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Two RNAi Complexes, RITS and RDRC, Physically Interact and Localize to Noncoding Centromeric RNAs

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Summary

RNAi-mediated heterochromatin assembly in fission yeast requires the RNA-induced transcriptional silencing (RITS) complex and a putative RNA-directed RNA polymerase (Rdp1). Here we show that Rdp1 is associated with two conserved proteins, Hrr1, an RNA helicase, and Cid12, a member of the polyA polymerase family, in a complex that has RNA-directed RNA polymerase activity (RDRC, RNA-directed RNA polymerase complex). RDRC physically interacts with RITS in a manner that requires the Dicer ribonuclease (Dcr1) and the Clr4 histone methyltransferase. Moreover, both complexes are localized to the nucleus and associate with noncoding centromeric RNAs in a Dcr1-dependent manner. In cells lacking Rdp1, Hrr1, or Cid12, RITS complexes are devoid of siRNAs and fail to localize to centromeric DNA repeats to initiate heterochromatin assembly. These findings reveal a physical and functional link between Rdp1 and RITS and suggest that noncoding RNAs provide a platform for siRNA-dependent localization of RNAi complexes to specific chromosome regions.

Introduction

RNA interference (RNAi) is a conserved silencing mechanism with roles in cellular defense against RNA viruses and transposons, the regulation of cell identity during development, and epigenetic control of chromatin structure (Hannon, 2002; Matzke et al., 2004; Mello and Conte, 2004; Tijsterman et al., 2002; Zamore, 2002). In most instances, RNAi functions at the posttranscriptional level to silence target mRNAs, but in several cases it acts at the DNA and chromatin level. For example, RNAi mediates DNA methylation and histone H3-K9 methylation in plants (Baulcombe, 2004; Matzke et al., 2001; Zilberman et al., 2003), DNA elimination in *Tetrahymena* (Mochizuki et al., 2002), and heterochromatin formation in centromeric DNA regions in *Drosophila*, human, and the fission yeast *Schizosaccharomyces pombe* (Fukagawa et al., 2004; Pal-Bhadra et al., 2004; Volpe et al., 2002).

Silencing by the RNAi pathway is initiated by double-stranded RNA (dsRNA), which is processed by the RNase III-like ribonuclease Dicer into ∼23 nucleotide small RNAs, called small interfering RNAs (siRNAs) (Bernstein et al., 2001; Elbashir et al., 2001; Fire et al., 1998; Hamilton and Baulcombe, 1999; Parrish et al., 2000; Zamore et al., 2000). SiRNAs direct gene silencing through the action of effector complexes. In posttranslational gene silencing, siRNAs target the RNA-induced silencing complex (RISC) to complementary mRNA by base-pairing interactions (Hammond et al., 2001). RISC contains a conserved protein called Argonaute that binds siRNA and mediates mRNA target recognition and inactivation (Hammond et al., 2001; Hutvagner and Zamore, 2002; Lingel et al., 2003; Meister et al., 2004; Song et al., 2004; Yan et al., 2003). In *C. elegans*, plants, and fungi, RNAi also requires RNA-directed RNA polymerases (RDRs, also called RdRPs), which are involved in siRNA and template-directed production of dsRNA (Baulcombe, 2004; Dalmay et al., 2000; Mello and Conte, 2004; Sijen et al., 2001). Almost nothing is known about how RDR activity is regulated and whether these enzymes interact with components of the RNAi pathway that are involved in dsRNA processing or target recognition.

S. pombe contains one gene for each Argonaute (*ago1*$^{+}$), Dicer (*dcr1*$^{+}$), and RNA-directed RNA polymerase (*rdp1*$^{+}$). The deletion of any of these genes results in disruption of heterochromatin at centromeric DNA regions and inefficient initiation of heterochromatin formation at the mating type loci (Hall et al., 2002; Volpe et al., 2002). In addition, similar to what has been observed in multicellular eukaryotes (Hannon and Rossi, 2004), the expression of hairpin RNAs can induce both transcriptional and posttranscriptional gene silencing in fission yeast by mechanisms that require the above RNAi components as well as the Clr4 histone H3 lysine 9 methyltransferase (Schramke and Allshire, 2003; Sigova et al., 2004).

RNAi-mediated heterochromatin assembly in *S. pombe* requires an RNAi effector complex, called RITS, which contains Ago1, the Chp1 chromodomain protein, and Tas3 (Verdel et al., 2004). RITS also contains siRNAs that match the sequence of repetitive DNA at the outer centromeric repeats where heterochromatin assembly is initiated (Reinhart and Bartel, 2002; Verdel et al., 2004). In cells that lack the Dicer enzyme, RITS is not loaded with siRNAs and does not associate with heterochromatic DNA regions. These observations support the proposal that RITS uses Dicer-produced siRNAs to target specific chromosome regions by a recognition mechanism that involves base-pairing interactions. In addition to siRNAs, the association of RITS with chromatin requires the Clr4 histone H3 lysine 9 (-K9) methyltransferase. Methylation of H3-K9 is thought to stabilize the association of RITS with chromatin by creating a binding site for the chromodomain of Chp1 (Partridge et al., 2002). However, it is unclear how the RITS complex targets specific chromosome regions for heterochromatin formation, although the initial localization of RITS has been proposed to involve base-pairing interactions between siRNAs and either centromeric repeat DNA or noncoding transcripts associated with these repeats (Ekwall, 2004; Verdel et al., 2004).

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Transcription from divergent promoters within the *S. pombe* outer centromeric repeats (*otr1*) produces complimentary RNAs that are more abundant in mutants that disrupt heterochromatin formation (Volpe et al., 2002). These centromeric RNAs are thought to base pair and produce dsRNA that is processed into siRNAs by Dicer (Reinhart and Bartel, 2002; Volpe et al., 2002). The initial siRNAs produced from these primary dsRNAs would be expected to load onto RITS and trigger heterochromatin assembly (Verdel et al., 2004). However, in this scenario, it is unclear why RNAi-mediated heterochromatin formation also absolutely requires an RNA-directed RNA polymerase (Rdp1). Similarly, it is unclear why hairpin-induced gene silencing requires Rdp1 even though the hairpins are transcribed from very strong promoters and should themselves be targets for Dicer (Schramke and Allshire, 2003; Sigova et al., 2004).

In this paper, we address the mechanism by which RITS targets specific chromosome regions and explore the requirement for Rdp1 in this process. We show that Rdp1 executes its functions in association with two highly conserved proteins, a putative RNA helicase (Hrr1, helicase required for RNAi-mediated heterochromatin assembly) and Cid12, a protein that belongs to the polyA polymerase/2′-5′ oligoadenylate synthetase family of enzymes. This complex, henceforth referred to as RDRC (RNA-directed RNA polymerase complex), has RNA template-dependent RNA polymerase activity and both its activity and associated subunits are required for association of siRNAs with RITS and for centromeric gene silencing. We also show that the RDRC and RITS complexes physically interact and this interaction requires the Dicer ribonuclease and the Clr4 histone H3 methyltranferase. These observations strongly suggest that little or no siRNAs are produced from primary centromeric transcripts in the absence of RDRC and that Clr4 regulates dsRNA and siRNA production by controlling the interaction of RDRC with RITS. Furthermore, using crosslinking and RNA immunoprecipitation experiments (RNA-IP), we provide direct evidence that RITS and RDRC physically interact with noncoding centromeric RNAs in an siRNA-dependent manner. Together with other results, these findings suggest that the recognition of nascent noncoding RNAs transcribed from centromeric repeats, by siRNA-programmed RITS, plays a crucial role in RNAi-mediated heterochromatin formation.

Results

Purification of the Fission Yeast RNA-Directed RNA Polymerase Complex

We constructed a strain that expressed a fully functional C-terminally TAP-tagged Rdp1 protein (Rdp1-TAP) and purified this protein as previously described (Verdel and Moazed, 2004). Analysis of the purification by polyacrylamide gel electrophoresis and mass spectrometry indicated that two other proteins specifically copurified with Rdp1-TAP (Figures 1A and 1D). A 120 kDa protein that co-migrated with Rdp1-CBP was identified as SPCC1739.03, a previously uncharacterized open reading frame, which has a high degree of sequence similarity to the Upf1 family of RNA helicases and is closely related to proteins in human, *N. crassa*, *Arabidopsis*, and *C. elegans* (Figure 1E; Dalmay et al., 2001). We have named this protein Hrr1 (*h*elicase *r*equired for *R*NAi-mediated heterochromatin assembly 1). The second protein, which migrated at 38 kDa, was identified as Cid12, a member of a highly conserved family of proteins that include the budding yeast Trf4/5 and the fission yeast Cid13 proteins, which have been implicated in mRNA polyadenylation (Figures 1A, 1D, and 1F; Read et al., 2002; Saitoh et al., 2002).

To further test if Hrr1 and Cid12 were associated with Rdp1 in a complex, we constructed strains that expressed C-terminally TAP-tagged versions of Hrr1 and Cid12 and purified each protein. Mass spectrometry analysis of excised gel bands and mixtures of purified proteins confirmed the results in Figure 1A: Rdp1 and Cid12 specifically copurified with Hrr1-TAP, and Rdp1 and Hrr1 copurified with Cid12-TAP (Figures 1B–1D). Together these purifications demonstrate that the fission yeast Rdp1, Hrr1, and Cid12 proteins are physically associated in a complex (termed RDRC). Surprisingly, we found a significant number of Ago1 peptides in the Hrr1-TAP purifications (Figure 1D), suggesting that the RDRC complex may interact physically with the Ago1-containing RITS complex (see below). However, the association of Ago1 with Hrr1-TAP was not detectable by silver staining (Figure 1B), indicating that only substoichiometric amounts of Ago1 associated with Hrr1.

In order to gain further insight into the architecture of RDRC, we purified Rdp1-TAP from cells that carried a deletion of $hrr1^+$ or $cid12^+$. Mass spectrometry analysis of Rdp1-TAP purifications from *hrr1*Δ or *cid12*Δ cells identified a large number of Rdp1 peptides (42% and 43% amino acid coverage, respectively) but none corresponding to the other subunit (Cid12 or Hrr1, respectively, Table 1). Similarly, purification of Cid12-TAP from *rdp1*Δ cells only identified Cid12 peptides (55% amino acid coverage). The absence of any peptides corresponding to the interacting partner together with the high degree of amino acid coverage for the purified TAP-tagged proteins in the LC-MS/MS analysis strongly suggests that the interacting partners are absent from each mixture analysis. We conclude that the integrity of RDRC requires all three of its subunits.

Surprisingly, Cid12-TAP purifications from *rdp1*Δ cells also contained nearly all subunits of the spliceosome with very high amino acid coverage (Table 1). Several spliceosome subunits were also detected in purifications of Cid12-TAP from a wild-type background but were represented with a small number of peptides (data not shown). We never detected spliceosome subunits in purifications of other subunits of RDRC (or RITS), suggesting that the Cid12-spliceosome interaction may be unrelated to the possible role of Cid12 in RNAi-mediated heterochromatin assembly.

Hrr1 and Cid12 Are Required for Centromeric Gene Silencing

To test whether $hrr1^+$ and $cid12^+$ are required for centromeric gene silencing, we replaced each gene with an antibiotic resistance marker (Kan^R) in a strain that harbors the $ura4^+$ reporter gene inserted at the innermost centromeric repeats (*imr1R::ura4*$^+$). As shown in Figure

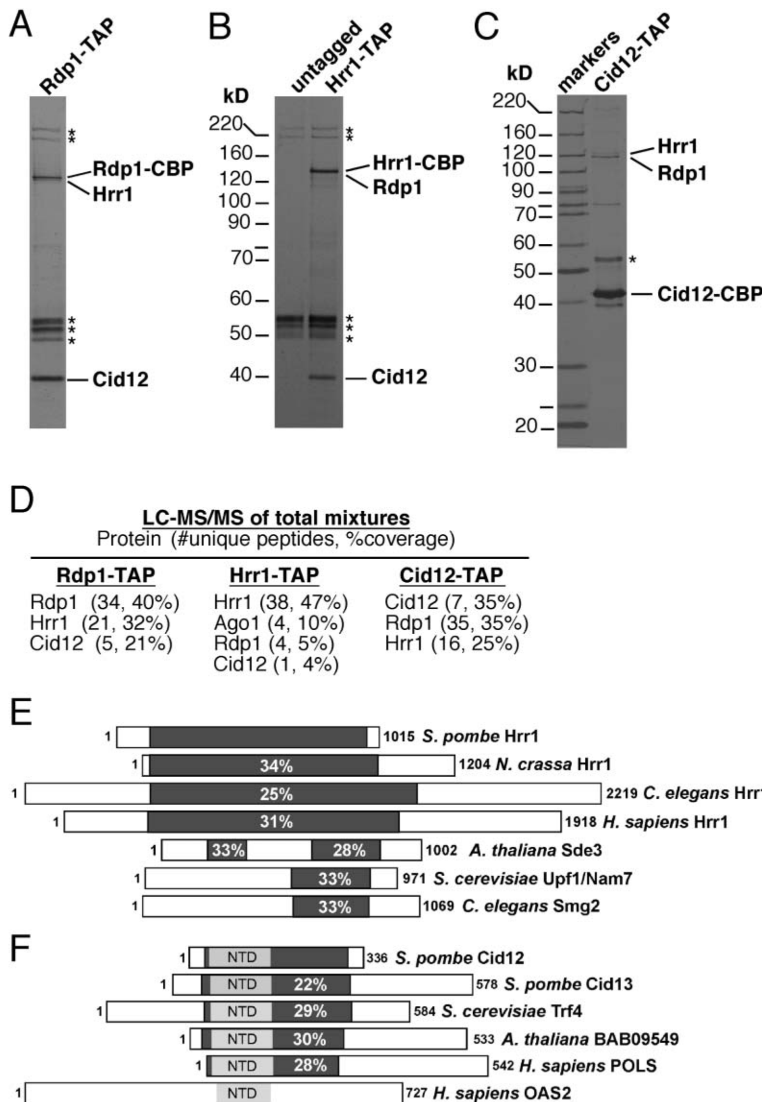

Figure 1. Purification of the Fission Yeast Rdp1 Complex and Identification of Its Associated Subunits

Silver-stained gels showing the purification of Rdp1-TAP (A), Hrr1-TAP (B), and Cid12-TAP (C) from strains SPY35, SPY150, and SPY273, respectively. A control purification from an untagged strain (SPY28) is shown in (B). Proteins identified by ms/ms mass spectrometry sequencing of gel bands are indicated. * denotes residual GST-TEV used for elution of bound proteins from the IgG-Sepharose column or background proteins.

(D) Results of tandem mass spectrometry sequencing of mixtures of proteins (LC-MS/MS) in each purifications are indicated as the number of unique peptides and percent coverage based on total amino acid number.

(E) Regions of sequence identity (indicated as percentages in shaded areas) between the *S. pombe* Hrr1 and apparent homologs in *N. crassa* (GB: CAD79665.1), *C. elegans* (GB: CAA93884.1), and *H. sapiens* (NP_066363.1); the region of similarity with the *A. thaliana* Sde3, the *S. cerevisiae* Upf1/nam7, and the *C. elegans* Smg2 is also highlighted.

(F) Regions of sequence identity between the *S. pombe* Cid12 and closest orthologs in other organisms. NTD, a nucleotidyltransferase domain found in the Cid family members, polyA polymerases, and 2′-5′-oligoadenylate synthetases.

2A, deletion of *hrr1*$^+$ or *cid12*$^+$ abolished silencing of the *ura4*$^+$*::imr1R* reporter gene to the same extent as previously observed for deletion of *sir2*$^+$ or *dcr1*$^+$ (Verdel et al., 2004; Volpe et al., 2002). Moreover, in *hrr1*Δ, *cid12*Δ, *clr4*Δ cells, transcription from promoters at the outermost centromeric repeat regions was derepressed (Figure 2B, compare lanes 5–7 with lanes 1 and 3), as has been previously shown for *rdp1*Δ and *dcr1*Δ cells (Figure 2B, lanes 2 and 4, respectively; Volpe et al., 2002).

The localization of RITS to centromeric repeats requires Rdp1 (Verdel et al., 2004). We therefore investigated whether the newly identified Rdp1 subunits, Hrr1 and Cid12, also affected the localization of RITS to centromeric DNA repeats. Using chromatin immunoprecipitation (ChIP) experiments, we compared the chromatin localization of RITS in *hrr1*Δ and *cid12*Δ cells to wild-type cells. Consistent with previous observations, we found that the Chp1 and Tas3 subunits of RITS specifically were localized to the centromeric *dg* and *dh* repeats (Figures 2C and 2D, lanes 2 and 3, respectively; Verdel et al., 2004). However, the association of Tas3 with both regions was reduced to background levels in *hrr1*Δ and *cid12*Δ cells (Figures 2C and 2D, lanes 4 and 5). The requirement of Hrr1 and Cid12 for centromeric gene silencing and localization of RITS to centromeric repeats, as well as their physical association with Rdp1, suggest that these proteins function together in a complex that mediates heterochromatin formation.

We found that compared to the Chp1 and Tas3 subunits of RITS (Figures 2C and 2D, lanes 2 and 3), the association of Rdp1-TAP or Hrr1-TAP with the centromeric *dg* or *dh* repeats was not significantly above background (Figures 2C and 2D, compare lanes 2, 3, 6, 7 with lane 1). It has previously been reported that Rdp1 is localized to centromeric DNA repeats (Volpe et al., 2002). Our ChIP experiments were performed using cells that had been crosslinked with 1% formaldehyde, whereas Volpe et al. (2002) used 3% formaldehyde for crosslinking. Using the crosslinking conditions described in Volpe et al. (2002), we observed a 2- to 3-fold enrichment of *cen* DNA in both Rdp1-TAP and Hrr1-TAP precipitates above the background signal for *fbp1*$^+$, an

Table 1. Mass Spectrometry Analysis of RITS and RDRC Complexes Purified from Wild-Type and Mutant Backgrounds

Genotype Protein	No. of peptides (coverage)
chp1-TAP	
Chp1	43 (34%)
Tas3	21 (50%)
Ago1	21 (38%)
Hrr1	11 (17%)
chp1-TAP, clr4Δ	
Chp1	33 (38%)
Tas3	17 (36%)
Ago1	16 (21%)
chp1-TAP, dcr1Δ	
Chp1	30 (37%)
Tas3	13 (22%)
Ago1	15 (20%)
rdp1-TAP	
Rdp1	40 (41%)
Hrr1	10 (15%)
Cid12	4 (19%)
rdp1-TAP, hrr1Δ	
Rdp1	45 (42%)
rdp1-TAP, cid12Δ	
Rdp1	47 (43%)
cid12-TAP, rdp1Δ	
Cid12	18 (55%)
Spliceosome[a]	

[a] Twenty-nine different spliceosomal proteins, represented with 3 to 51 peptides for each protein, were identified.

active euchromatic gene (data not shown). Nonetheless, in parallel experiments the enrichment of *cen* DNA in Rdp1-TAP and Hrr1-TAP precipitations was generally about 5-fold lower compared to Chp1-TAP and Tas3-TAP precipitations. The lower enrichment of *cen* DNA in Rdp1/Hrr1 precipitations compared to Chp1/Tas3 precipitations may reflect either a lower occupancy for RDRC on *cen* DNA or a more peripheral association with chromatin.

The RDRC and RITS Complexes Physically Interact

Our mass spectrometry analysis of Hrr1-TAP purifications revealed that Ago1, previously identified as a subunit of the RITS complex (Verdel et al., 2004), was associated with this helicase (Figure 1D). We further investigated the nature of this interaction using a combination of mass spectrometry analysis of mixtures of purified proteins and immunoprecipitation experiments. First, we repeated Chp1-TAP purifications under less stringent washing conditions to determine whether Hrr1 or other components of RDRC, which might be less tightly associated with RITS, could be identified in these purifications. Mass spectrometry of Chp1-TAP purifications using these conditions uncovered many peptides for each Chp1, Tas3, and Ago1 (34%, 50%, and 38% amino acid coverage, respectively; Table 1), confirming the previously reported identity of the core subunits of RITS. These purifications also identified 11 Hrr1 peptides (17% amino acid coverage) in the mixture of proteins specifically associated with Chp1, providing further evidence for an interaction between RITS and an RDRC subunit. Parallel purifications from an untagged control strain did not identify any of the above proteins.

The simplest explanation for these observations is that RDRC and RITS are dynamic complexes that weakly interact through their Hrr1 and Ago1 subunits, respectively. The detection of the more peripherally associated subunits of each complex may be below the sensitivity of our LC-MS/MS analysis. However, it is also possible that Ago1 is a subunit of both RITS and RDRC, and similarly Hrr1 is a subunit of both complexes, but that the two complexes do not physically interact. In order to distinguish between these possibilities, we tested whether Chp1 and Rdp1, which were not identified together in the LC-MS/MS analysis of each purified protein, coimmunoprecipitated. As shown in Figure 3A, Rdp1-13myc coprecipitated with Chp1-TAP (lane 6), and reciprocally, Chp1-13myc coprecipitated with Rdp1-TAP (Figure 3B, lane 4), indicating that the RDRC and RITS complexes associate.

RITS-RDRC Interaction Requires Dcr1 and Clr4

We hypothesized that the interaction of RITS with RDRC might be analogous to the interaction of a "priming complex" with a polymerase. If true, we would expect siRNAs to be required for the RITS-RDRC interaction. Consistent with this hypothesis, deletion of *dcr1*$^+$, which is required for siRNA production, abolished the interaction of the Tas3-TAP with both Rdp1-myc13 and Hrr1-myc13 (Figure 3C, compare lane 3 with 5 and lane 7 with 9, respectively) and Chp1-TAP with Hrr1 (Table 1). Consistent with previous observations, deletion of *dcr1*$^+$ had no effect on integrity of the RITS complex (Table 1; Verdel et al., 2004).

Clr4 is also required for siRNA generation and localization of Chp1 to centromeric DNA repeats (Partridge et al., 2002; Schramke and Allshire, 2003). We therefore examined the possible requirement of Clr4 for the association of RITS with RDRC. We found that the purification of Chp1-TAP from *clr4Δ* cells, under conditions identical to those used for Chp1-TAP purification from wild-type cells, consistently identified only Ago1 and Tas3 but no Hrr1 (Table 1). Furthermore, we used immunoprecipitation assays to examine the requirement for Clr4 in RITS-RDRC interactions. Consistent with the mass spectrometry results, both Rdp1-myc13 and Hrr1-myc13 coprecipitated with Tas3-TAP, but this coprecipitation was abolished or reduced in *clr4Δ* cells (Figure 3C, compare lane 3 with 4 and lane 7 with 8, respectively). These results demonstrate that the Clr4 histone H3-K9 methyltransferase is required for efficient interaction of RITS with RDRC.

RITS Purified from RDRC Mutant Backgrounds Lacks siRNAs

Transcription from divergent promoters at centromeric repeats has been proposed to produce complementary RNAs that can base pair to form dsRNA, which is then processed into siRNAs by Dcr1 (Reinhart and Bartel, 2002; Volpe et al., 2002). Loss of RDRC activity should result in a failure in synthesis of additional dsRNA and subsequent generation of more siRNAs. According to this scenario, some primary siRNAs should be produced independently of Rdp1. To determine to what extent RDRC is required for the formation of the RITS-siRNA ribonucleoprotein complex, we purified RITS from wild-

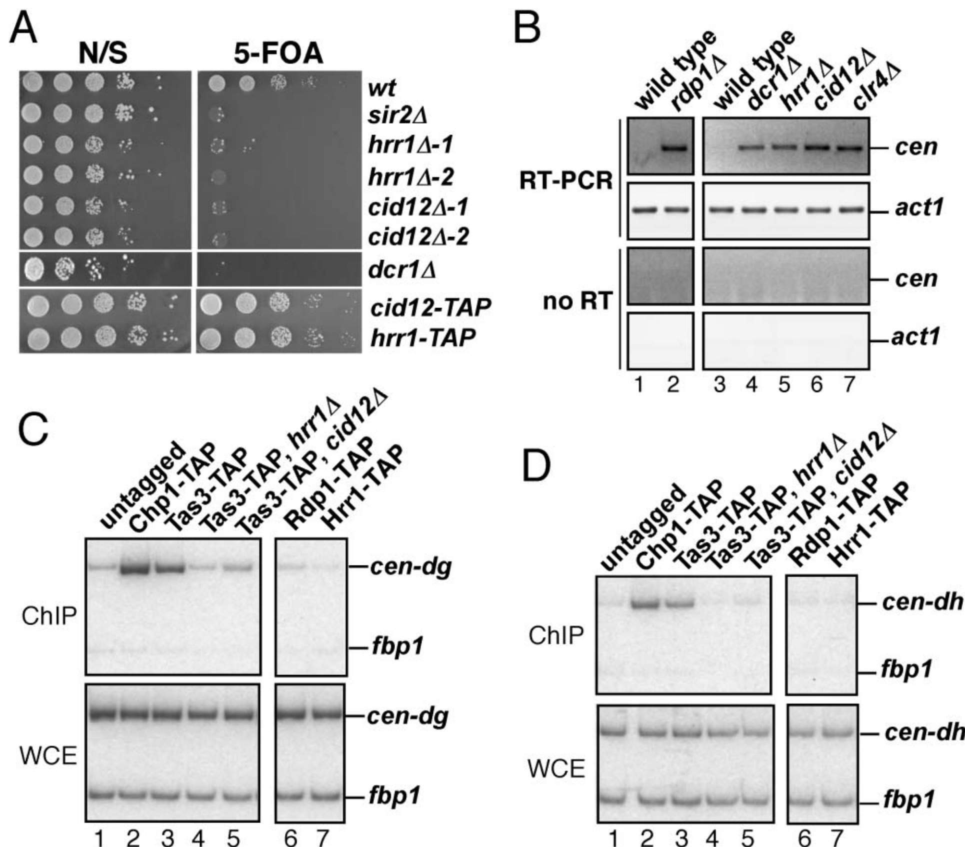

Figure 2. Components of the Rdp1 Complex Are Required for Centromeric Silencing and Association of RITS with Centromeric Repeats

(A) Silencing of a *ura4*$^+$ reporter gene inserted at the innermost centromeric repeats (*imr1R:: ura4*$^+$) is lost in *hrr1*Δ and *cid12*Δ cells to the same extent as that observed for *sir2*Δ and *dcr1*Δ cells. Two independent *hrr1* and *cid12* deletions are shown.

(B) RT-PCR assays showing that the centromeric repeat transcripts (*cen*), but not actin mRNA (*act1*), become more abundant in *hrr1*Δ, *cid12*Δ, and *clr4*Δ cells (compare lanes 5–7 with lane 3), similar to what has been previously described for *rdp1*Δ*, ago1*Δ, and *dcr1*Δ cells (lanes 2 and 4; Volpe et al., 2002).

(C and D) ChIP experiments showing that the localization of Tas3-TAP to the centromeric *dg* (*cen-dg*) and *dh* (*cen-dh*) repeats requires Hrr1 and Cid12 (lanes 3–5). Under these conditions, unlike Chp1-TAP and Tas3-TAP, Rdp1-TAP and Hrr1-TAP are not significantly enriched at *cen-dg* and *cen-dh* (compare lanes 2 and 3 with lanes 6 and 7). *fbp1*, an active euchromatic gene used as an internal control.

type and mutant cells and 3′-end labeled any associated siRNAs using 5′-[^{32}P]-pCp and T4 RNA ligase (Verdel et al., 2004). We found that RITS purified from *rdp1*Δ, *hrr1*Δ, or *cid12*Δ cells lacked detectable siRNAs (Figure 3D, compare lane 3 with lanes 4–6), similar to RITS purified from *dcr1*Δ cells (Verdel et al., 2004; Figure 3D, lanes 2 and 8). In contrast, deletion of *swi6*$^+$, which encodes the histone H3-K9-methyl binding protein, or the histone deacetylase Sir2, had little or no effect on the level of RITS-associated siRNAs (Figure 3D, lanes 10 and 11). None of the above mutations affected the levels of Chp1-TAP or the integrity of the RITS complex (Figure 3E, Table 1, and data not shown). Finally, RDRC did not contain detectable levels of siRNAs, as would be expected from the association of substoichiometric levels of Ago1 with this complex (data not shown).

Previously it has been reported that Clr4 is required for the generation of siRNAs from a hairpin transcript (Schramke and Allshire, 2003). Consistent with this observation, we found that deletion of *clr4*$^+$ results in greatly reduced levels of RITS bound siRNAs (Figure 3C, lane 9; Noma et al., 2004). Together these results suggest that RDRC plays a major role in either the generation of siRNAs or in mediating their association with RITS. Furthermore, the requirement of Clr4 for the association of RDRC and RITS complexes (Figure 3C, Table 1), and generation of an siRNA loaded RITS, suggests that the Clr4-dependent localization of RITS to chromatin may be required for dsRNA synthesis and siRNA production.

The RDRC and RITS Complexes Are Associated with Noncoding Centromeric Transcripts

The physical association of RITS with Rdp1, which is predicted to use noncoding centromeric RNAs as templates for the synthesis of dsRNA, suggested that both RITS and RDRC may localize to noncoding centromeric RNAs (see Figure 4A). In order to test this possibility, we used an RNA immunoprecipitation (RNA-IP) protocol in which the association of specific RNAs with a protein of interest is determined following immunoprecipitation from in vivo formaldehyde crosslinked cells and RT-PCR analysis (Gilbert et al., 2004; Hurt et al., 2004). We tested whether components of RITS and RDRC preferentially

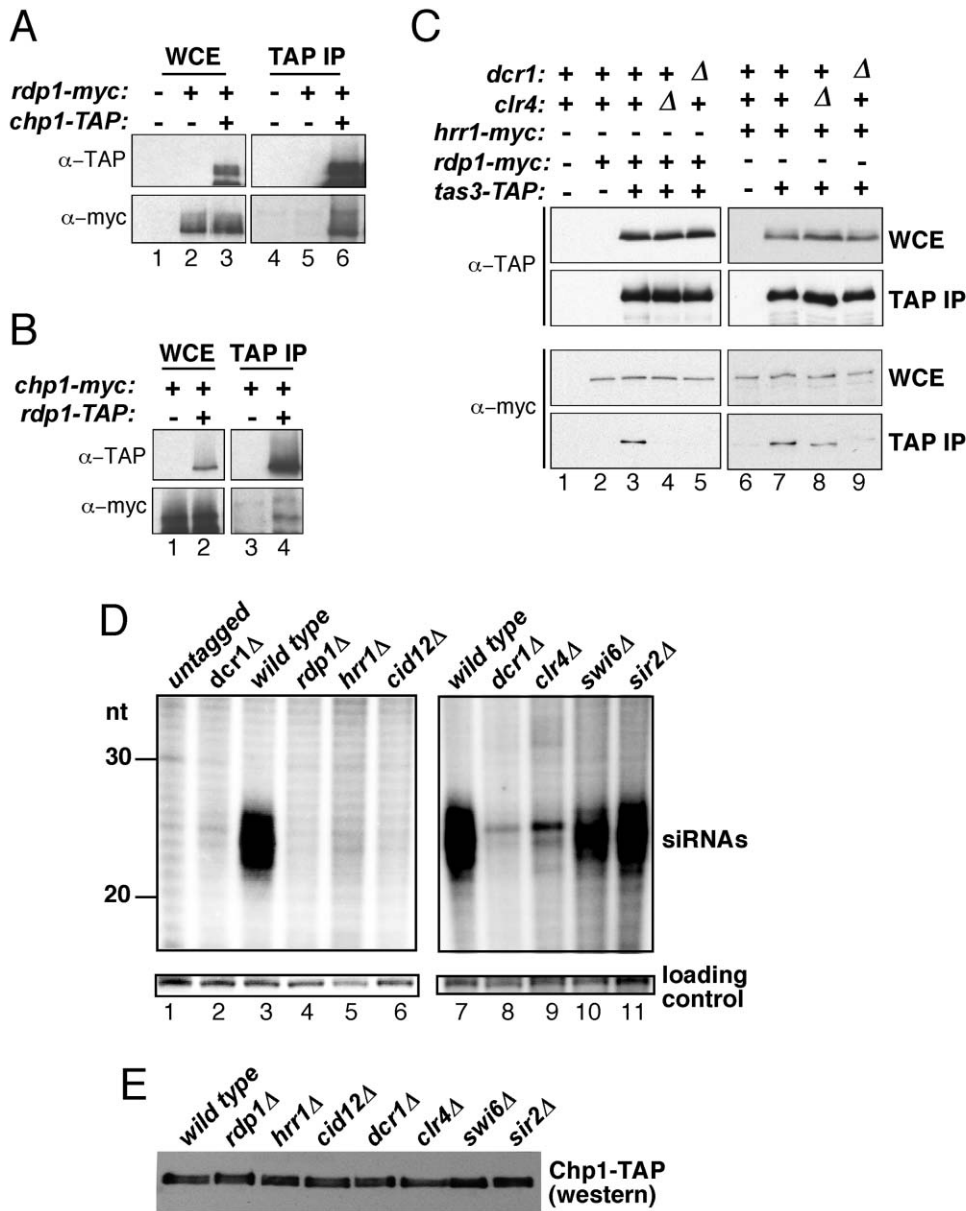

Figure 3. Physical Association of RDRC with RITS and the Requirement for RDRC and Clr4 in siRNA Production

Western blots showing that Rdp1-myc13 coprecipitates with Chp1-TAP (A) and that Chp1-myc13 coprecipitates with Rdp1-TAP (B). (C) Rdp1-myc13 (lanes 1-3) and Hrr1-myc13 (lanes 6 and 7) coprecipitate with Tas3-TAP in a Clr4- and Dcr1-dependent manner (lanes 4 and 5 and 8 and 9, respectively). (D) Components of RDRC and Clr4 are required for the association of siRNAs with RITS; in contrast, RITS-siRNA association is independent of Swi6 and Sir2. (E) Western blots showing that Chp1-TAP is stable in the mutant strains used for the isolation of RITS and siRNA labeling in (D).

associated with noncoding centromeric (*cen*) RNAs as compared to actin mRNA (*act1*). We detected little or no *cen* RNA in RT-PCR amplifications from total RNA prepared from crosslinked cells, consistent with the previously reported low abundance of these RNAs (Volpe et al., 2002; Figure 4B, lanes 1–3 and Figure 4C, lanes 1 and 2). However, in Chp1-TAP and Tas3-TAP precipitates, we observed a large enrichment of *cen* RNA compared to the untagged control (Figure 4B, compare lane 4 with lanes 5 and 6, ∼8- and 14-fold enrichment, respectively). In contrast, the more abundant *act1* mRNA was not significantly enriched in precipitates from Chp1-TAP and Tas3-TAP compared to the untagged control (Figure 4B, lanes 4–6). No signal was detected in the absence of reverse transcriptase, indicating that the observed amplification was not due to the presence of residual DNA in the immunoprecipitates (Figure 4B, lower panels). We also performed RNA-IP experiments from a strain that produces a functional myc3-Ago1 protein. Myc3-Ago1 immunoprecipitates much less efficiently than Chp1-TAP or Tas3-TAP (unpublished data); nonetheless, *cen* RNAs were enriched in the myc3-Ago1 immunoprecipitates, but to a lower level than that observed for Chp1-TAP and Tas3-TAP precipitates (Figure 4C, ∼2-fold enrichment). To determine the nature of RNAs that were associated with the RITS complex, we

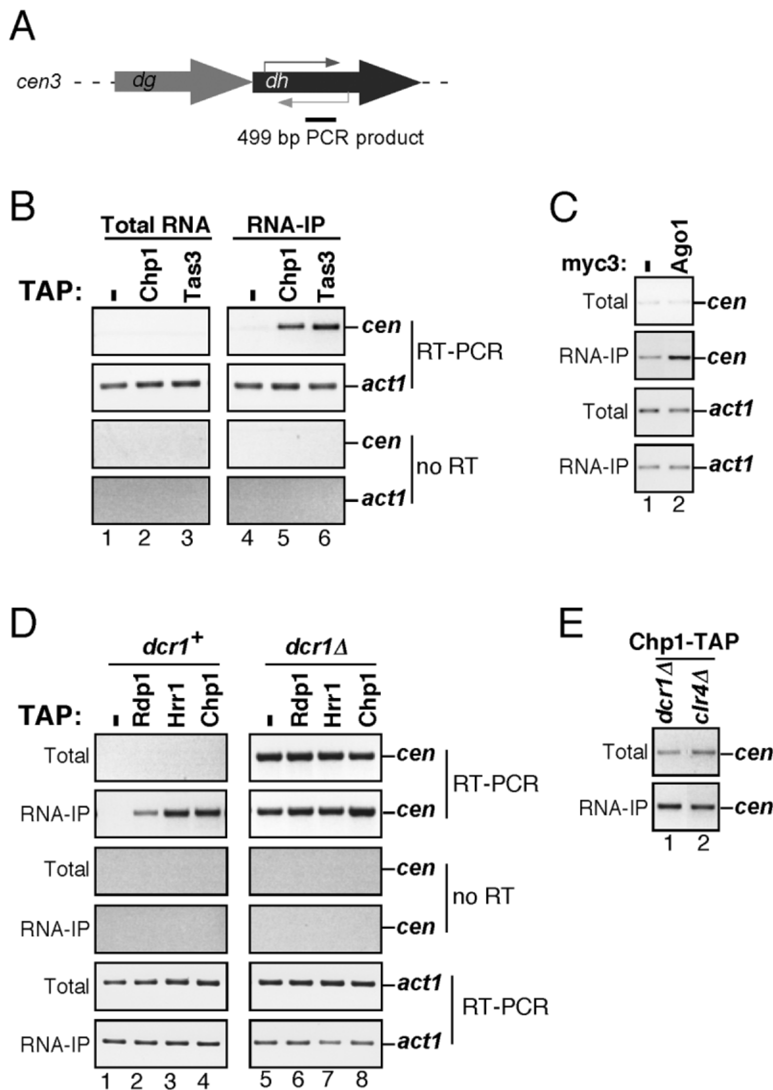

Figure 4. RITS and RDRC Localize to Noncoding Centromeric Transcripts

(A) Schematic diagram of the *S. pombe* centromeric *dg* and *dh* repeats. Small arrows indicated forward and reverse noncoding transcripts. The location of the 499 bp product amplified by oligos in RT-PCR is indicated below the map (Volpe et al., 2002).

(B) RNA-IP experiments showing that noncoding centromeric RNAs (*cen*), but not actin mRNA (*act1*), are enriched in Chp1-TAP and Tas3-TAP precipitations.

(C) Enrichment of *cen* but not *act1* RNA in myc3-Ago1 immunoprecipitations.

(D) The association of cen RNA with Rdp1-TAP, Hrr1-TAP, and Chp1-TAP is reduced to background levels in *dcr1*Δ cells (compare lanes 1–4 with lanes 5–8 in total and RNA-IP panels).

(E) In *clr4*Δ cells (lane 2), the association of *cen* RNAs with Chp1-TAP is similar to the background level observed in *dcr1*Δ cells (lane 1).

treated immunoprecipitated RNAs with single-strand-specific ribonucleases (A and T1) prior to RT-PCR. This treatment abolished the *cen* RNA signal, suggesting that RITS-associated RNAs are primarily single stranded (data not shown). These results show that the RITS complex is associated specifically with noncoding RNAs that are transcribed from divergent promoters within the centromeric repeats.

We next tested whether RDRC subunits were also localized to *cen* RNAs. We observed a weak, but reproducible, association of Rdp1-TAP with *cen* RNAs (Figure 4D, lane 2, ∼4-fold enrichment). Hrr1-TAP was associated with *cen* RNAs to the same extent as Chp1-TAP and Tas3-TAP (Figure 4D, lanes 3 and 4; Figure 4B, lanes 5 and 6), and Cid12-TAP associated with these RNAs to a similar extent as Rdp1-TAP (data not shown).

We have previously shown that the localization of RITS to centromeric DNA regions requires Dicer, suggesting that siRNAs are required for targeting RITS to specific DNA regions (Verdel et al., 2004). In order to determine whether the localization of RITS and RDRC to *cen* RNAs is similarly Dicer dependent, we carried out RNA immunoprecipitations from *dcr1*Δ cells. In *dcr1*Δ cells, *cen* RNAs are derepressed and are present at 8- to 10-fold higher levels than in *dcr1*$^+$ cells (Volpe et al., 2002; Figure 4D, compare left and right panels). Because background signal in RNA-IP experiments is directly proportional to the relative abundance of the RNA (Gilbert et al., 2004), and *cen* RNAs are more abundant in *dcr1*Δ cells, we used untagged *dcr1*Δ cells as control for background signal in these experiments (Figure 4D, lane 5). As shown in Figure 4D, in *dcr1*Δ cells, *cen* RNAs were not significantly enriched in Rdp1-TAP, Hrr1-TAP, or Chp1-TAP precipitations compared to the untagged control (Figure 4D, compare lanes 6–8 with lane 5). Furthermore, we observed no significant association of Chp1-TAP with *cen* RNAs in *rdp1*Δ, *hrr1*Δ, *cid12*Δ, or *clr4*Δ cells above that observed in *dcr1*Δ (Figure 4E, data not shown). We conclude that components of the RITS and RDRC complexes localize to *cen* RNAs in a Dcr1- and Clr4-dependent fashion.

RDRC and RITS Are Preferentially Localized to the Nucleus

We next determined the subcellular localization of components of the RITS and RDRC complexes. Of the six

proteins that constitute these complexes, only the Chp1 localization pattern is known (Doe et al., 1998; Sadaie et al., 2004). We inserted 13 myc epitopes at the C terminus of the Chp1, Tas3, Rdp1, Hrr1, and Cid12 subunits of the two complexes and determined their subcellular localization by indirect immunofluorescence. Ago1-myc13 was not functional, but we constructed a strain that expressed a functional amino-terminally tagged Ago1 (myc3-Ago1) for these experiments (also used in Figure 4C). Consistent with previous observations, Chp1-myc13 localized to the nucleus in a speckled pattern of about four foci, which represent the different heterochromatic chromosome regions (Doe et al., 1998; Figures 5P–5R). As expected from the results described above, the Tas3 subunit of RITS displayed a staining pattern similar to Chp1 (Figures 5S–5U), providing evidence that the RITS complex is predominantly localized to the nuclear subdomain characteristic of heterochromatin proteins. Of the three subunits of RDRC, Hrr1-myc13 was predominantly nuclear (Figures 5J–5L), while Cid12-myc13 overlapped the DAPI-stained nuclear area but also was present to a lesser extent in the cytoplasm (Figures 5M–5O). Finally, consistent with its lower abundance compared to Hrr1 and Cid12, the Rdp1-myc13 signal was only weakly detectable above background. However, most of this signal overlapped the nucleus (Figures 5A–5F). Moreover, an overexpressed Rdp1-myc13 protein was localized to the nucleus in most cells but also was present in the cytoplasm, especially of mitotic cells (Figures 5G–5I, data not shown). We observed only weak staining above background for myc3-Ago1, which was present in a speckled pattern in the nucleus and throughout the cytoplasm (data not shown). These results are consistent with chromatin immunoprecipitation experiments showing that the RITS complex is specifically localized to heterochromatic DNA region (Verdel et al., 2004), and that Rdp1 is enriched at these regions, albeit to a lesser extent (Volpe et al., 2002; A.V. and D.M., unpublished observations).

RDRC Has RNA-Directed RNA Synthesis Activity

Rdp1 is a member of the family of cellular RDRs that are conserved in many organisms including fungi, plants, and *C. elegans* (reviewed in Baulcombe [2004]). Previously, RDR activity has been demonstrated for recombinant Qde1, a member of this family that is required for RNAi-related quelling in *N. crassa*, and for a tomato RDR (Makeyev and Bamford, 2002; Schiebel et al., 1998). We were interested in investigating the possible enzymatic activity of the fission yeast Rdp1 complex.

We tested whether full-length Rdp1-TAP or two C-terminal truncations that removed portions of the conserved region, believed to be part of the active site of the enzyme, had RNA synthesis activity, using a 500 nucleotide single-stranded RNA template corresponding to the *Photius pyralis* luciferase (*Pp*-luciferase) gene. Rdp1-TAP and its C-terminal truncations, Rdp1-Δ314-TAP and Rdp1-Δ45-TAP, were expressed to similar levels and were purified with similar yields (Figures 6A and 6B). RNA synthesis reactions were carried out using these purified proteins in the presence of single-stranded template RNA, ^{32}P-UTP, and all four unlabeled ribonucleotides as described previously (Makeyev and Bamford, 2002). Rdp1-TAP synthesized two labeled RNA species of approximately 175 and 500 nucleotides (Figure 6C, lanes 3 and 4). The size of the larger product is consistent with the transcription of complementary RNA corresponding to the full length of the template RNA. We are uncertain of the nature of the smaller 175 nucleotide product but it may result from premature termination. As controls, we observed no activity in reactions that lacked the RNA template (Figure 6C, lane 7). Moreover, the addition of a complementary RNA oligonucleotide as a primer had no effect on the efficiency of the reaction (Figure 6C, compare lanes 3 and 4). This template-dependent RNA synthesis activity was abolished with the C-terminal truncation of Rdp1 (Rdp1-Δ314), indicating that the activity was intrinsic to Rdp1 (Figure 6C, lane 5). Purified Rdp1 complexes also had RNA-directed RNA polymerase activity using an RNA template produced from the *Renilla* luciferase gene, which is unrelated to the *Pp*-luciferase used above (data not shown), indicating that activity was independent of the primary sequence of the RNA template.

As positive controls for the above experiments, we used the *N. crassa* Qde1 and the bacteriophage $\phi 6$ RNA-directed RNA polymerases (Makeyev and Bamford, 2002). Using the luciferase RNA template, these enzymes synthesized RNA products which were similar in length to those synthesized by Rdp1-TAP (Figure 6D).

To facilitate the detection of Rdp1 activity, we constructed strains that overproduced Rdp1-TAP proteins from the *nmt1* promoter and examined their activities. We observed a large increase in RNA synthesis activity with Rdp1-TAP protein overexpressed from the *nmt1* promoter (Figure 6E, compare lanes 3 with 4) and could detect this activity with enzyme immobilized on IgG-Sepharose beads. The activity of overexpressed Rdp1-TAP was abolished with the truncation of either 314 or 45 amino acids from the C terminus of Rdp1 and required an RNA template (Figure 6D, compare lanes 4–6 and left panel with right panel). Moreover, the activity of the overexpressed Rdp1-TAP was not affected by deletion of *cid12*$^{+}$ (Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/6/789/DC1/). Since Cid12 is required for the association of Hrr1 with Rdp1 (Table 1), we conclude that the integrity of the RDRC complex is not required for its RNA synthesis activity. The deletion analysis then indicates that the RNA synthesis activity observed in our experiments is intrinsic to Rdp1. Finally, loss of enzymatic activity for the above Rdp1 truncations correlated with the loss of centromeric silencing (Figure 6F). We also examined the activity of an Rdp1 active site point mutation and found that it lacked activity in vitro and silencing function in vivo (T. Sugiyama et al., submitted). Together these results suggest that the activity of Rdp1 is required for RNAi-mediated transcriptional gene silencing.

Discussion

The biochemical analysis presented in this study provides insight into the relationship between two key protein complexes that mediate RNAi-dependent heterochromatin assembly in fission yeast and suggests a mechanism for their association with specific chromosome regions (Figure 7). The major findings in this study are as follows: (1) the fission yeast Rdp1 is associated

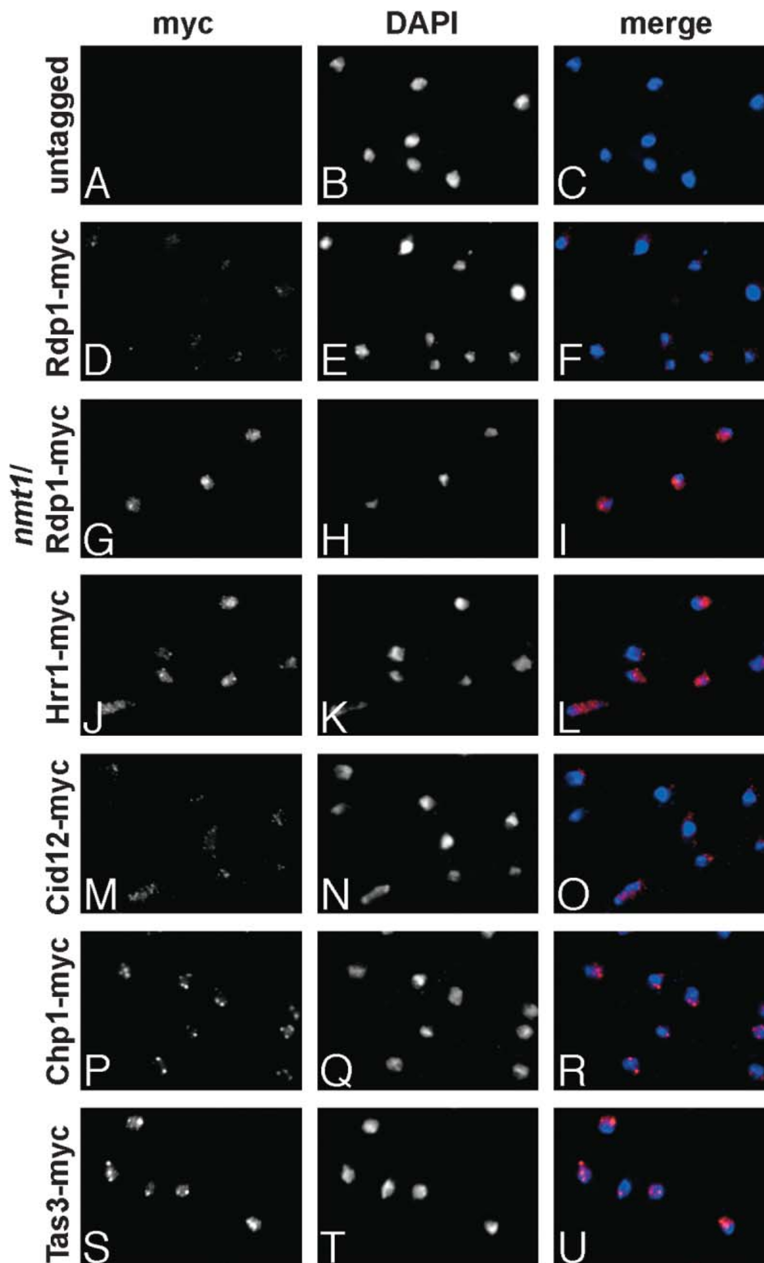

Figure 5. Immunofluorescence Experiments Showing the Subcellular Localization of the Subunits of RITS and RDRC (Tagged with 13 myc Epitopes)

Strains are described in Supplemental Table S1.

with two highly conserved proteins in a complex, termed RDRC, which has RNA-directed RNA synthesis activity, (2) the RDRC and RITS complexes associate together in a Dcr1- and Clr4-dependent manner, suggesting that both siRNA-based target recognition and chromatin association are involved in mediating this interaction, and (3) the components of the RDRC and RITS complexes associate with noncoding centromeric RNA in a Dcr1- and Clr4-dependent manner. We discuss the implications of these findings for the mechanism of RNAi-dependent heterochromatin assembly.

Association of RITS with RDRC and Its Possible Role in Regulation of dsRNA and siRNAs Production

The biochemical analysis presented in this study uncovers a network of protein-protein interactions that physically link the RITS and RDRC complexes (summarized in Figure 7A). The association of RDRC with the RITS complex is Dcr1 and Clr4 dependent and correlates with the presence of siRNAs in RITS (Figure 3). What is the biological significance of these observations? The in vivo RNA synthesis activity of RDRC must be tightly regulated so that only specifically selected RNAs are targeted for dsRNA synthesis. The source of this specificity ultimately must reside in siRNAs that target specific chromosome regions for assembly into heterochromatin. A possible explanation for our observations is that RITS acts as a "priming complex" for the dsRNA synthesis activity of RDRC, in a fashion analogous to mechanisms that regulate the dsRNA synthesis activity of viral RDRs. Viral RDRs often require specific proteins that bind to viral RNA sequences and act as "primers" for the initiation of RNA synthesis by recruiting the RDR to its RNA template (van Dijk et al., 2004). The Dcr1-

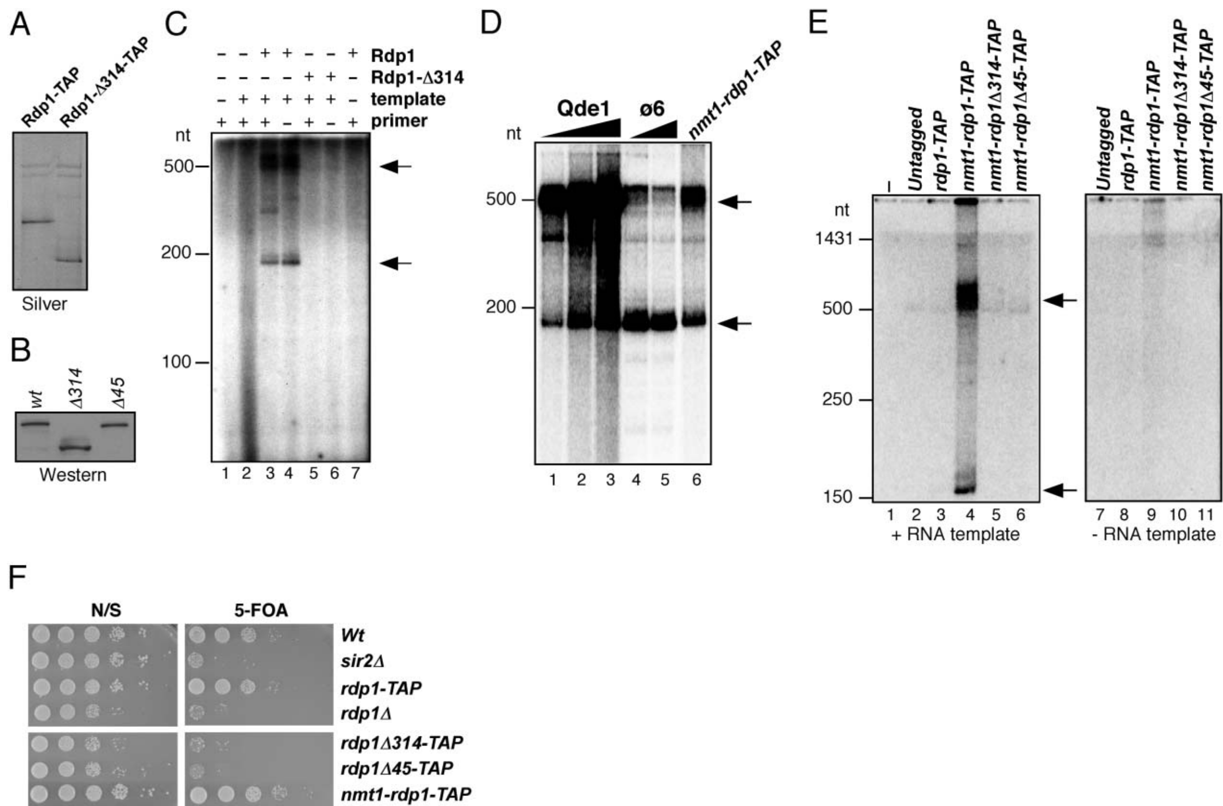

Figure 6. RDRC Has RNA-Directed RNA Polymerase Activity

(A) Purification of full-length Rdp1 (Rdp1-TAP) and its C-terminal truncation (Rdp1-Δ314).

(B) Rdp1-Δ314 and a smaller C-terminal truncation (Rdp1-Δ45) are expressed at wild-type levels.

(C) RNA template-dependent RNA synthesis activity of Rdp1-TAP using a 503-nucleotide *Pp*-luciferase RNA (lane 4); no activity was observed in the absence of the purified enzyme (lanes 1 and 2), in the absence of the *Pp*-luciferase RNA template (lanes 7), or when Rdp1-TAP was replaced with Rdp1-Δ314 (lanes 5 and 6). The addition of a 23-nucleotide primer complementary to the 3′ end region of *Pp*-luciferase had little or no effect on RNA synthesis activity (lane 3).

(D) The *N. crassa* Qde1 and the bacteriophage ϕ6 RDRs were used as positive controls with the *Pp*-luciferase RNA template (used in [C] and [E]) and produced similar size RNA products (lanes 1–6).

(E) Reactions were carried out as in (C) but with overexpressed full-length or Rdp1 truncations immobilized on IgG-Sepharose beads.

(F) Silencing of *imr1R:: ura4*$^{+}$ is lost in *rdp1-Δ45* and *rdp1-Δ314*, whereas full-length Rdp1-TAP displays wild-type levels of silencing. Arrows highlight the position of Rdp1-synthesized RNAs.

dependent association of RDRC and RITS complexes with each other and with centromeric RNAs suggests that RITS may direct specific dsRNA synthesis by acting as a priming complex that recruits RDRC and promotes its assembly on target RNAs (Figure 7B). In this model, siRNAs would act as specificity factors for dsRNA synthesis by RDRC—whether or not RDRC extends the 3′ end of RITS bound siRNAs or begins RNA synthesis in a nearby region on the RNA template. In our in vitro reactions, the presence of an RNA primer had no significant effect on the RNA synthesis activity of RDRC. Similarly, the RNA template-directed activity of the *Neurospora* Qde1 RDR is largely primer independent (Makeyev and Bamford, 2002). Both these observations suggest that an RNA primer (or an siRNA) is unable to stimulate RNA-directed RNA polymerase activity by itself and may require a priming complex to promote dsRNA synthesis. The ultimate test of this priming hypothesis requires the demonstration that an siRNA-programmed RITS can trigger dsRNA synthesis on specific RNA templates by RDRC.

RDRs have been proposed to perform two distinct functions in RNA silencing. The first involves using siRNAs as primers to synthesize dsRNAs, which serve to amplify the RNAi response and allow spreading of RNAi silencing (Sijen et al., 2001). Spreading refers to the ability of exogenously introduced siRNAs to stimulate the generation of new siRNAs, beyond the sequences to which they are complementary, in a phenomenon termed transitive RNAi (Baulcombe, 2004; Sijen et al., 2001). The second role of RDRs has been proposed to involve using aberrant cellular transcripts, associated with repeated transgenes, as templates to synthesize dsRNA to initiate the RNAi response (Baulcombe, 2004). In the first case, some siRNAs must be present in the absence of RDR, whereas in the second case the generation of initial siRNAs absolutely requires an RDR enzyme.

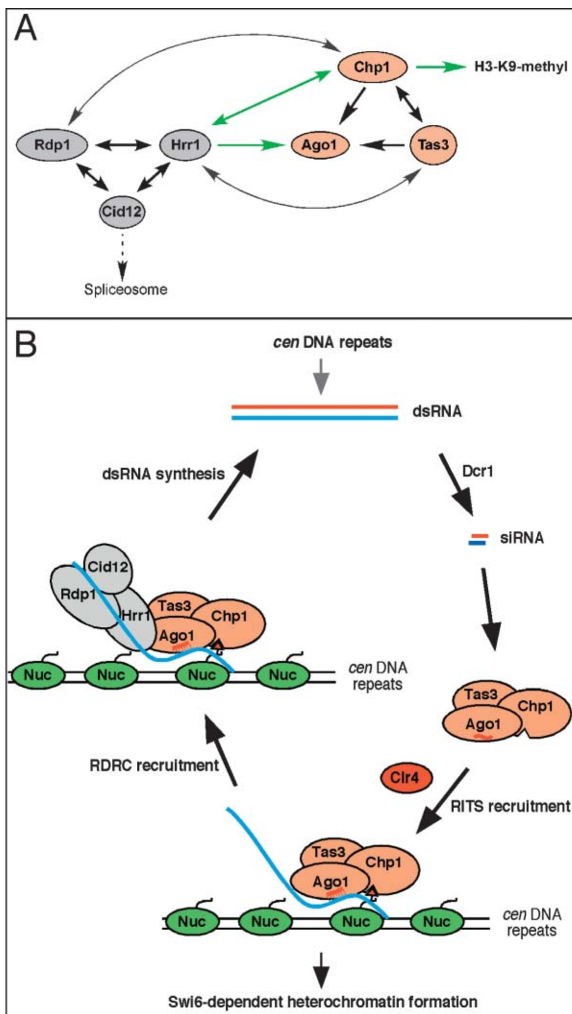

Figure 7. RNAi Complexes and Model for Their siRNA-Driven Association with Specific Chromosome Regions

(A) The network of protein-protein interactions involving the RITS and RDRC complexes. Straight arrows, interactions identified by mass spectrometry of gel bands or mixtures of purified proteins; curved arrow, interactions found in coimmunoprecipitation assays; green arrows, interactions that are Dcr1 and Clr4 dependent. The association with the spliceosome was Cid12 specific.

(B) Model for RITS-mediated initiation of heterochromatin assembly and its relationship to RDRC. See text for details.

We were unable to detect any siRNAs associated with RITS complexes purified from *rdp1*Δ, *hrr1*Δ, or *cid12*Δ cells, above the background level in *dcr1*Δ cells, suggesting that in fission yeast little or no siRNAs are produced in cells that lack RDRC. Moreover, previously it has been reported that Rdp1 is required for the production of siRNAs from a hairpin RNA transcribed from a strong *nmt* promoter (Schramke and Allshire, 2003). Together, these results argue that RDRC is required for the efficient generation of siRNAs. DsRNAs resulting from the pairing of complementary centromeric transcripts may be poor substrates for Dcr1, or may base pair too inefficiently, to produce enough dsRNA and siRNA for detection in our assays. An alternative explanation for the apparent absolute requirement of RDRC in generation of siRNAs is that RDRC may be required for loading dsRNA, its own products or those resulting from the pairing of complementary cen transcripts, onto the Dcr1 ribonuclease.

Our results also provide a possible explanation for the role of Clr4 in siRNA generation (Noma et al., 2004; Schramke and Allshire, 2003; this study). In *clr4*Δ cells, the association of RITS with Rdp1 and with centromeric RNAs is abolished. If RITS is in fact a priming complex that recruits RDRC to RNA templates, the failure to localize RITS to *cen* RNAs would result in a failure to recruit RDRC to initiate dsRNA synthesis and siRNA production (Figure 7B).

Association of Rdp1 with Hrr1 and Cid12

RDRs play a central role in RNAi-mediated gene silencing in plants, fungi, and *C. elegans* (Baulcombe, 2004; Sijen et al., 2001). Several viral RDRs have been previously purified and characterized (van Dijk et al., 2004). Purification of RDRC provides the first example of a cellular RDR complex and shows that the fission yeast RDR is associated with two highly conserved proteins that are also required for RNAi-mediated centromeric gene silencing. Although the exact role of Hrr1 and Cid12 is still unclear, their primary sequence offers clues to their physiological roles. Hrr1 shares a high degree of similarity to the DEAD box RNA helicases that belong to the Smg2/Upf1 family helicases (Figure 1E). Genetic studies have uncovered a requirement for members of the Smg2 family of RNA helicases in the RNAi response in several other organisms, including *Arabidopsis*, *N. crassa*, *Drosophila*, and *C. elegans*. The *C. elegans* Smg2 helicase is required for nonsense-mediated mRNA decay and RNAi (Domeier et al., 2000). However, the *C. elegans* helicase that is the most closely related to Hrr1 is an uncharacterized open reading frame (Figure 1E). The *Arabidopsis* Sde3 RNA helicase, a member of the Smg2 family, is required for dsRNA-induced RNAi-mediated silencing of a GFP transgene and has been proposed to function in the same genetic pathway as Sde1, an *Arabidopsis* RDR (Dalmay et al., 2001). The association of Hrr1 with Rdp1 and its requirement for RNAi-mediated centromeric silencing suggest that this conserved helicase may promote dsRNA synthesis by increasing the processivity of Rdp1 on its RNA templates.

Cid12 belongs to a family of proteins that contain a nucleotidyltransferase domain (NTD) that is weakly similar to the NTD found in both polyA polymerases (PAPs) and mammalian oligoadenylate synthetases (OASs) (Figure 1F; Justesen et al., 2000; Saitoh et al., 2002). PolyA polymerase activity for two members of the Cid family (the fission yeast Cid1 and Cid13) has been observed in vitro (Read et al., 2002; Saitoh et al., 2002). One possibility is that Cid12-mediated adenylation of RDRC-produced dsRNAs, directly or indirectly, contributes to further processing of these dsRNAs into siRNAs. In the case of OAS enzymes, the synthesis of a 2′-5′ linked oligoA tail creates a signal that recruits and activates RNase L, which degrades mRNAs and promotes apoptosis (Justesen et al., 2000). Thus, an intriguing alternative possibility is that Cid12-mediated oligoadenylation contributes to a later step in the assem-

bly process that stabilizes the association of RITS with chromatin, namely the recruitment of Clr4 via Rik1. The Rik1 protein physically interacts with Clr4 and is required for the recruitment of Clr4 to chromatin (Partridge et al., 2002; Sadaie et al., 2004). Rik1 also has a putative RNA binding domain that is similar to the RNA binding domain of cleavage polyadenylation specificity factor A (CPSF-A). CPSF-A recognizes the AAUAAA polyadeylation signal and is required for polyadenylation of most mRNAs (Barabino et al., 2000). We speculate that recognition of a Cid12-synthesized oligoA tail by the CPSF-A domain of Rik1 may provide a physical connection between RNAi complexes and enzymes that mediate heterochromatin-specific modifications.

Localization of RITS and RDRC to Noncoding Centromeric RNAs

Our observations suggest that the recognition of nascent transcripts by siRNA-programmed RITS mediates the initial localization of RITS to specific chromosome regions (Figure 7B). If this RNA-RNA recognition model is correct, the centromeric transcripts associated with RITS must be chromatin bound and in close proximity to their DNA targets in order to be able to achieve site-specific initiation. Two lines of evidence suggest that the associations of RITS and RDRC with centromeric RNAs observed in our experiments involve nascent or chromatin bound rather than mature cytoplasmic transcripts. First, subunits of both RITS and RDRC are localized predominantly to the nucleus, arguing against their association with mature cytoplasmic transcripts. Moreover, both Rdp1 and Hrr1 localize to centromeric DNA repeats, albeit with a lower efficiency than components of the RITS complex (Volpe et al., 2002; A.V. and D.M., unpublished data). This lower level of association may reflect lower RDRC DNA occupancy but is also consistent with a more peripheral association with chromatin that may be mediated through the interaction of RDRC with *cen* RNAs rather than DNA or histone tails. Second, and perhaps more importantly, the Clr4 histone methyltransferase, which is required for the localization of RITS to centromeric DNA repeats, is also required for the association of RITS with noncoding *cen* RNAs as well as for RITS-RDRC association. Clr4 methylates histone H3 at K9 and is thought to stabilize the binding of RITS to H3-K9-methyl via the chromodomain of Chp1, which in vitro binds specifically to H3-K9-methylated peptides (Partridge et al., 2002). These results argue for synergy between chromatin and RNA association and strongly suggest that RITS-*cen* RNA association involves nascent transcripts (Figure 7B). Thus, noncoding RNAs may play multiple roles in RNAi-mediated heterochromatin assembly, including the production of dsRNA and siRNA, siRNA-directed localization of RITS, and possibly the recruitment of chromatin-modifying complexes.

Experimental Procedures

Strain and Plasmid Construction

Strains used in this study are listed in Supplemental Table S1 on the *Cell* website. Deletion, overexpression, and epitope-tagged strains were generated using a single-step gene replacement strategy. Genes encoding hygromycin B phosphotransferase (*hph*) and nourseothricin N-acetyl transferase (*nat*) were subcloned from pAG32 and pAG25, respectively, as described in Supplemental Data. Cells were transformed with PCR-amplified fragments containing kanamycin, hygromycin, or nourseothricin resistance cassettes flanked by 80-nucleotide sequences homologous to the insertion region; positive transformants were selected by growth on medium containing the appropriate antibiotic and confirmed by PCR and immunoblotting (Bahler et al., 1998; Supplemental Data online).

Silencing Assays

Silencing of centromeric *ura4*$^{+}$ strains were performed as described (Verdel et al., 2004).

Cell Growth, Affinity Purification, and Protein Identification

Rdp1-TAP, Hrr1-TAP, Cid12-TAP, Chp1-TAP, and Tas3-TAP were purified from wild-type or mutant backgrounds, and associated proteins were identified by mass spectrometry of individual gel bands and trichloroacetic acid-precipitated pellets of total mixtures, as described previously with modifications described in Supplemental Data (Verdel and Moazed, 2004).

RDR Activity Assays

RDR activity assays were performed mainly as described by Makeyev and Bamford (2002). Briefly, assays were conducted in 10 μl reactions containing 50 mM HEPES-NaOH pH7.6, 20 mM ammonium acetate, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, 1 mM GTP, 0.2 mM CTP, 0.2 mM UTP, 0.4 units/μl RNase Inhibitor, 5.6% (W/V) PEG4000, and 0.2–0.3 μCi/μl [α-32P] UTP (3000 Ci/mmol, NEN Life Science). 80–250 μg/ml single-stranded RNA template were present in each reaction. Single-stranded RNA was made from the coding sequence of *Photius pyralis* luciferase (*Pp*-luciferase) present in pGL3-Basic vector (Promega). The region corresponding to nucleotides 94–597 of the luciferase coding sequence was amplified by PCR with the forward primer also containing the minimal promoter for T7 RNA polymerase. Single-stranded RNA was produced and purified using T7 polymerase and the MEGAscript kit (Ambion) as described by the manufacturer. Single-stranded RNA was also produced from the *Renilla* luciferase gene, which is unrelated to the *Pp*-luciferase gene.

Three to five microliters of native complexes containing wild-type or mutant Rdp1, isolated by tandem affinity purification, were added per reaction. Recombinant Qde1 and ϕ6 polymerase were used at final concentrations of 0.6–2.5 mg/ml and 1000–2000 units/ml, respectively. For the ϕ6 RDR assays the reaction was supplemented with 2 mM $MnCl_2$. When indicated, primer complementary to the RNA template was added to the reaction at a final concentration of 10 μg/ml and incubated at room temperature for 10 min before the RDRs were added. RDR reactions were incubated 1–2 hr at 30°C. Reaction products were treated with 20 μg proteinase K and 0.5% SDS at 60°C for 15 min, and RNA was isolated by phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. RNA was separated on 6.5 or 15% urea-acrylamide gel and the products of RDR activity were visualized by autoradiography and/or exposure to a phosphorimager screen.

Small-scale RDR assays using overexpressed and immunoprecipitated Rdp1 proteins were conducted as follows. One hundred milliliter culture at an OD600 of 2.5 to 3.0 was harvested and washed once in cold lysis buffer (6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1% NP-40, 2 mM EDTA, 2 mM EGTA, 50 mM sodium fluoride, 0.1 mM sodium vanadate). Cells were resuspended in one volume of cold lysis buffer, which was supplemented just before use with 1 mM DTT, 1 μg/ml of leupeptin, pepstatin, and bestatin, 1 mM benzamidine, and 1 mM PMSF. Cold glass beads were added to the cells and cells were lysed by bead beating twice for 30 s with a 2 min rest on ice between the bead beating steps. After centrifugation at 3000 g for 5 min at 4°C, the supernatant was recovered and mixed with 60 μl of 1:1 IgG-Sepharose slurry. The mixture was incubated for 2 hr at 4°C with constant rotation. Sepharose beads were washed three times in lysis buffer and once in 50 mM HEPES-NaOH pH7.6, 20 mM ammonium acetate, 5 mM MgCl2, 0.1 mM EDTA. Three to six microliters of the IgG-Sepharose beads containing immobilized Rdp1 or its C-terminal deletions was then used for RDR activity assays as described above.

Immunoprecipitation Assays

Cell extracts were obtained by bead lysis in immunoprecipitation buffer (6 mM Na_2HPO_4, 4 mM $NaH_2PO_4 \cdot H_2O$, 200 mM $NaC_2H_3O_2$, 0.25% NP-40, 5 mM Mg $C_2H_3O_2$, 2 mM EDTA, 1 mM EGTA, 50 mM NaF, 50 mM Na_3VO_4, 5% glycerol, 2 mM PMSF, 1 μg/ml pepstatin, 1 μg/ml bestatin, 1 μg/ml leupeptin, and Roche Complete EDTA-free protease inhibitor cocktail). Lysates were cleared by centrifugation and normalized using Bio-Rad Protein Assay before precipitation with 10 μl IgG Sepharose 6 Fast Flow beads (Amersham Biosciences) for 2 hr at 4°C. Beads were then washed four times in immunoprecipitation buffer, transferred to new tubes, and washed two more times with immunoprecipitation buffer lacking NP-40 and protease inhibitors. Immunoprecipitated complexes and input extracts were analyzed by standard immunoblotting procedures using horseradish peroxidase-conjugated anti-peroxidase (PAP, Sigma) and monoclonal anti-myc 9E10 antibody (BabCo).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed, using primers listed in Supplemental Table S2 online, as described previously (Huang and Moazed, 2003), except that cells were crosslinked with 1% or 3% formaldehyde for 30 min. ^{32}P-labeled PCR products were separated on 6% acrylamide gels. After drying, gels were exposed to X-ray film and a phosphorimager screen for quantification.

RNA Immunoprecipitation (RNA-IP)

RNA-IP experiments were performed as previously described from 50 ml cultures grown in rich medium to OD600 of 1.5–2 with the following modifications (Gilbert et al., 2004; Hurt et al., 2004). Whole-cell extract, prepared from formaldehyde crosslinked cells, were supplemented with $MgCl_2$ and $CaCl_2$ to final concentrations of 25 and 5 mM, respectively, and treated with 700 units DNase I per ml for 1 hr at 30°C. We found that high concentrations of DNase I were required to completely remove centromeric DNA from whole-cell extracts. RT-PCR was performed using the one-step RT-PCR kit from Qiagen with centromeric dh primers described previously (Volpe et al., 2002; Supplemental Table S2). Reaction products were separated on 2.2% Agarose TAE gels containing 2.5 mg per liter ethidium bromide. Approximate quantification was based on visual inspection of band intensities of 2-fold serial dilutions. For the detection of *cen* and *act1* RNAs after the reverse transcriptase step, 31–35 and 25 PCR cycles were performed, respectively.

SiRNA Labeling

SiRNAs present in RITS were isolated from final eluates of tandem affinity purifications and labeled mainly as previously described and detailed in Supplemental Data (Verdel et al., 2004; Verdel and Moazed, 2004).

Immunofluorescence Localization

Indirect immunofluorescence staining was conducted using 13myc epitope tagged strains and monoclonal anti-myc 9E10 antibody primary antibody (BabCo) and rhodamine-conjugated goat anti-mouse secondary antibody (Jackson Immunochemicals) as described in Supplemental Data.

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References

Bahler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., 3rd, Steever, A.B., Wach, A., Philippsen, P., and Pringle, J.R. (1998). Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast *14*, 943–951.

Barabino, S.M., Ohnacker, M., and Keller, W. (2000). Distinct roles of two Yth1p domains in 3′-end cleavage and polyadenylation of yeast pre-mRNAs. EMBO J. *19*, 3778–3787.

Baulcombe, D. (2004). RNA silencing in plants. Nature *431*, 356–363.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363–366.

Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D.C. (2000). 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.

Dalmay, T., Horsefield, R., Braunstein, T.H., and Baulcombe, D.C. (2001). SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in Arabidopsis. EMBO J. *20*, 2069–2078.

Doe, C.L., Wang, G., Chow, C., Fricker, M.D., Singh, P.B., and Mellor, E.J. (1998). The fission yeast chromo domain encoding gene chp1(+) is required for chromosome segregation and shows a genetic interaction with alpha-tubulin. Nucleic Acids Res. *26*, 4222–4229.

Domeier, M.E., Morse, D.P., Knight, S.W., Portereiko, M., Bass, B.L., and Mango, S.E. (2000). A link between RNA interference and nonsense-mediated decay in Caenorhabditis elegans. Science *289*, 1928–1931.

Ekwall, K. (2004). The RITS complex-A direct link between small RNA and heterochromatin. Mol. Cell *13*, 304–305.

Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. *15*, 188–200.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806–811.

Fukagawa, T., Nogami, M., Yoshikawa, M., Ikeno, M., Okazaki, T., Takami, Y., Nakayama, T., and Oshimura, M. (2004). Dicer is essential for formation of the heterochromatin structure in vertebrate cells. Nat. Cell Biol. *6*, 784–791.

Gilbert, C., Kristjuhan, A., Winkler, G.S., and Svejstrup, J.Q. (2004). Elongator interactions with nascent mRNA revealed by RNA immunoprecipitation. Mol. Cell *14*, 457–464.

Hall, I.M., Shankaranarayana, G.D., Noma, K., Ayoub, N., Cohen, A., and Grewal, S.I. (2002). Establishment and maintenance of a heterochromatin domain. Science *297*, 2232–2237.

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science *286*, 950–952.

Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R., and Hannon, G.J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. Science *293*, 1146–1150.

Hannon, G.J. (2002). RNA interference. Nature *418*, 244–251.

Hannon, G.J., and Rossi, J.J. (2004). Unlocking the potential of the human genome with RNA interference. Nature *431*, 371–378.

Huang, J., and Moazed, D. (2003). 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.

Hurt, E., Luo, M.J., Rother, S., Reed, R., and Strasser, K. (2004). Cotranscriptional recruitment of the serine-arginine-rich (SR)-like proteins Gbp2 and Hrb1 to nascent mRNA via the TREX complex. Proc. Natl. Acad. Sci. USA *101*, 1858–1862.

Hutvagner, G., and Zamore, P.D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. Science *297*, 2056–2060.

Justesen, J., Hartmann, R., and Kjeldgaard, N.O. (2000). Gene structure and function of the 2′-5′-oligoadenylate synthetase family. Cell. Mol. Life Sci. *57*, 1593–1612.

Lingel, A., Simon, B., Izaurralde, E., and Sattler, M. (2003). Structure and nucleic-acid binding of the Drosophila Argonaute 2 PAZ domain. Nature *426*, 465–469.

Makeyev, E.V., and Bamford, D.H. (2002). Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. Mol. Cell *10*, 1417–1427.

Matzke, M.A., Matzke, A.J., Pruss, G.J., and Vance, V.B. (2001). RNA-based silencing strategies in plants. Curr. Opin. Genet. Dev. *11*, 221–227.

Matzke, M., Aufsatz, W., Kanno, T., Daxinger, L., Papp, I., Mette, M.F., and Matzke, A.J. (2004). Genetic analysis of RNA-mediated transcriptional gene silencing. Biochim. Biophys. Acta *1677*, 129–141.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol. Cell *15*, 185–197.

Mello, C.C., and Conte, D., Jr. (2004). Revealing the world of RNA interference. Nature *431*, 338–342.

Mochizuki, K., Fine, N.A., Fujisawa, T., and Gorovsky, M.A. (2002). Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in tetrahymena. Cell *110*, 689–699.

Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D., and Grewal, S.I. (2004). RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. Nat. Genet. *36*, 1174–1180.

Pal-Bhadra, M., Leibovitch, B.A., Gandhi, S.G., Rao, M., Bhadra, U., Birchler, J.A., and Elgin, S.C. (2004). Heterochromatic silencing and HP1 localization in Drosophila are dependent on the RNAi machinery. Science *303*, 669–672.

Parrish, S., Fleenor, J., Xu, S., Mello, C., and Fire, A. (2000). Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. Mol. Cell *6*, 1077–1087.

Partridge, J.F., Scott, K.S., Bannister, A.J., Kouzarides, T., and Allshire, R.C. (2002). cis-acting DNA from fission yeast centromeres mediates histone H3 methylation and recruitment of silencing factors and cohesin to an ectopic site. Curr. Biol. *12*, 1652–1660.

Read, R.L., Martinho, R.G., Wang, S.W., Carr, A.M., and Norbury, C.J. (2002). Cytoplasmic poly(A) polymerases mediate cellular responses to S phase arrest. Proc. Natl. Acad. Sci. USA *99*, 12079–12084.

Reinhart, B.J., and Bartel, D.P. (2002). Small RNAs correspond to centromere heterochromatic repeats. Science *297*, 1831.

Sadaie, M., Iida, T., Urano, T., and Nakayama, J. (2004). A chromodomain protein, Chp1, is required for the establishment of heterochromatin in fission yeast. EMBO J. *23*, 3825–3835.

Saitoh, S., Chabes, A., McDonald, W.H., Thelander, L., Yates, J.R., and Russell, P. (2002). Cid13 is a cytoplasmic poly(A) polymerase that regulates ribonucleotide reductase mRNA. Cell *109*, 563–573.

Schiebel, W., Pelissier, T., Riedel, L., Thalmeir, S., Schiebel, R., Kempe, D., Lottspeich, F., Sanger, H.L., and Wassenegger, M. (1998). Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. Plant Cell *10*, 2087–2101.

Schramke, V., and Allshire, R. (2003). Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. Science *301*, 1069–1074.

Sigova, A., Rhind, N., and Zamore, P.D. (2004). A single Argonaute protein mediates both transcriptional and posttranscriptional silencing in Schizosaccharomyces pombe. Genes Dev. *18*, 2359–2367.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell *107*, 465–476.

Song, J.J., Smith, S.K., Hannon, G.J., and Joshua-Tor, L. (2004). Crystal structure of Argonaute and its implications for RISC slicer activity. Science *305*, 1434–1437.

Tijsterman, M., Ketting, R.F., and Plasterk, R.H. (2002). The genetics of RNA silencing. Annu. Rev. Genet. *36*, 489–519.

van Dijk, A.A., Makeyev, E.V., and Bamford, D.H. (2004). Initiation of viral RNA-dependent RNA polymerization. J. Gen. Virol. *85*, 1077–1093.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science *303*, 672–676.

Verdel, A., and Moazed, D. (2004). Isolation and labeling of siRNAs associated with the RNAi effector complex. RITS. Meth Enzymol, in press.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science *297*, 1833–1837.

Yan, K.S., Yan, S., Farooq, A., Han, A., Zeng, L., and Zhou, M.M. (2003). Structure and conserved RNA binding of the PAZ domain. Nature *426*, 468–474.

Zamore, P.D. (2002). Ancient pathways programmed by small RNAs. Science *296*, 1265–1269.

Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell *101*, 25–33.

Zilberman, D., Cao, X., and Jacobsen, S.E. (2003). ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science *299*, 716–719.