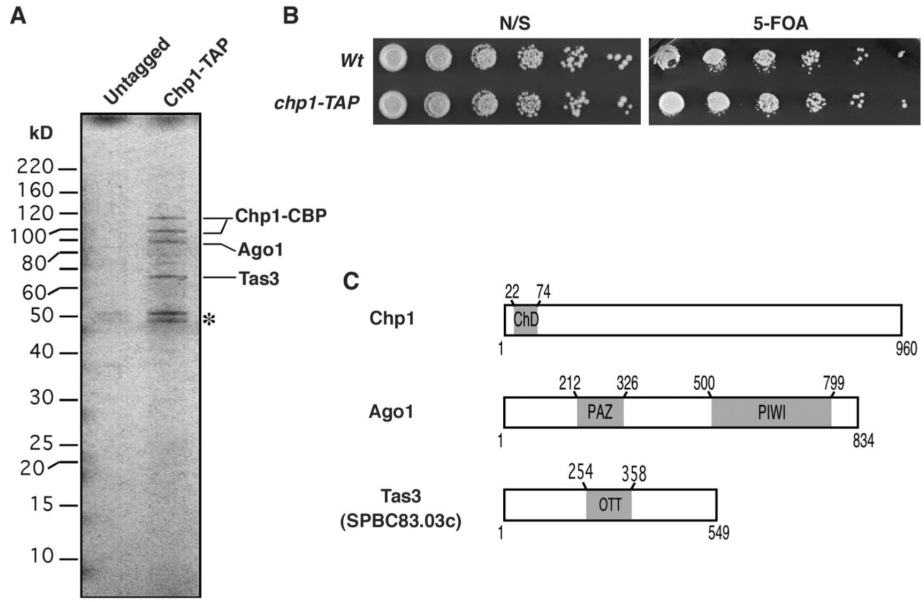


Fig. 1. Purification of Chp1-TAP and identification of associated proteins. Extracts from a Chp1-TAP strain and an untagged control strain were purified by the TAP procedure and applied to a 4 to 12% polyacrylamide gel, which was stained with colloidal Coomassie blue (**A**). The bands in the Chp1-TAP purification were excised from the gel and sequenced by tandem mass spectrometry (*22*). The identity of each band is based on multiple sequenced peptides and is indicated on the right. *Residual GST-TEV, the protease used for elution from the first affinity column. (**B**) The Chp1-TAP protein was fully functional for silencing of a centromeric *imr::ura4*$^{+}$ reporter gene as indicated by wild-type levels of growth on 5-FOA medium, which only allows growth when *ura4*$^{+}$ is silenced. N/S, nonselective medium. (**C**) Schematic diagram showing the subunits of the RITS complex and their conserved motifs. The chromodomain (ChD) in Chp1, the PAZ and PIWI domains in Ago1, and a region of sequence similarity between Tas3 and the mouse OTT (ovary testis transcribed) protein are indicated.

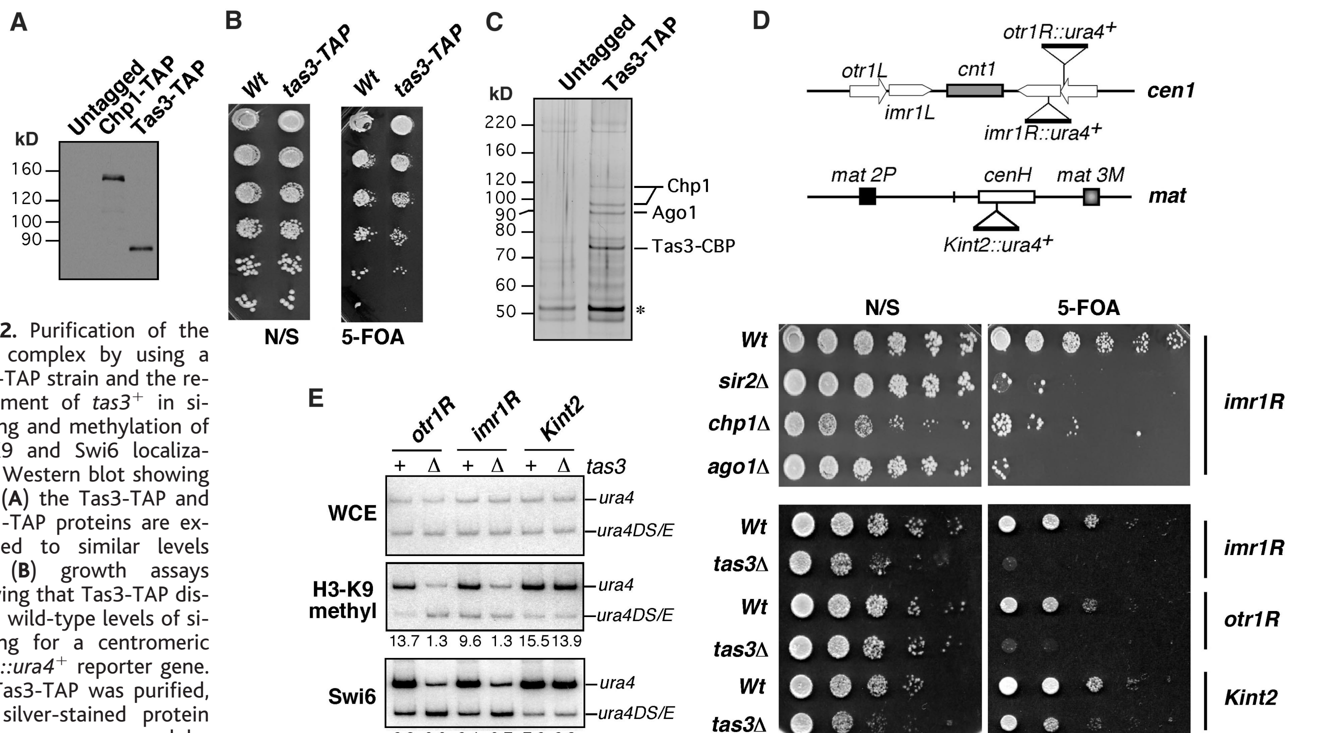


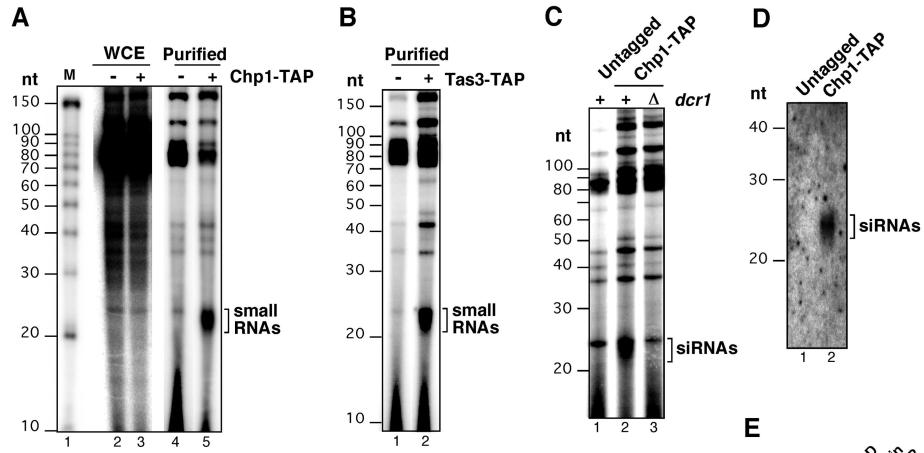
Fig. 2. Purification of the RITS complex by using a Tas3-TAP strain and the requirement of *tas3*$^{+}$ in silencing and methylation of H3-K9 and Swi6 localization. Western blot showing that (**A**) the Tas3-TAP and Chp1-TAP proteins are expressed to similar levels and (**B**) growth assays showing that Tas3-TAP displays wild-type levels of silencing for a centromeric *imrIR::ura4*$^{+}$ reporter gene. (**C**) Tas3-TAP was purified, and silver-stained protein bands were sequenced by tandem mass spectrometry. *GST-TEV. (**D**) In *tas3*Δ cells, silencing of a *ura4*$^{+}$ reporter gene inserted at the centromeric repeats (*imr1R::ura4*$^{+}$ and *otr1R::ura4*$^{+}$) is lost, but silencing of the same reporter gene at the silent mating-type interval (*Kint2::ura4*$^{+}$) is unaffected. Loss of silencing in *sir2*Δ, *chp1*Δ, and *ago1*Δ is shown for comparison. Loss of silencing results in loss of growth on counterselective 5-FOA medium. (**E**) ChIP experiments showing that in *tas3*Δ cells methylation of histone H3-K9 and localization of Swi6 to a *ura4*$^{+}$ reporter gene inserted at *otr1R* and *imr1R* centromeric repeats is abolished. In contrast, deletion of *tas3*$^{+}$ has little or no effect on H3-K9 methylation and Swi6 localization (*Kin2::ura4*$^{+}$). ChIP analysis and quantification were performed as described previously (*26*). The ratios of *ura4*$^{+}$ or *cen* signals to *ura4DS/E*-minigene signal present in the immunoprecipitated DNA (ChIP) and whole-cell extracts (WCE) were used to calculate fold enrichment shown underneath each lane.

(*17*). Here, we address the key question of how small RNAs generated by the RNAi machinery initiate heterochromatin assembly in fission yeast.

To identify factors important for RNAi-mediated targeting of heterochromatin complexes, we reasoned that such factor(s) would act in early steps in heterochromatin assembly and would be required for the establishment of heterochromatin-specific histone modification patterns. The Chp1 protein binds to centromeric repeats and is required for methylation of histone H3-K9 and for localization of Swi6 (*3*, *18*). Moreover, the phenotypes displayed by *chp1*Δ strains are identical to RNAi mutants. To test whether Chp1 provides a physical and functional link between RNAi and heterochromatin assembly, we used a tandem affinity purification procedure (TAP) and a TAP tag to identify factors that interact with Chp1 (Fig. 1). Several protein species of about 65, 90, 100, and 120 kD were specifically purified from the Chp1-TAP strain (Fig. 1A). Mass spectrometry of excised gel bands, as well as protein mixtures, identified the 120- and 100-kD bands as Chp1, the 90-kD band as Ago1, and the 65-kD band as SPBC83.03c, a previously uncharacterized protein (Fig. 1, A and C; table S1; figs. S1 and S2), which we named Tas3 (targeting complex subunit 3). The ratio of the 120- and 100-kD bands varies from experiment to experiment, which suggests that the 100-kD protein is a degradation product of Chp1.

To verify that Chp1, Ago1, and Tas3 are associated together in a complex, we constructed an *S. pombe* strain that produced a fully functional Tas3-TAP protein (Fig. 2, A and B). Affinity purification followed by mass spectrometry sequencing identified Ago1 and Chp1 as Tas3-associated proteins (Fig. 2C, table S1). N- or C-terminally tagged Ago1 proteins were not functional in centromeric silencing and were not used for purification experiments. However, identical purification profiles of Chp1-TAP and Tas3-TAP suggests that Chp1, Ago1, and Tas3 are associated together in a complex, which we have named RITS.

Chp1, as well as Ago1 and other components of the RNAi pathway, have previously been shown to be required for the assembly of heterochromatin and silencing of reporter genes inserted within heterochromatic domains (*9*, *10*, *19*, *20*). A *tas3* deletion strain carrying the $ura4^+$ reporter gene inserted at innermost (*imr*) or outermost (*otr*) centromeric repeats of chromosome 1 (*imr1R::ura4*$^+$ and *otr1R::ura4*$^+$, respectively) displayed a loss of silencing of both reporter genes (Fig. 2D) to an extent similar to that of the deletion of *sir2*, *chp1*, or *ago1* (Fig. 2D) (*9*, *10*, *19*, *21*). Further, chromatin immunoprecipitation (ChIP) showed that Tas3 was required for H3-K9 methylation and Swi6 localization of a $ura4^+$ reporter gene inserted at each of the above loci (Fig. 2E).



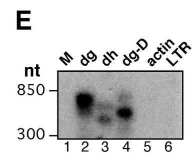
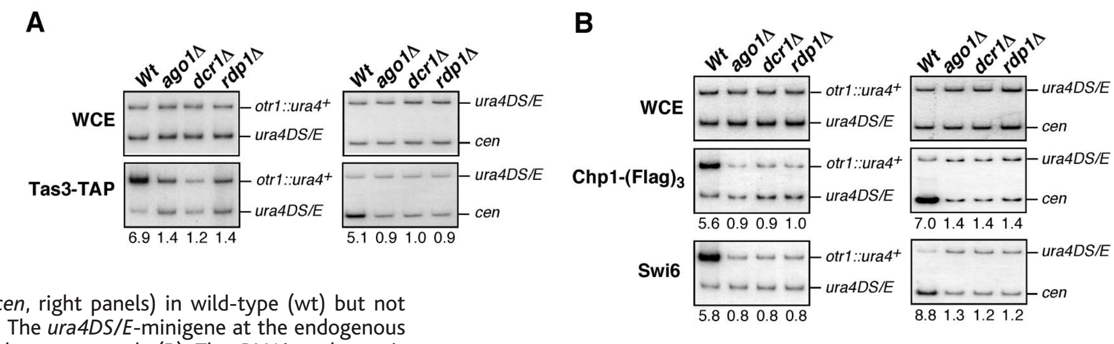


Fig. 3. Dicer-dependent association of RITS with siRNAs. (**A**) Small RNAs of ~22 to 25 nt copurify with Chp1-TAP. RNAs isolated from untagged control (–) and Chp1-TAP (+) strains were 3′ end-labeled with [5′-^{32}P]pCp and separated on 15% denaturing urea polyacrylamide gel. Lane 1, [γ-^{32}P]ATP–labeled RNA markers (Ambion); lanes 2 and 3, labeling of RNA from whole-cell extract (WCE) (~1/2500 of input); lanes 3 and 4, labeling of RNAs after purification. Bracket on the right side indicates the position of small RNAs specifically associated with Chp1-TAP. (**B**) Copurification of small RNAs with Tas3-TAP. (**C**) No small RNAs are associated with RITS purified from *dcr1*Δ cells. Parallel purifications were performed from an untagged (control, lane 1) strain as well as *chp1-TAP, dcr1*$^+$ (lane 2) and *chp1-TAP, dcr1*Δ (lane 3) cells, and the associated RNAs were [5′-^{32}P]pCp labeled (compare lanes 2 and 3, bracket). (**D**) Northern blot showing that siRNAs associated with RITS hybridize to ^{32}P-labeled probes corresponding to centromeric repeat sequences. RNA from untagged control (lane1) and Chp1-TAP cells (lane 2), purified as described in (B), was separated on a denaturing gel and electrotransferred to a nylon membrane (*22*). DNA oligonucleotides with sequence complementary to the 12 heterochromatic siRNAs identified by Reinhart and Bartel (*16*) were 5′ labeled with [γ-^{32}P]ATP and used as probes for the Northern blot. (**E**) Southern blot showing that RITS contains siRNAs complementary to the outer centromeric repeats (*otr*). *dg* (lanes 2 and 4) and *dh* (lane 3) repeats, *actin* (lane 5), and LTRs (lane 6) were amplified by polymerase chain reaction (PCR) from genomic DNA, separated on 1.1% agarose gel, and transferred to nylon membrane. ^{32}P-labeled RITS siRNAs, obtained by labeling RNAs as described in (A), were separated on a denaturing urea gel, eluted, and used as probes for the blot.

As is the case for RNAi mutants (*10*), deletion of $tas3^+$ had little or no effect on silencing or localization of H3-K9 methylation and Swi6 to the $ura4^+$ reporter gene inserted at the *mat* locus (*Kint2::ura4*$^+$) (Fig. 2, D and E). The similarity in phenotypes displayed by *tas3*Δ, *chp1*Δ, and RNAi mutants underscores the importance of Tas3 interaction with Chp1 and the role of the RITS complex in RNAi-mediated heterochromatin assembly.

Members of the Argonaute family of proteins constitute the core subunit of RISC, which is associated with small RNA molecules that target it to specific mRNAs (*12*, *13*). To determine whether the RITS complex is associated with small RNA molecules, we subjected Chp1-TAP or control purifications to phenol-chloroform extraction and precipitated the aqueous phase of the extraction containing any nucleic acid. The precipitated material was then labeled with [5′-^{32}P]pCp and T4 RNA ligase (*22*). As shown in Fig. 3A, Chp1-TAP is specifically associated with small RNA molecules ranging in size from ~22 to 25 nt. In contrast, the predominant RNA species prepared from a whole-cell extract (total RNA) are 70 to 100 nt in size, most likely representing transfer RNA (tRNA) and 5*S* RNA (Fig. 3A). RNA species, mainly in the size range of abundant tRNAs, as well as a small amount of an RNA species of ~25 nt, were present in both the untagged control and Chp1-TAP purification and represent nonspecific background binding (Fig. 3A, lanes 2 to 4). Similar results were obtained when the RITS complex was purified from a strain producing Tas3-TAP (Fig. 3B).

siRNAs are produced by the ribonuclease Dicer (*12*, *13*). We purified the RITS complex from a strain that carried a deletion of $dcr1^+$, the only *S. pombe* gene that codes for Dicer. Deletion of $dcr1^+$ resulted in a loss of small RNA species that specifically copurify with Chp1-TAP but had no effect on the presence of nonspecific RNA species, which were also present in the untagged control purification (Fig. 3C). These results indicated that the small RNA species specifically associated with RITS are siRNAs that are produced in a Dcr1-dependent manner.



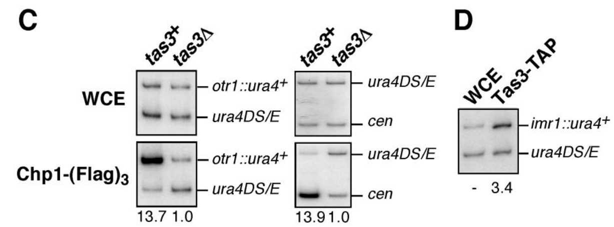


Fig. 4. The RNAi pathway is required for localization of RITS to heterochromatin. (**A**) ChIP experiments showing that Tas3-TAP is localized to centromeric heterochromatin in an RNAi-dependent manner. Tas3-TAP is associated with *ura4*$^+$ inserted at the *otr* centromeric repeats (*otr1::ura4*$^+$, left panels) and with native centromeric repeat sequences (*cen*, right panels) in wild-type (wt) but not *ago1*Δ, *dcr1*Δ, or *rdp1*Δ cells. The *ura4DS/E*-minigene at the endogenous euchromatic location is used as a control. (**B**) The RNAi pathway is required for the localization of Chp1-(Flag)$_3$ to centromeric heterochromatin. (**C**) Tas3 is required for the localization of Chp1-(Flag)$_3$ to heterochromatin. Immunoprecipitations were carried out using a Flag-specific antibody from *tas3*$^+$ and *tas3*Δ cells. (**D**) Tas3 is associated with *ura4*$^+$ inserted at the *imr* centromeric region (*imr1::ura4*$^+$). WCE, whole-cell extract. Fold enrichment values are shown underneath each lane.

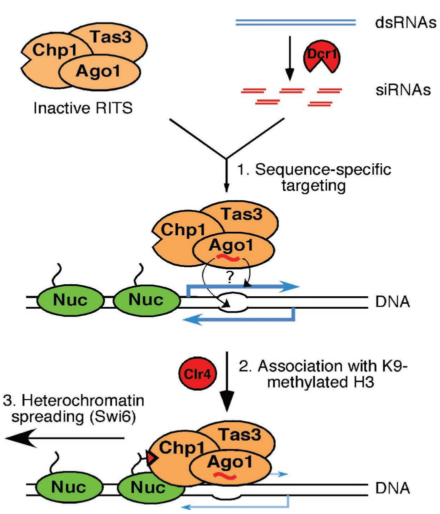


Fig. 5. A model for siRNA-dependent initiation of heterochromatin assembly by RITS. The RITS complex is programmed by Dcr1-produced siRNAs to target specific chromosome regions by sequence-specific interactions involving either siRNA-DNA or siRNA-nascent transcript (blue arrows) base pairing. Nuc, nucleosome; red triangle, K9-methylation on the amino terminus of histone H3. See text for further discussion and references.

Sequencing of small RNAs from *S. pombe* has identified a series of small RNA species that are complementary to the centromeric repeat sequences (*16*). These small RNAs have been termed heterochromatic siRNAs and are clustered at two regions within the centromeric repeats, the *dh* repeats and a region immediately downstream of the *dg* repeats. Centromeric siRNAs have been proposed to function in sequence-specific targeting of homologous DNA regions (i.e., centromeric repeats) for heterochromatin assembly. To determine whether siRNAs associated with RITS originate from centromeric repeats, we first analyzed RITS-associated RNAs on a Northern blot probed with a mixture of oligonucleotides derived from the centromeric repeats. These oligonucleotides were specifically designed to hybridize to siRNAs previously identified by Reinhart and Bartel (*16*). The ^{32}P-labeled oligonucleotide probes specifically hybridized to RNA species of ~22 to 25 nt in size present in the Chp1-TAP purification but not with nonspecific RNAs present in the untagged control purification (Fig. 3D).

As a second test for the identities of the siRNAs associated with RITS, we labeled RITS-associated siRNAs with [5′-^{32}P]pCp, then gel purified and used them to probe a Southern blot containing equal amounts of DNA fragments (ranging in size from 300 to 700 base pairs) corresponding to the *dg* and *dh* centromeric repeats, the region downstream of *dg* repeats to which siRNAs map (designated *dg-D*), retrotransposon long terminal repeats (LTRs) that have been shown to mediate RNAi-dependent gene silencing (*17*), and DNA fragments corresponding to actin and molecular size markers. The labeled siRNAs specifically hybridized to *dg*, *dh*, and *dg-D* centromeric sequences (Fig. 3E). No hybridization was detected to LTR, actin, or DNA size markers (Fig. 3E). Our inability to detect hybridization of RITS-associated siRNAs with LTR sequences may be due to a relatively lower abundance of LTR siRNAs compared with siRNAs that originate from the centromeric repeats. Together, these experiments show that RITS is associated with siRNAs that originate from processing of centromeric dsRNA transcripts.

We next used *S. pombe* strains that produced either Tas3-TAP or Chp1-Flag to determine the in vivo chromatin localization of the RITS complex and the requirement for the RNAi pathway in its localization. It has previously been shown that Chp1 localizes to the centromeric repeat regions and together with the Clr4 methyltransferase is required for H3-K9 methylation and Swi6 localization (*3*). ChIP experiments showed that Tas3-TAP is similarly localized to a *ura4*$^+$ reporter gene inserted within in the *otr* centromeric repeat region (*otr1::ura4*$^+$) and centromeric repeat sequences but not to the control mini-*ura4* (*ura4DS/E*) gene at the endogenous euchromatic location (Fig. 4). Tas3-TAP, like Chp1 (*18*), is also localized to the *imr* centromeric repeats (Fig. 4D). Furthermore, deletion of *ago1*$^+$, *dcr1*$^+$, or *rdp1*$^+$ abolished the association of Chp1-Flag and Tas3-TAP with *otr1::ura4*$^+$, as well as with centromeric repeat sequences (Fig. 4, A and B). These results indicated that the RNAi pathway is required for association of the Chp1 and Tas3 subunits of RITS with heterochromatic DNA regions. Our purification of the RITS complex from *dcr1*Δ cells showed that the protein subunits of the complex remained associated together in the absence of siRNAs (fig. S4). The purification results, together with the ChIP analysis, indicate that the "empty" RITS complex is inactive and can only associate with its chromosomal target after it is programmed by siRNAs.

We further tested whether Tas3 was required for the localization of Chp1-Flag to each of the above regions. Deletion of *tas3*$^+$ abolished the association of Chp1-Flag with *otr1::ura4*$^+$, as well as with native *cen* sequences (Fig. 4C). These results support the biochemical identification of Tas3 as an integral subunit of RITS and indicate that it plays an essential role in localizing the complex to heterochromatin.

Our analysis suggests a remarkably direct role for the RNAi machinery in heterochromatin assembly. By analogy to RISC complexes, which use small RNAs as guides to target specific mRNAs for degradation or translational repression, we propose that RITS uses siRNAs to recognize and to bind to specific chromosome regions so as to initiate heterochromatic gene silencing (Fig. 5). Four lines of evidence support this view. First, RITS contains Ago1, the *S. pombe* homolog of the Argonaute family of proteins, which form the common subunit of RISC complexes purified from different organisms and are thought to be directly responsible for target recognition (*12*). Second, RITS is associated with siRNAs that require Dcr1 for their formation and originate from heterochromatin repeat regions. Thus, this complex contains the expected specificity determinants, i.e., siRNAs, which in other systems have been shown to direct target recognition (*14*, *15*, *23*, *24*). Third, at least two subunits of the RITS complex, Chp1 and Tas3, are specifically associated with the expected heterochromatic DNA regions, which suggests that the complex localizes directly to its target DNA. Fourth, in addition to Ago1, RITS contains a chromodomain protein, Chp1, which is localized throughout heterochromatic DNA regions (*18*) (Fig. 4) and requires the methyltransferase Clr4 and histone H3-K9 methylation for localization to chromatin (*3*, *18*). Thus, RITS contains both a subunit (Ago1) that binds to siRNAs and can function in RNA or DNA targeting by sequence-specific pairing interaction and a subunit (Chp1) that associates with specifically modified histones and may be involved in further stabilizing its association with chromatin (Fig. 5).

Mechanisms analogous to the RITS-mediated targeting of heterochromatin complexes are likely to be conserved in other systems. For example, in *Tetrahymena*, genomewide DNA elimination during macronucleus development requires an Argonaute family protein, Twi1, and a chromodomain protein, Pdd1, both of which are also required for H3-K9 methylation and accumulation of small RNAs corresponding to target sequences (*8*, *25*). Similarly, in *Drosophila* repeat-induced transcriptional gene silencing requires an Argonaute family protein, Piwi, and a chromodomain protein, Polycomb (*7*). Our results support the hypothesis that Argonaute proteins form the core subunit of a number of different effector complexes that use sequence-specific recognition to target either RNA or DNA.

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Kinesin Walks Hand-Over-Hand

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Kinesin is a processive motor that takes 8.3-nm center-of-mass steps along microtubules for each adenosine triphosphate hydrolyzed. Whether kinesin moves by a "hand-over-hand" or an "inchworm" model has been controversial. We have labeled a single head of the kinesin dimer with a Cy3 fluorophore and localized the position of the dye to within 2 nm before and after a step. We observed that single kinesin heads take steps of 17.3 ± 3.3 nm. A kinetic analysis of the dwell times between steps shows that the 17-nm steps alternate with 0-nm steps. These results strongly support a hand-over-hand mechanism, and not an inchworm mechanism. In addition, our results suggest that kinesin is bound by both heads to the microtubule while it waits for adenosine triphosphate in between steps.

Conventional kinesin (referred to simply as kinesin) is a highly processive, dimeric motor that takes 8.3-nm steps along microtubules (*1–3*). Kinesin transports a variety of cargo, including membranous organelles, mRNA, intermediate filaments, and signaling molecules (*4*). Mutations in a neuron-specific conventional kinesin have been linked to neurological diseases in humans (*5*).

Kinesin is a homodimer with identical catalytic cores (heads) that bind to microtubules and adenosine triphosphate (ATP) (*6*). Each head is connected to a "neck-linker," a mechanical element that undergoes nucleotide-dependent conformational changes that enable motor stepping (*7*). The neck linker is in turn connected to a coiled coil that then leads to the cargo-binding domain (*8*). In order to take many consecutive steps along the microtubule without dissociating, the two heads must operate in a coordinated manner, but the mechanism has been controversial. Two models have been postulated: the hand-over-hand "walking" model in which the two heads alternate in the lead (*7*), and an inchworm model in which one head always leads (*9*).

The hand-over-hand model predicts that, for each ATP hydrolyzed, the rear head moves twice the center of mass, whereas the front head does not translate. For a single dye on one head of kinesin, this leads to a prediction of alternating 16.6-nm and 0-nm translation of the dye (Fig. 1A). In contrast, the inchworm model predicts a uniform translation of 8.3 nm for all parts of the motor, which is equal to the center-of-mass translation (Fig. 1A). In addition, each model makes predictions about rotation of the stalk. The inchworm model predicts that the stalk does not rotate during a step. A symmetric version of the hand-over-hand model, in which the kinesin-microtubule complex is structurally identical at the beginning of each ATP cycle, predicts that the stalk rotates 180 degrees, whereas an asymmetric hand-over-hand

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