RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production

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In fission yeast, factors involved in the RNA interference (RNAi) pathway including Argonaute, Dicer, and RNA-dependent RNA polymerase are required for heterochromatin assembly at centromeric repeats and the silent mating-type region. Previously, we have shown that RNA-induced initiation of transcriptional gene silencing (RITS) complex containing the Argonaute protein and small interfering RNAs (siRNAs) localizes to heterochromatic loci and collaborates with heterochromatin assembly factors via a self-enforcing RNAi loop mechanism to couple siRNA generation with heterochromatin formation. Here, we investigate the role of RNA-dependent RNA polymerase (Rdp1) and its polymerase activity in the assembly of heterochromatin. We find that Rdp1, similar to RITS, localizes to all known heterochromatic loci, and its localization at centromeric repeats depends on components of RITS and Dicer as well as heterochromatin assembly factors including Clr4/Suv39h and Swi6/HP1 proteins. We show that a point mutation within the catalytic domain of Rdp1 abolished its RNA-dependent RNA polymerase activity and resulted in the loss of transcriptional silencing and heterochromatin at centromeres, together with defects in mitotic chromosome segregation and telomere clustering. Moreover, the RITS complex in the *rdp1* mutant does not contain siRNAs, and is delocalized from centromeres. These results not only implicate Rdp1 as an essential component of a self-enforcing RNAi loop but also ascribe a critical role for its RNA-dependent RNA polymerase activity in siRNA production necessary for heterochromatin formation.

RNA interference | *Schizosaccharomyces pombe*

Heterochromatin plays an important role in the regulation of transcriptional repression, recombination and chromosome segregation (1). In the fission yeast *Schizosaccharomyces pombe*, large stretches of heterochromatin are present at centromeres, telomeres, and the silent mating-type (*mat*) region (1). Heterochromatin formation at these loci involves conserved pathways of histone modifications, including deacetylation and methylation on key histone residues. For example, Clr4, a mammalian Suv39 homolog, specifically methylates histone H3 on lysine 9 (H3–K9) at loci targeted for heterochromatin formation (2). H3–K9 methyl marks have been shown to act as binding sites for chromodomain proteins such as Swi6 (an HP1 homolog) (3).

RNA interference (RNAi) is a gene silencing mechanism that utilizes siRNAs generated from the cleavage of dsRNAs to target the destruction or translational inhibition of mRNAs (4). Genetic and biochemical studies from diverse species have revealed that an RNase III-type endonuclease, Dicer, processes dsRNAs into siRNAs, which in turn associate with RNA-induced silencing complex (RISC) to direct degradation of cognate mRNAs. RNAi factors have also been implicated in transposon suppression (5–7), programmed genome rearrangements (8), virus resistance (9, 10), germ line development (11), and stem cell maintenance (12).

The RNAi pathway also plays an important and conserved role in heterochromatin assembly and proper segregation of chromosomes (8, 13–20). Deletions of RNAi components including Argonaute (Ago1), Dicer (Dcr1), or RNA-dependent RNA polymerase (Rdp1) in *S. pombe* impair epigenetic silencing at centromeres and the initiation of heterochromatin assembly at the *mat* locus, resulting in a loss of H3–K9 methylation and Swi6 localization from these loci (13, 14). An RNAi effector complex (RITS) consisted of a chromodomain protein Chp1, Tas3, Ago1, and small interfering RNAs (siRNAs) was recently shown to be necessary for heterochromatin assembly (21). RITS localizes to all known heterochromatic loci and acts primarily *in cis* to promote transcriptional and posttranscriptional silencing (22). Importantly, a mutation in the Chp1 chromodomain that has been shown to bind methylated H3–K9, or deletion of H3–K9 methyltransferase *clr4*, not only prevents RITS from binding to chromatin but also leads to the loss of siRNAs copurifying with RITS (22). These analyses imply a self-enforcing loop mechanism in which siRNAs generated by the RNAi pathway help target heterochromatin proteins such as Clr4 to homologous sequences. Clr4-mediated H3–K9 methylation anchors RITS to chromatin, allowing the RNAi machinery to act in cis to process nascent transcripts and efficiently generate siRNAs for the targeting of heterochromatin complexes. As per this model, RITS may be a component of a larger RNAi complex assembled on chromatin, along with Rdp1 and Dcr1, which directly couples siRNA generation with heterochromatin assembly (22).

RNA-dependent RNA polymerases (RdRPs), which are believed to play an important role in the RNAi process, have been identified in several species including plants (23, 24), fungi (14, 25, 26), and *Caenorhabditis elegans* (11, 27), and are required for a variety of different cellular functions. For example, in *Arabidopsis* SDE1/SGS2/RDR6 is necessary for posttranscriptional gene silencing (PTGS) and virus resistance, and RDR2 is required for the production of siRNAs from endogenous transcripts (9, 10). In *C. elegans*, RRF-1 and EGO-1 are essential for secondary siRNA production from RNAi-targeted transcripts in somatic cells and germ-line development, respectively (11, 27), and Sad1 in *Neurospora crassa* is required for silencing unpaired DNA during meiosis (28). It is believed that RdRPs generate dsRNAs from single-stranded transcripts either by *de novo* second-strand synthesis or by relying on siRNAs to prime transcription (27, 29, 30). Thus, RdRP activity may initiate RNAi/PTGS and/or dramatically enhance RNAi response. However, no RdRP homologs have been identified in *Drosophila* and mammals, leading to the suggestion that RNAi can occur in the absence of RdRP activity.

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Abbreviations: RNAi, RNA interference; RISC, RNA-induced silencing complex; RITS, RNAi effector complex; siRNA, small interfering RNA; RdRP, RNA-dependent RNA polymerase; ChIP, chromatin immunoprecipitation; IF, immunofluorescence.

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In *S. pombe*, *rdp1* deletion results in loss of pericentric heterochromatin (14). However, it is not known whether the RdRP activity of Rdp1 is essential for heterochromatin formation. In this study, we demonstrated that Rdp1 possesses RdRP activity that is indispensable for centromeric silencing, heterochromatin assembly, chromosome segregation, and telomere clustering during mitosis. In addition, we found that components of RITS and heterochromatin machinery cooperate to recruit Rdp1 to centromeres. Our analyses suggest that Rdp1 is an essential component of a self-enforcing RNAi loop that couples the generation of siRNAs with heterochromatin assembly.

Materials and Methods

Strains and Culture Conditions. Standard conditions were used for growth, sporulation and tetrad analysis. *rdp1-3HA* and *rdp1-TAP* strains were prepared by PCR-based C-terminal tagging. To create *rdp1*^{D903A}*-3HA* and *rdp1*^{D903A}*-TAP* strains, a wild-type strain was transformed with a PCR-derived *rdp1* coding region containing a D903A mutation. Transformants were screened by PCR and the mutation was confirmed by sequencing.

Measurement of Rdp1 Activity. Whole cell extracts (WCEs) were prepared from cells overexpressing *rdp1-TAP* and *rdp1*^{D903A}*-TAP* under the control of *nmt1* promoter. Rdp1-TAP and Rdp1^{D903A}-TAP proteins were then affinity-purified by using IgG-Sepharose and Calmodulin-Sepharose. RdRP activity assay was performed as described (29) (M. Motamedi, A.V., S. Colmenares, and D.M., unpublished data).

Chromatin Immunoprecipitation (ChIP) and Immunofluorescence (IF). ChIP and IF were performed as described (31).

siRNA Purification and pCp Labeling. Chp1–3FLAG was purified by using FLAG immunoaffinity purification protocol as described (32). siRNAs in the purified fractions were recovered by phenol-chloroform extraction and ethanol precipitation. siRNAs were 3′-end-labeled with [5′-^{32}P]pCp by using T4 RNA ligase at 4°C for 24 h and resolved by electrophoresis on a 10% denaturing acrylamide gel (21). ^{32}P-labeled Decade Markers (Ambion) were used as RNA size markers.

Results and Discussion

Rdp1 Localizes to Constitutive Heterochromatic Domains. We have previously shown that Ago1 and other RITS components bind stably to all known constitutive heterochromatic domains in *S. pombe* genome including the *mat* locus, centromeres, and telomeres (21, 22). Likewise, Rdp1 has been found to associate with centromeres (14). We performed ChIPs to determine whether Rdp1 associates with other heterochromatic loci. Our analysis revealed that not only Rdp1 could readily be detected at centromeric repeats, it was preferentially enriched at *cenH*, the site of heterochromatin nucleation at the *mat* locus, and at telomeres (Fig. 1*A*). Based on this analysis, Rdp1 associates with known sites of heterochromatin formation in the fission yeast genome, similar to RITS components (22). Moreover, these data are consistent with our previous model that the RNAi machinery is a stable component of heterochromatin that processes "aberrant" transcripts into siRNAs for the targeting of heterochromatin complexes (22).

Rdp1 Localization at Centromeres Depends on the Heterochromatin and RNAi Machinery. Although the mechanisms of heterochromatin assembly at the *mat* locus and telomeres are genetically more complex, RNAi mutants display severe defects in heterochromatin assembly at centromeres (13, 14, 22, 33). To explore factors involved in the targeting of Rdp1 to heterochromatic loci, we investigated the effects of RITS components on Rdp1 localization at centromeres. ChIP analysis revealed that deletion

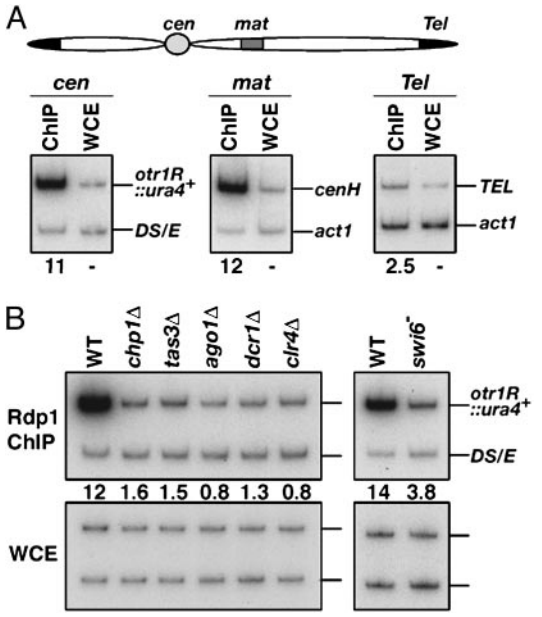

Fig. 1. Rdp1 localization at centromeres depends on RITS, Dcr1, and heterochromatin assembly factors. (*A*) Rdp1 associates with constitutive heterochromatic domains. Schematic diagrams show centromere (*cen*), telomeres (*TEL*), and the *mat* locus (*mat*) (see also Fig. 7, which is published as supporting information on the PNAS web site). Rdp1 localization at *ura4*$^+$ gene inserted at the outer centromeric repeat (*otr1R*::*ura4*$^+$), heterochromatin-nucleation center (*cenH*) at the *mat* locus, or telomere associated sequence (*TEL*) was tested by ChIP. Relative enrichment values are shown underneath each lane. Rdp1 enrichment at *TEL* was relatively lower compared to *otr1R*::*ura4*$^+$ and *cenH* but was reproducible. (*B*) ChIP analysis of Rdp1 at centromeric repeats in strains lacking one of RITS components, Dcr1, Clr4, or Swi6 protein.

of RITS components prevented the association of Rdp1 with centromeric chromatin (Fig. 1*B*). Dcr1-generated siRNAs are believed to be essential for locus-specific H3–K9 methylation, which promotes stable binding of RITS to chromatin (22). Loss of Dcr1 or Clr4 results in delocalization of the RITS complex from centromeres (21, 22). We asked whether Rdp1 localization at centromeres would be affected in the absence of Dcr1 or Clr4. As expected, loss of either Dcr1 or Clr4 completely abolished the recruitment of Rdp1 to centromeres. We also investigated the possible involvement of Swi6, a key component of centromeric heterochromatin, in Rdp1 localization at centromeres. Because Swi6 acts downstream of H3–K9 methylation, mutations in *swi6* do not cause any detectable change in H3–K9 methylation or binding of RITS to chromatin (2, 22). Interestingly, we found that Rdp1 localization at centromeres was severely compromised in a *swi6* mutant strain (Fig. 1*B*), suggesting that Swi6 could aide the localization of Rdp1 at centromeres. These results together with previous findings indicate that not only Rdp1 is required for RITS localization at centromeres (21), but conversely, Rdp1 depends on RITS to associate with centromeric chromatin, implying that Rdp1 and RITS might colocalize at centromeric loci together as a single unit.

Mutation Within the RdRP Domain of Rdp1 Abolishes Silencing and Heterochromatin Formation at Centromeres. Rdp1 along with other RNAi components regulates heterochromatin assembly at centromeres and the *mat* locus (13, 14). However, the exact function of Rdp1 in this process is not well understood. It is known that tomato and *Neurospora* Rdp1 homologs possess RdRP activity *in vitro*, and mutations of the catalytic RdRP domain abolish the RNAi response (23, 29). Alignment of amino acid sequences between *S. pombe* Rdp1 and its putative homologs in other

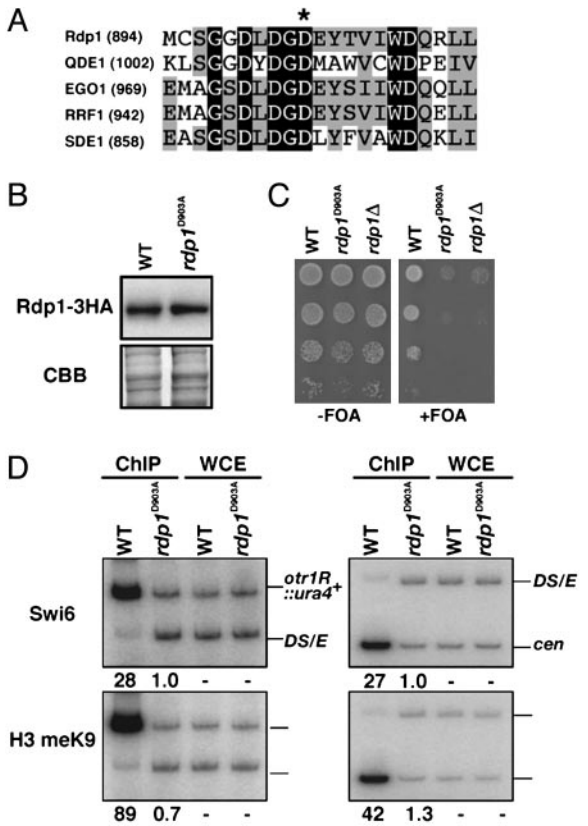

Fig. 2. Mutation of RdRP domain of Rdp1 abolishes silencing and heterochromatin formation at centromeres. (*A*) Protein sequence alignment of the catalytic domain of *S. pombe* Rdp1 and its homologs *N. crassa* QDE-1, *C. elegans* EGO-1, *C. elegans* RRF-1, and *A. thaliana* SDE1/SGS2. Black shading, invariant residues; gray shading, conserved residues. The aspartate residue essential for RdRP activity is marked with an asterisk. (*B*) Expression of Rdp1–3HA and Rdp1^{D903A}-3HA proteins. Whole cell extracts (WCE) from *rdp1–3HA* and *rdp1*^{D903A}*-3HA* strains were examined by Western blotting. (*Upper*) Western blotting using anti-HA antibody. (*Lower*) CBB staining. (*C*) Derepression of *otr1R*::*ura4*$^+$ in *rdp1*^{D903A} cells. Serial dilutions of wild-type and mutant strains containing *otr1R*::*ura4*$^+$ were spotted on nonselective (−FOA) or counterselective (+FOA) plates and incubated at 30°C for 3 days before photography. (*D*) ChIP analyses of Swi6 and H3–K9 methylation in *rdp1*^{D903A} mutant strain. Relative enrichment of Swi6 and H3–K9 methylation at *otr1R*::*ura4*$^+$ and centromeric repeats sequences (*cen*) adjacent to the *otr1R*::*ura4*$^+$ insertion site were analyzed by ChIP and are shown underneath each lane.

organisms revealed several conserved residues within the RdRP domain including a key aspartate (D903) residue known to be critical for the RdRP catalytic activity of *Neurospora* QDE-1 (29) (Fig. 2*A*). We determined the role of the RdRP catalytic domain of Rdp1 by mutating the corresponding aspartate residue to alanine (D903A). Immunoblotting assay showed that the protein level of the Rdp1 mutant (Rdp1^{D903A}) is comparable to that of wild-type Rdp1, suggesting that the D903A mutation does not affect the stability of the mutant protein (Fig. 2*B*).

We next investigated the effect of the D903A mutation on the function of Rdp1 in maintaining centromeric silencing and heterochromatin formation. Interestingly, *rdp1*^{D903A} mutant similar to *rdp1*Δ strain displayed a complete loss of centromeric silencing as indicated by the derepression of a *ura4*$^+$ reporter gene inserted at centromeric repeats (*otr1R*::*ura4*$^+$) in these two mutants (Fig. 2*C*). To test whether the loss of silencing in mutant cells is caused by defects in heterochromatin assembly at centromeres, we performed ChIPs to examine the status of H3–K9 methylation and Swi6 localization at centromeric repeats in *rdp1*^{D903A} cells. We found that similar to *rdp1*Δ cells localization of both methylated H3–K9 and Swi6 at centromeric *otr1R*::*ura4*$^+$ was severely affected in *rdp1*^{D903A} cells (Fig. 2*D*). These results demonstrated that the RdRP domain of Rdp1 is essential for the functioning of Rdp1 in heterochromatin assembly at centromeres.

***rdp1*^{D903A} Cells Display Defects in Proper Chromosome Segregation and Telomere Clustering.** RNAi-dependent heterochromatin formation is tightly linked to centromere function and proper segregation of chromosomes in *S. pombe*. Mutant strains that are defective in centromeric silencing usually exhibit lagging chromosomes, higher minichromosome loss rates, and increased sensitivity to microtubule-destabilizing drugs such as thiabendazole (TBZ) (15, 16, 34). Because *rdp1*^{D903A} mutant displayed silencing defect at centromeres (Fig. 2*C*), we assessed whether chromosome segregation was also disrupted in this mutant. As expected, serial dilution analysis confirmed that the *rdp1*^{D903A} mutation, like the *rdp1*Δ, rendered the cells hypersensitive to TBZ, indicating that chromosome segregation is not robust in these mutant cells (Fig. 3*A*). To directly observe the process of mitotic chromosome segregation in wild-type and mutant cells, we performed IF with anti-tubulin antibody to visualize microtubules and observed chromosomes by staining with DAPI. We found that *rdp1* mutants have a significantly higher percentage (≈20%) of cells with lagging chromosomes during late anaphase than in the wild-type strain (<1%) (Fig. 3*B*). These results demonstrated that the RdRP domain of Rdp1 is critical for Rdp1 role in heterochromatin formation as well as proper centromere function.

In *S. pombe*, redundant pathways mediate the formation of heterochromatin at telomeres. However, RNAi mutants including *rdp1*Δ are defective in telomere clustering, although silencing of marker genes inserted at telomeres is apparently intact (15). To test whether the D903A mutation would affect the role of Rdp1 in telomere clustering during mitosis, we performed co-IF of Swi6 with a telomere-binding protein Taz1. In wild-type cells, we observed one to five Swi6 foci, which colocalized with Taz1 (Fig. 3 *C* and *D*). In contrast, a noticeably larger fraction of *rdp1*^{D903A} cells exhibited an increased number of Swi6 foci, and most of these Swi6 foci still colocalized with Taz1, suggesting a declustering of telomeres even though the localization of telomeres to the nuclear periphery was unaffected (Fig. 3 *C* and *D*). These results indicate that mitotic telomere clustering depends on an intact RdRP domain of Rdp1. In light of our results showing Rdp1 localization at telomeres (Fig. 1*A*), together with previous findings of RITS presence at telomeres (22, 33), it is likely that the catalytic activity of Rdp1 is essential for the functioning of RNAi at telomeres. How a mutation in the RdRP domain of Rdp1 affects telomere clustering is unclear. It is possible that RNA intermediates produced by Rdp1 (see below) and the RNAi machinery at telomeres are essential for higher-order chromatin organization and telomeric clustering.

The RdRP Domain Confers Rdp1 with RNA-Dependent RNA Polymerase Activity That Is Essential for Generation of RITS-Associated siRNAs. To test whether Rdp1 has RdRP activity and whether the D903A substitution abolishes this activity, we affinity-purified Rdp1 proteins from Rdp1-TAP and Rdp1^{D903A}-TAP strains and measured their primer-independent (*de novo*) RNA polymerase activity. We detected three different RNA products in reactions containing wild-type Rdp1 (Fig. 4*A*; M. Motamedi, A.V., S. Colmenares, and D.M., unpublished data). It is thought that the 500-nt species derives from *de novo* second-strand RNA synthesis, and the smear bands >500 nt might be a product of a "back-priming" reaction in which the 3′ end of the template strand folds back to prime second-strand synthesis. The short 180-nt RNA band is probably a result of premature transcription termination. This profile of Rdp1 activity is similar to that of

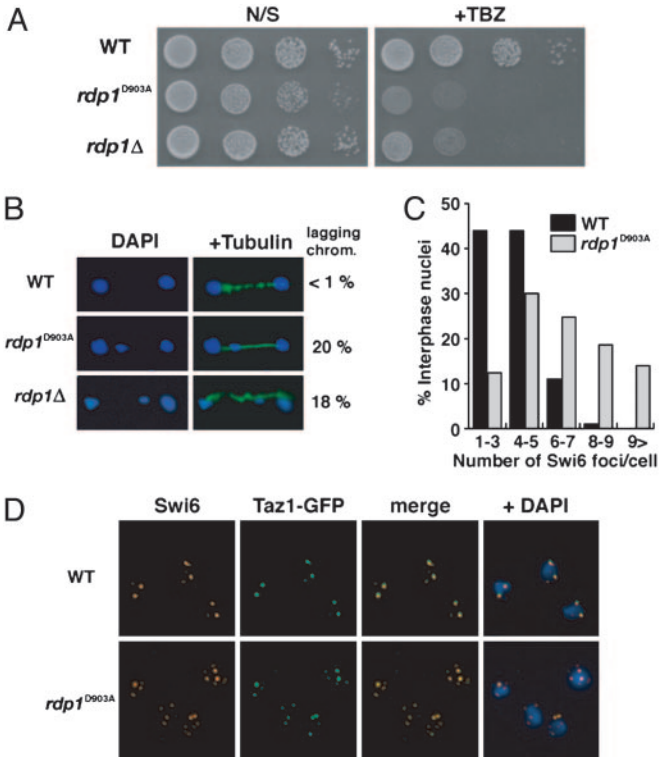

Fig. 3. *rdp1* mutants display defects in chromosome segregation and telomere clustering. (*A*) *rdp1* mutants are hypersensitive to TBZ. Wild-type and mutant strains were grown in YEA medium, and serial 10-fold culture dilutions were spotted on YEA (N/S) or YEA containing 10 μg/ml TBZ (+TBZ) plates and incubated at 30°C for 3 days. (*B*) Mitotic chromosome segregation is impaired in *rdp1* mutants. Chromosome segregation during late anaphase was analyzed by DAPI staining and IF using the anti-tubulin TAT1 antibody. The percentage of cells showing lagging chromosomes (numbers shown on the right) was determined by microscopic inspection of 100 or more cells for each strain. (*C* and *D*) *rdp1* mutants are defective in telomere clustering. Shown are deconvoluted images of wild-type and *rdp1* mutant subjected to IF with Swi6 antibody and Taz1-GFP together with DAPI staining. A graph displaying the frequency of Swi6 foci observed per cell is shown on the right. More than 100 cells were counted for each strain.

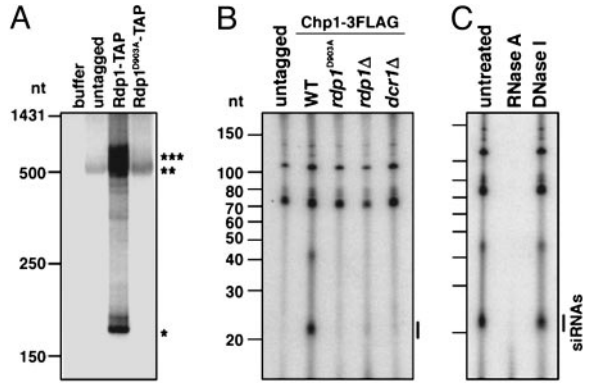

Fig. 4. Rdp1 possesses RdRP activity that is essential for generation of RITS-associated siRNAs. (*A*) Rdp1 has RdRP activity that is abolished in Rdp1^{D903A} cells. Purified Rdp1 and Rdp1^{D903A} proteins were incubated with *in vitro*-transcribed luciferase single-stranded transcripts, NTPs, and [γ-^{32}P]UTP and resolved on a denaturing gel. **, Full-length luciferase RNA; ***, longer product; *, short transcript probably caused by premature termination. (*B*) RdRP activity is essential for RITS-associated siRNAs. Chp1–3FLAG was purified from indicated strains. siRNAs were recovered by phenol-chloroform extraction, labeled with [5′-^{32}P]pCp, and separated on a denaturing gel. (*C*) Purified RITS contains mostly RNA species. [5′-^{32}P]pCp-labeled siRNAs prepared from *chp1–3FLAG* strain were treated either with RNase A (10 μg/ml) or DNase I (0.25 units/μl) at 30°C for 30 min and analyzed on a denaturing gel.

Neurospora QDE-1 (29). In contrast, the RdRP activity of Rdp1^{D903A} was reduced to nearly background levels (Fig. 4*A*), suggesting that the conserved RdRP domain of Rdp1 is essential for Rdp1 catalytic activity. The effect of D903A mutation on the primer-dependent RdRP activity could not be tested because our preliminary analysis suggests that primer-dependent activity of Rdp1 is much weaker than its primer-independent activity.

Loss of RdRP activity in Rdp1^{D903A} protein could explain the phenotypes observed in Rdp1^{D903A} mutant cells, in that Rdp1 could aide in the synthesis of dsRNAs that serve as a source for Dcr1-mediated amplification of siRNAs. These siRNAs are believed to promote the targeting of heterochromatin complexes and are an essential part of the self-enforcing loop required for heterochromatin formation at centromeres (22). Loss of RdRP activity in Rdp1^{D903A} mutant would short-circuit this loop by preventing continual siRNAs synthesis needed for the targeting of heterochromatin assembly complexes to centromeres (22).

The RITS complex contains siRNAs corresponding to known targets of RNAi-mediated heterochromatin assembly (21). Our previous work suggests that RITS-associated siRNAs are derived primarily from the RNAi machinery acting in cis, probably by directly processing nascent transcripts (22). Deletion of either *dcr1* or *clr4* results in a loss of siRNAs from the RITS complex even though the protein components of RITS could still assemble as a complex (22). We asked whether the RdRP activity of Rdp1 is crucial for the synthesis of RITS-associated siRNAs by assaying for the presence of siRNAs copurifying with the RITS complex from *rdp1^{D903A}* cells. We found that, whereas siRNAs could be readily detected in the affinity-purified fraction of RITS from wild-type cells, there were no detectable RITS-associated siRNAs present in *rdp1^{D903A}*, *rdp1*Δ, or *dcr1*Δ cells (Fig. 4*B*). Because RITS associates with chromatin, and T4 RNA ligase can also ligate DNA, we confirmed that RNA copurifying with RITS were not contaminating genomic DNA by subjecting the purified RITS fraction from wild-type strain to enzymatic digestion with either RNase A or DNase I. As expected, most bands copurifying with RITS, including the ≈22-nt siRNAs band, completely disappeared after the treatment with RNase A but not with DNase I (Fig. 4*C*). Therefore, we conclude from these analyses that the RdRP activity of Rdp1 is essential for the generation of RITS-associated siRNAs.

RITS and Rdp1 Localization at Centromeres Requires Rdp1 Catalytic Activity. Efficient siRNA production is essential for H3–K9 methylation and RITS localization at centromeres. Loss of RdRP activity of Rdp1 impaired both siRNA production and centromeric H3–K9 methylation, suggesting that RITS localization at centromeres might also be compromised in *rdp1*^{D903A} mutant. We explored this possibility by examining the localization of RITS components at centromeres in *rdp1*^{D903A} cells. Although all three components of RITS (Ago1, Chp1, and Tas3) are found to be dramatically enriched at *otr1R*::*ura4*$^{+}$ and centromeric repeats in wild-type cells, these proteins completely fail to localize to these centromeric loci in *rdp1*^{D903A} cells (Fig. 5*A*). Taken together, our analyses support the idea that RdRP activity of Rdp1 is essential for efficient generation of siRNAs, which in turn help target H3–K9 methylation and RITS to centromeres.

Deletion of any component of RITS abolishes Rdp1 localization at centromeres (Fig. 1*A*), and the RdRP activity of Rdp1 is required for RITS localization to centromeric chromatin (Fig. 5*A*). We asked whether the RdRP activity of Rdp1 itself is necessary for Rdp1 localization. Remarkably, we found that the lack of RdRP activity indeed prevents Rdp1 from localizing to

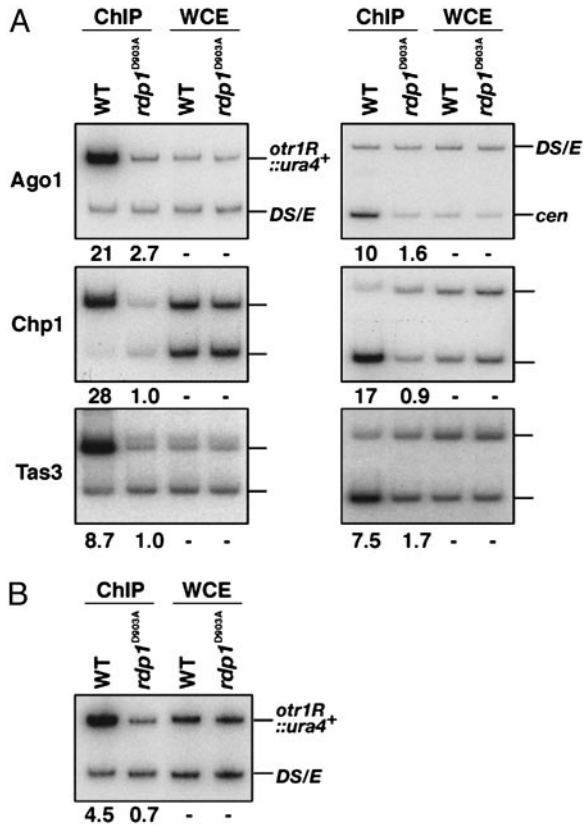

Fig. 5. RdRP activity is necessary for RITS and Rdp1 localization at centromeres. (*A*) RITS components fail to localize at centromeres in *rdp1*^{D903A} cells. Relative enrichment of Ago1, Chp1, and Tas3 at *otr1R*::*ura4*$^{+}$ and centromeric repeat sequences (*cen*) was analyzed by ChIP. (*B*) Rdp1^{D903A} fails to associate with centromeres. Wild-type Rdp1 and Rdp1^{D903A} localization at *otr1R*::*ura4*$^{+}$ was analyzed by ChIP. Relative enrichment values are shown.

centromeres (Fig. 5*B*). These results reinforce our conclusions that RITS and Rdp1 interdependently colocalize at centromeres (Fig. 1*A*) and that the presence of these factors at centromeric repeats likely promotes the processing of nascent transcripts into siRNAs, which are required for the targeting of heterochromatin complexes to centromeres. H3–K9 methylation by Clr4 at centromeres in turn stabilizes RITS and Rdp1 to chromatin.

Rdp1 Is an Essential Component of a Self-Enforcing Loop Linking siRNA Generation to Heterochromatin Formation. Based on our recent analyses, we proposed that the RNAi machinery acts primarily in cis whereby chromatin-bound RITS cooperates with Rdp1 and Dcr1 to process nascent transcripts to promote the efficient generation of siRNAs (22). In this study, we have shown that the RITS complex and Rdp1 mutually support their localization at centromeres. Deletion of any components of the RITS complex or Dcr1 abolishes Rdp1 localization at centromeric repeats. More importantly, we demonstrated that the RdRP activity of Rdp1 has an essential role in the generation of RITS-associated siRNAs and that a mutation in the catalytic domain of Rdp1, which abolishes its RdRP activity results in a complete loss of RITS as well as H3–K9 methylation and Swi6 at centromeric loci. Our analysis also suggests that centromeric localization of Rdp1 requires factors involved in heterochromatin assembly such as Clr4 and Swi6. Taken together, these results lend further support to the idea that RNAi-mediated heterochromatin assembly at centromeres occurs via a self-enforcing loop mechanism rather than a linear cascade (Fig. 6) (22), and reveal an essential and specific function for Rdp1 in this process.

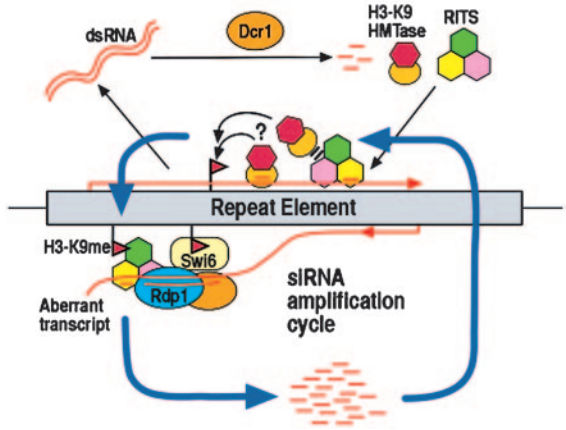

Fig. 6. Rdp1 is an essential component of a self-enforcing RNAi loop mediating heterochromatin formation. dsRNAs produced from bidirectional transcription of repetitive sequences serve as the initial source for Dcr1-processed siRNAs. These primary siRNAs are targeted back to the repetitive sequences, either by RITS or an unidentified complex before or in concert with heterochromatin assembly factors such as Clr4 to methylate H3–K9, which helps anchor RITS to chromatin. The rest of the RNAi machinery, including Rdp1, can assemble on RITS at heterochromatic loci. Ago1-bound siRNAs may assist in directing Rdp1 to the correct nascent single-stranded transcript for dsRNA synthesis, which requires RdRP activity. Dcr1 may be recruited to the *cis*-acting RNAi machinery to process Rdp1-generated dsRNAs into secondary siRNAs that serve as amplification signals to further recruit of heterochromatin assembly factors. Our model predicts that in the absence of a target locus, siRNAs generation induced by an ectopic hairpin RNA construct would be inefficient. Swi6 also binds to methyl H3–K9 marks, and in addition to mediating the spreading of heterochromatin, might have a role in stabilizing Rdp1 at heterochromatic loci.

During the *de novo* assembly of heterochromatin structures, dsRNAs generated by bidirectional transcription of centromeric repeats (14, 22) are recognized and cleaved by Dcr1 into siRNAs (Fig. 6). This initial step is most likely mediated by trans-acting RNAi factors similar to the processing of diffusible dsRNAs produced from ectopically introduced hairpin constructs (35, 36). siRNAs are incorporated into RITS and/or an unknown complex that mediates the recruitment of heterochromatin assembly factors such as Clr4 to homologous target loci, presumably through interactions with nascent transcripts. Once recruited, however, Clr4-mediated establishment of stable methyl marks on H3–K9 creates binding sites for Swi6 and the RITS complex, which in turn promote the recruitment of other RNAi components such as Rdp1 and possibly Dcr1 to the target loci, allowing the RNAi machinery to operate in cis to process nascent transcripts into siRNAs. This *in situ* production of secondary siRNAs results in amplification of the silencing signal that feeds back through a self-enforcing loop to trigger further recruitment of heterochromatin proteins and RNAi components to centromeric repeats. According to our model, the role of Rdp1 within this heterochromatin-forming loop is to convert rare "aberrant" transcripts that escape transcriptional silencing into dsRNAs in cis for sustained production of secondary siRNAs. In this respect, it should be noted that the amplification of siRNAs is a common feature of RNAi in plants and *C. elegans* (27, 30), and that the production of siRNAs from endogenous transcripts in *Arabidopsis* requires an RdRP homolog (10).

Several lines of evidence indicate that *de novo* heterochromatin formation at *S. pombe* centromeres by the trans-acting RNAi machinery (37, 38) or the silencing induced by the ectopically expressed hairpin RNAs is a highly stochastic and inefficient process (35, 36). Furthermore, it has been reported that accumulation of siRNAs in cells expressing hairpin mRNAs requires

Rdp1 (35, 36), indicating that Dcr1 and Rdp1 might collaborate to synthesize siRNAs. It is possible that Dcr1 preferentially targets Rdp1-generated dsRNAs compared to dsRNAs produced by bidirectional transcription of centromeric repeats or hairpin mRNAs. Moreover, chromatin-bound RITS could facilitate the recruitment of Rdp1 and Dcr1 into the same RNAi complex at the target locus for the efficient processing of transcripts into siRNAs. Indeed, recent studies suggest that *Drosophila* Dicer associates with the Argonaute-containing RISC complex (39, 40). Furthermore, in *Dictyostelium*, the DExD helicase domain associated with Dicer in most other species is present instead on the RdRPs, leading to the suggestion that Dicer and RdRP form a complex, and that the helicase domain may be present on either protein to carry out its function (26). The findings that Ago1 homologs in other systems can directly bind to siRNAs and that Ago1-containing RITS complex associates with siRNAs strongly implicate Ago1 as the RITS component that directly bind to siRNAs (21, 41). Our observation of RITS-dependent localization of Rdp1 at centromeres suggests that an Ago1-siRNAs subcomplex of RITS capable of hybridizing to target transcripts could be involved in guiding Rdp1 to its intended template for second-strand synthesis.

This study provides evidence showing that the RdRP activity of Rdp1 is crucial for RNAi-mediated heterochromatin assembly in *S. pombe* and further supports the existence of a self-enforcing RNAi loop in which RNAi components, including RITS and Rdp1, assemble on chromatin to collaborate in the generation of secondary siRNAs that act as amplification signals for the targeting of heterochromatin complexes to specific loci. We expect that future analysis of this pathway in *S. pombe* will yield important insights regarding the role of RNAi in epigenetic control of the genome.

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