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Coupling of Double-Stranded RNA Synthesis and siRNA Generation in Fission Yeast RNAi

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SUMMARY

The fission yeast centromeric repeats are transcribed and ultimately processed into small interfering RNAs (siRNAs) that are required for heterochromatin formation. siRNA generation requires dsRNA synthesis by the RNA-directed RNA polymerase complex (RDRC) and processing by the Dicer ribonuclease. Here we show that Dcr1, the fission yeast Dicer, is physically associated with RDRC. Dcr1 generates siRNAs in an ATP-dependent manner that requires its conserved N-terminal helicase domain. Furthermore, C-terminal truncations of Dcr1 that abolish its interaction with RDRC, but can generate siRNA in vitro, abolish siRNA generation and heterochromatic gene silencing in vivo. Finally, reconstitution experiments show that the association of Dcr1 with RDRC strongly stimulates the dsRNA synthesis activity of RDRC. Our results suggest that heterochromatic dsRNA synthesis and siRNA generation are physically coupled processes. This coupling has implications for *cis*-restriction of siRNA-mediated heterochromatin assembly and for mechanisms that give rise to siRNA strand polarity.

INTRODUCTION

RNA interference (RNAi) is a highly conserved mechanism of gene regulation mediated by base-pairing interactions of small interfering RNAs (siRNAs) with target RNAs (Hamilton and Baulcombe, 1999; Martinez et al., 2002; Zamore et al., 2000). Targeting by siRNAs results in either translational inhibition or degradation of complementary mRNAs in a posttranscriptional mode of RNAi (Doench et al., 2003; Fire et al., 1998; Hammond et al., 2000; Zeng et al., 2002). siRNAs also function in initiation and maintenance of silent chromatin via the recruitment of histone modifying enzymes to targeted loci (Verdel et al., 2004; Volpe et al., 2002). Central to these processes is siRNA generation by RNase III-like Dicer enzymes (Bernstein et al., 2001; Ketting et al., 2001), inactivation of cognate RNA targets by Argonaute (Hammond et al., 2001), and siRNA amplification that in some systems requires an RNA-directed RNA polymerase (RdRP) (Sijen et al., 2001).

In fission yeast, RNAi requires *dcr1*$^+$, *ago1*$^+$, and *rdp1*$^+$, which encode Dicer, Argonaute, and RdRP, respectively. These core components of the RNAi pathway are all required for heterochromatin formation and transgene silencing at pericentric repeats (Volpe et al., 2002). The outer centromeric repeats are characterized by H3K9 methylation, binding of the HP1 homolog Swi6 protein, and localization of an Ago1-containing complex termed RNA-induced initiation of transcriptional gene silencing (RITS), which contains the heterochromatic chromodomain protein Chp1, siRNAs, and Tas3, a protein of unknown function (Allshire et al., 1994; Partridge et al., 2002; Verdel et al., 2004). RITS targets nascent noncoding centromeric RNAs presumably through base-pairing interactions of bound siRNAs complementary to centromeric sequences (Verdel et al., 2004). This hypothesis is supported by the finding that tethering of RITS to the native euchromatic *ura4*$^+$ transcript is sufficient to induce silencing, recruit heterochromatic proteins, and generate siRNAs (Buhler et al., 2006). Additionally, RITS binds another complex, termed RNA-dependent RNA polymerase complex (RDRC), that contains Rdp1, a putative helicase Hrr1, and a poly(A) polymerase family member Cid12 (Motamedi et al., 2004). RDRC similarly binds centromeric RNAs and is thought to be recruited by RITS to initiate double-stranded RNA (dsRNA) synthesis at nascent centromeric transcripts. Previous observations suggest that Dcr1 may also directly interact with these RNAi complexes. For example, siRNA-mediated gene silencing in fission yeast is *cis*-restricted (Buhler et al., 2006), suggesting that siRNAs are produced at the site of dsRNA synthesis on the chromosome. Furthermore, in fission yeast and several other systems, siRNAs display strand polarity such that only the antisense siRNA, corresponding to the RNA strand synthesized by RdRP, is loaded onto Argonaute (Buhler et al., 2007; Lee and Collins, 2006; Sijen et al., 2001). However, no physical interaction between Dcr1 and other RNAi complexes has been reported.

Long dsRNA is cleaved by Dicer from the ends into ~22 nt duplexes with 2 nt 3′ OH overhangs (Bernstein et al., 2001; Knight and Bass, 2001; Provost et al.,

2002a; Zhang et al., 2002). Cleavage is mediated by two highly conserved RNase III modules that each cut a single strand of dsRNA as part of an intramolecular dimer (Zhang et al., 2004). The signature overhang is generated by the staggered positions of the RNase III domains around the dsRNA groove, as shown by the crystal structure of a Giardia Dicer protein. More importantly, the Giardia Dicer structure shows the adjacent PAZ domain, which binds the ends of the dsRNA, to be positioned from the RNase III catalytic center at a distance that corresponds to the length of a 25 bp RNA duplex (Macrae et al., 2006). Therefore it has been proposed that the Dicer PAZ domain acts as part of a molecular ruler that measures the dsRNA substrate for processing into siRNAs of defined size. In fission yeast, Dcr1 lacks an apparent PAZ domain, thus raising the question of how siRNAs of defined size are generated in centromeric RNAi.

Most Dicer enzymes contain an N-terminal helicase/ATPase domain, and the cleavage activity of some Dicers is stimulated by ATP (Bernstein et al., 2001; Hutvagner et al., 2001; Nykanen et al., 2001). However, Giardia Dicer lacks an ATPase domain, and in vitro, human Dicer can generate siRNA in an ATP- and ATPase domain-independent manner (Macrae et al., 2006; Zhang et al., 2004). Instead, the dsRNA cleavage activity of human Dicer is dependent on the PAZ domain and a C-terminally located dsRNA-binding motif (Zhang et al., 2004). Fission yeast Dcr1 also contains an N-terminal helicase/ATPase domain, as well as a long C-terminal tail that bears no obvious similarity to the canonical dsRNA-binding motif. The role(s) of these domains in Dcr1 function and RNAi-mediated centromeric gene silencing is unknown.

In this study, we show that in addition to RNase III domains, which directly mediate dsRNA cleavage, the helicase/ATPase domain of fission yeast Dcr1 is essential for RNAi-mediated gene silencing. In contrast to Dicers that use the PAZ domain for siRNA size specificity, Dcr1 generates siRNA in an ATP-dependent manner that requires its helicase/ATPase domain. Furthermore, Dcr1 physically associates with the RDRC complex via its C-terminal domain, which also contains dsRNA-binding activity. Truncations of this C-terminal domain abolish Dcr1-RDRC association, siRNA generation, and gene silencing in vivo. Our findings suggest that the initiation and accurate control of a transcriptional mode of RNAi require the coupling of siRNA generation by Dcr1 to dsRNA synthesis by the RDRC complex at noncoding centromeric RNAs.

RESULTS

Dcr1 Mutants and Centromeric Silencing

Alignment of three Dicers from fission yeast, human, and Giardia shows the distribution and conservation of common domains (Figure 1A). Notably, no obvious homology to the PAZ domain or canonical dsRNA-binding motif has been detected in fission yeast Dcr1, whereas the largely uncharacterized helicase domain is conserved. Loss of *dcr1*$^+$ function results in loss of centromeric silencing, as evidenced by accumulation of noncoding centromeric transcripts and derepression of a *ura4*$^+$ reporter gene inserted in the *dg/dh* centromeric repeats (Volpe et al., 2002). To determine if the helicase and C-terminal domains of Dcr1 are required for RNAi, site-specific mutations in each domain were generated and tested for centromeric silencing (Figures 1B and 1C). A lysine to alanine substitution at position 38 corresponds to the ATPase Walker A motif of the helicase domain. Truncations of variable lengths were also generated at the C-terminal tail region. TAP tags fused to either the N- or C terminus of Dcr1 confirmed that these mutations do not affect Dcr1 protein levels (data not shown). Growth assays on 5-fluoro-orotic acid (5-FOA), which negatively selects against *cen::ura4*$^+$ reporter gene expression, were then used to assess the effects of Dcr1 mutations on RNAi-mediated centromeric silencing.

As shown in Figure 1C, both K38A and C-terminal truncation mutants of *dcr1* were defective for centromeric silencing. This silencing defect was comparable to that observed in *dcr1Δ* cells or cells carrying an aspartate to alanine substitution at position 937 (D937A) at the catalytic site of the Dcr1 RNase IIIa domain (Figures 1B and 1C). Consistent with a defect in RNAi, northern blot analysis showed loss of centromeric siRNAs in cells carrying each of the *dcr1* mutants that was comparable to that of *dcr1Δ* cells (Figure 1D). Therefore, the helicase, RNase III, and C-terminal domains of Dcr1 are all critical for centromeric silencing and RNAi in vivo.

Dcr1 Activity In Vitro

In vitro enzymatic studies of human Dicer have shown that ATP and an ATP-binding site at its N-terminal helicase region are dispensable for siRNA production. Meanwhile, mutation of Dicer C-terminal dsRNA-binding motif abrogates in vitro activity. Because Dcr1 clearly requires its helicase and C-terminal domains in vivo, we also wished to analyze the enzymatic activity of purified Dcr1 from fission yeast. Immunoprecipitated HA-tagged Dcr1 has previously been shown to display RNase activity that results in a ladder of cleavage products (Provost et al., 2002b). In order to understand how fission yeast Dcr1 generates siRNA of defined size, and to determine whether loss of silencing in various mutants correlates with defects in siRNA generation activity, we examined the activity of purified wild-type and mutant Dcr1 proteins.

Overexpressed wild-type and mutant Dcr1 proteins were immunopurified with a 3XFLAG epitope fused to the Dcr1 N terminus from fission yeast. A 160 kDa band corresponding to the full-length Dcr1 was visible after Coomassie staining without any other major protein except for a 75 kDa band that commonly contaminates FLAG purifications (Figure 2A). The concentrations of purified wild-type and mutant Dcr1 were estimated by comparison to BSA standards and tested for cleavage of 100 nt dsRNAs corresponding to centromeric repeat sequence. The dsRNA was designed to mimic

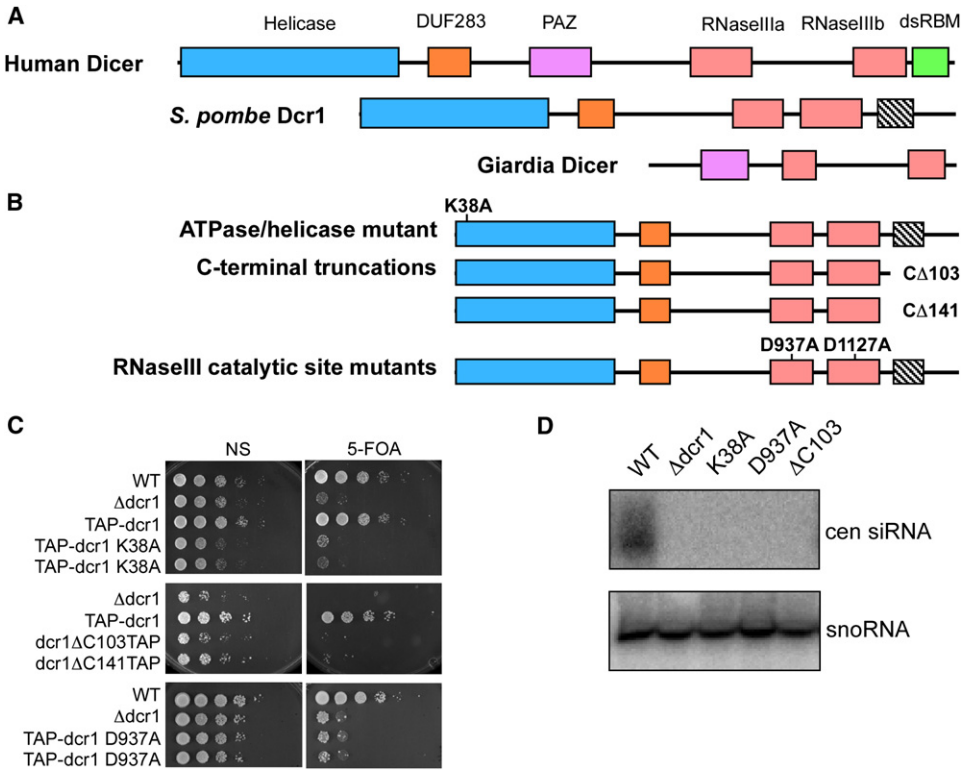

Figure 1. Dcr1 N- and C-Terminal Regions Are Required for Centromeric Silencing and siRNA Production

(A) Comparison of domain structures of human, fission yeast, and Giardia Dicers.

(B) Schematic diagrams showing the mutated sites at the helicase domain and in each or both RNase III catalytic centers. C-terminal truncations removed all or the last 103 amino acids of the region to the C terminus of the RNase IIIb domain.

(C) Growth assays on nonselective (NS) medium and medium supplemented with 5-fluoro-orotic acid (5-FOA) showing the effects of Dcr1 mutations on silencing of a *ura4*$^+$ transgene inserted in the centromeric inner repeat (*imr1R::ura4*$^+$). The wild-type parental strain and a *dcr1Δ* strain serve as controls.

(D) Northern blot showing loss of centromeric siRNAs in mutant *dcr1* cells.

a predigested Dicer substrate by containing 2 nt 3′ overhangs and optimized for sensitivity of the cleavage activity by radiolabeling at both 5′ ends followed by gel purification (Figure 2B).

3XFLAG-Dcr1 displays Mg^{2+}-dependent double-stranded ribonuclease activity that produces ~22 nt products consistent with the size of siRNAs (Figure 2C, compare lanes 1 and 2 with 7–10). In contrast to the discrete cleavage products produced by a similarly purified Giardia FLAG-Dicer or a commercially available human Dicer (Figure 2C, lanes 3–6 and 12), the primary fission yeast Dcr1 products varied in size and were accompanied by a ladder of minor cleavage products (~15–35-mer). The generation of such a ladder from an end-labeled template indicated that Dcr1 cuts its substrate internally as well as from the ends of the dsRNA (Figure 2C, lanes 7–10, and Figure 2D). This may reflect either an actual in vivo function or a requirement for an additional factor to load Dcr1 at the ends of dsRNA. Time-course experiments also showed that ~22 nt RNA species are generated within several seconds of Dcr1 addition to the substrate and are therefore primary products of Dcr1 cleavage activity (Figure 2D).

To test if the ~22 nt products were dependent on Dcr1 ribonuclease activity, catalytic site mutations of either or both RNase III domains were tested. The double mutant was completely defective for dsRNA cleavage, but unlike previous findings for human Dicer (Zhang et al., 2004), single catalytic site mutations of RNase IIIa or RNase IIIb domains retained dsRNA cleavage activity (Figure 3A, compare lanes 4–6 with lanes 7–9 and 10–12; Figure S1A in the Supplemental Data available with this article online). Moreover, native gel separation of Dcr1 products using internally labeled RNA substrates showed that Dcr1 generated duplex RNA cleavage products (Figure S1B). To determine if Dcr1 possesses specificity for terminal dsRNA structures, blunt-ended dsRNA was also tested for Dcr1 cleavage. Dcr1 generated similarly sized RNA cleavage products from either blunt ends or 2 nt 3′ overhangs. In contrast, Giardia Dicer produced larger siRNAs from blunt ends, probably due to the primary role of its PAZ domain in mediating size specificity (Figure S2A). Consistent with dsRNA termini-independent cleavage activity, Dcr1 did not display any significant homology with other PAZ domains and exhibited at best

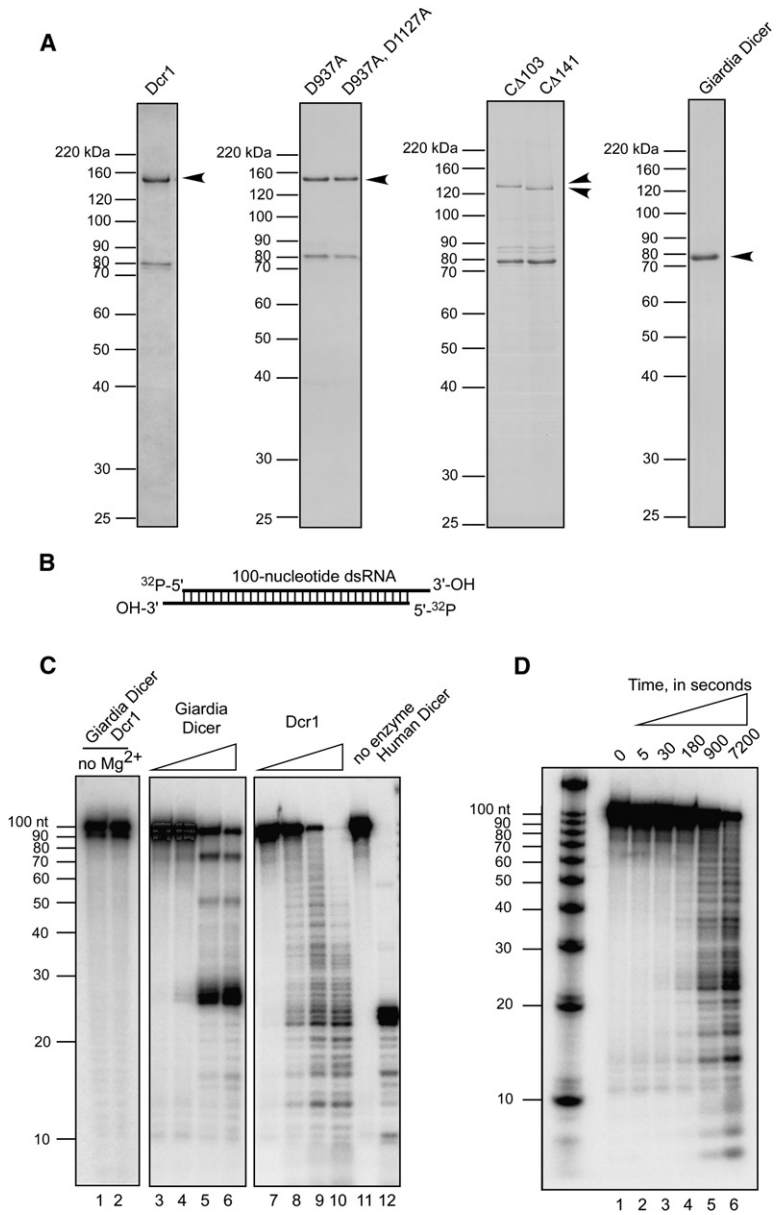

Figure 2. Dcr1 Cleaves dsRNA into siRNA-Sized Products

(A) Colloidal Coomassie staining showing FLAG-purified wild-type and mutant Dcr1 proteins.

(B) Diagram of the 100 nt dsRNA substrate used for Dcr1 cleavage assays.

(C) dsRNA cleavage assays compare increasing concentrations of Giardia Dicer with Dcr1 (4, 20, 100, and 500 ng); both enzymes require Mg^{2+}.

(D) Time-course experiment showing that Dcr1 generates the 22–24 nt product within ~30 s after the initiation of the cleavage reaction. RNA was separated in 13%–15% polyacrylamide-urea gels. All reactions were performed in the presence of ATP.

a poor sequence similarity with the conserved PAZ domain variant that includes *Arabidopsis* DCL1 (Figure S2B).

As shown in Figure 1, truncation of the C terminus of Dcr1 resulted in loss of centromeric gene silencing and siRNA generation. Consistent with a direct role in siRNA generation, truncations of Dcr1 removing the C-terminal 103 or 141 amino acids (CΔ103 and CΔ141, respectively) were defective for in vitro dsRNA cleavage. The CΔ141 protein showed no detectable activity (Figure 3A, lanes 16–18), but Dcr1-CΔ103 retained partial activity (Figure 3A, lanes 13–15). Secondary structure analysis of this C-terminal region predicted a divergent dsRNA-binding domain (residues 1262–1341) (Figure 3B). Several known domain templates were also used to model this region, but the final Dcr1 C-terminal structure shown is based on the *X. laevis* dsRNA-binding domain and in accord with Verify 3D evaluation (Eisenberg et al., 1997) (Figure 3B). Consistent with this prediction, we found that full-length Dcr1, but not its C-terminal truncations, possessed dsRNA-binding activity in mobility gel shift assays (Figure 3C, compare lane 2 with 5 and 6). This binding was independent of Mg^{+2} cation and was also observed for catalytically inactive Dcr1 (Figure 3C, lane 4). These results suggest that the C terminus of fission yeast Dcr1 contains a noncanonical dsRNA-binding domain, which contributes to dsRNA cleavage activity. Although Dcr1-CΔ103 did not appear to associate with dsRNA in the gel shift assay (Figure 3C), it displayed some cleavage

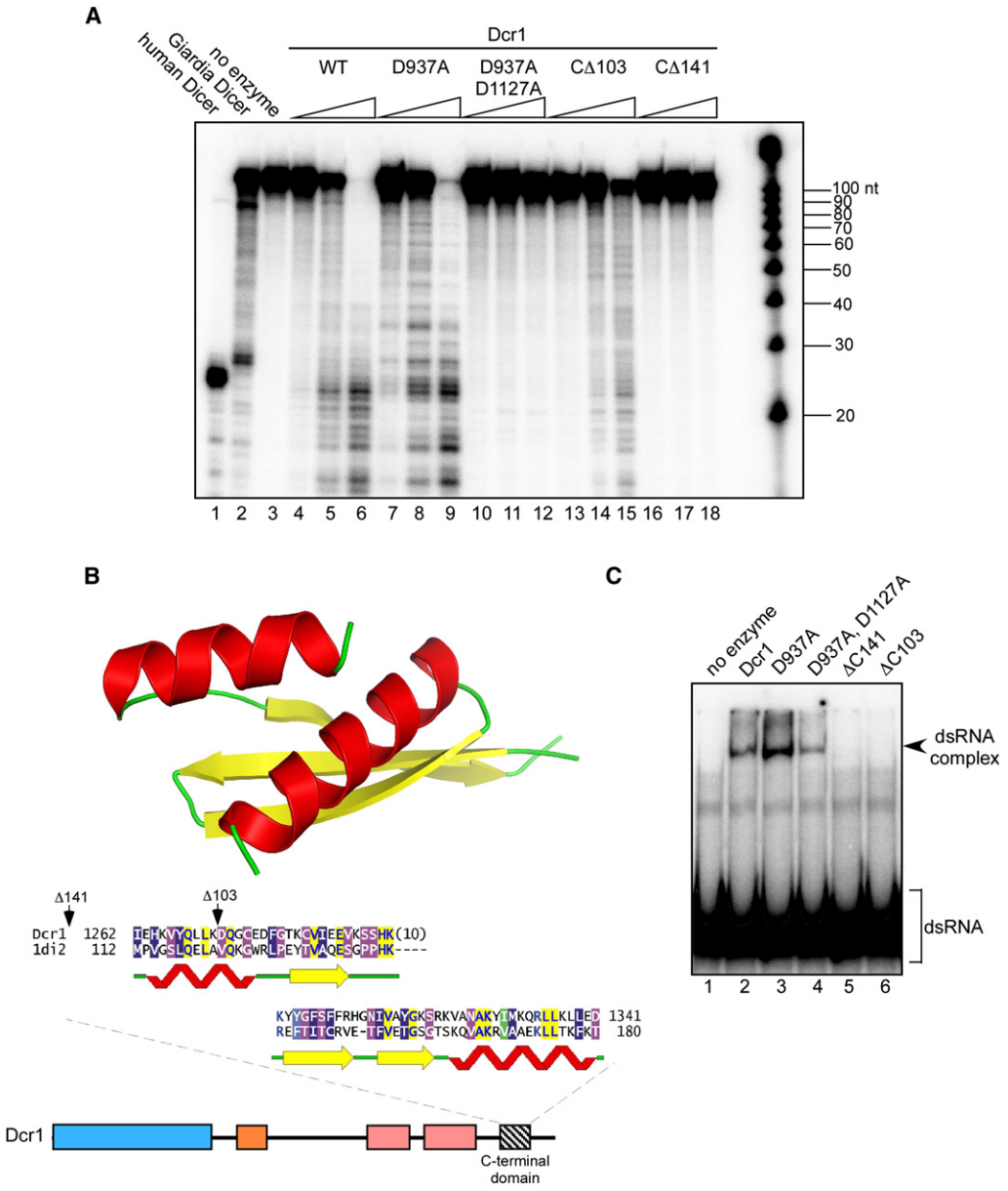

Figure 3. RNase III and dsRNA-Binding Activities of Dcr1

(A) dsRNA cleavage activity of 25, 100, and 400 ng of Dcr1 protein was compared between wild-type, RNase IIIa mutant D937A, RNase III double-mutant D937A, D1127A, and the C-terminal truncations CΔ141 and CΔ103. Reactions were carried out in the presence of Mg^{2+} and ATP.

(B) Secondary structure analysis of Dcr1 C-terminal domain (residues 1262–1341) reveals an αββββα motif characteristic of dsRNA-binding domains. A 3D model of this region is presented based on the *X. laevis* dsRNA-binding domain. Secondary structure elements from the template are shown schematically below its sequence.

(C) dsRNA-binding activity of 100 ng of the indicated wild-type and mutant Dcr1 protein at 4°C and in the absence of ATP and Mg^{2+}. Samples were separated in a 5% native polyacrylamide gel.

activity (Figure 3A, lanes 13–15), suggesting that it may retain some dsRNA binding activity that helps target it to its substrate.

The in vivo effects of the helicase mutation on silencing and siRNA generation suggested that Dcr1 is an ATP-dependent enzyme (Figures 1B–1D). In fact, in the absence of ATP, Dcr1 displayed only nonspecific dsRNA cleavage activity, whereas ATP had no effect on the activity of Giardia Dicer (Figure 4A, compare lanes 2 and 3 with 4 and 5). Moreover, purified Dcr1 K38A helicase mutant displayed weak internal dsRNA cleavage with little or no enrichment of the ~22 nt siRNA products generated by the wild-type protein (Figure 4B, compare lane 2 with lanes 6 and 7). This defect is attributable to loss of ATPase

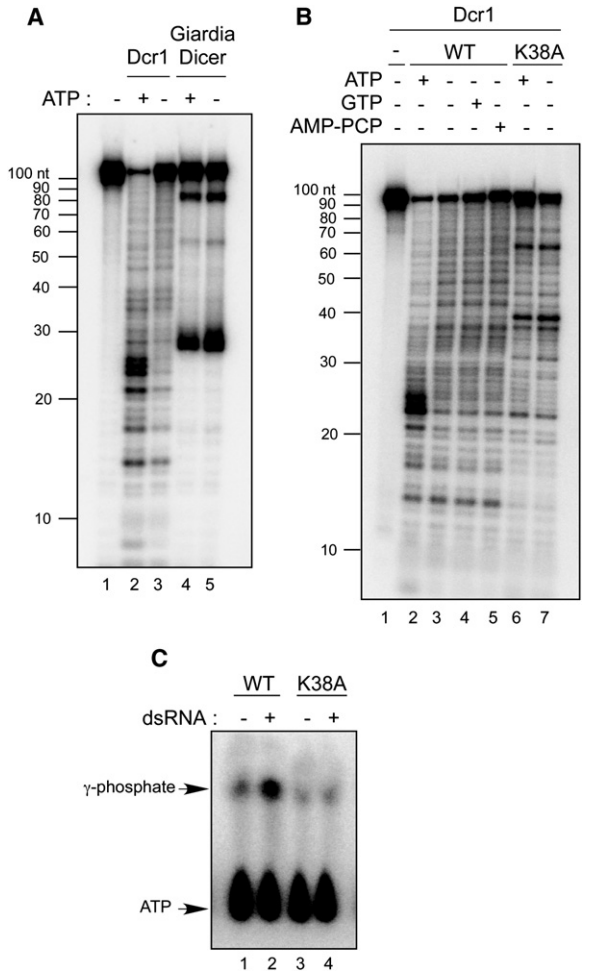

Figure 4. Dcr1 dsRNA Cleavage Requires ATP and the Helicase Domain

(A) Cleavage assay showing siRNA generation by Dcr1, but not Giardia Dicer, is dependent on ATP.

(B) siRNA generation requires ATP hydrolysis as a nonhydrolyzable AMP-PCP or GTP could not replace ATP; the K38A helicase mutant does not generate siRNAs.

(C) dsRNA stimulates the release of radiolabeled γ-phosphate from ATP by wild-type Dcr1, but not by the K38A mutant.

activity, as wild-type Dcr1 specifically fails to generate this product in the absence of ATP while retaining its internal cleavage activity (Figure 4A, lanes 1 and 2, and Figure 4B, lanes 2 and 3). Neither GTP nor the nonhydrolyzable ATP analog AMP-PCP could functionally replace ATP (Figure 4B, compare lane 2 with lanes 4 and 5). Moreover, ATP hydrolysis by Dcr1, but not the K38A mutant, was induced by addition of dsRNA (Figure 4C). These results indicated that fission yeast Dcr1 requires ATP hydrolysis to properly load onto and cleave dsRNA ends. This is in stark contrast to Giardia Dicer activity and previous results showing that human Dicer dsRNA cleavage occurs independently of ATP (Provost et al., 2002a) (Figure 4A, lanes 4 and 5). An ATP-independent form of dsRNA termini degradation activity was also observed for the Dcr1-K38A mutant that was consistent with the appearance of internal cleavage products, though the seemingly less random cleavage pattern suggests the mutation alters substrate accessibility (Figure 4A, lanes 6 and 7).

Association of Dcr1 with RDRC

In some organisms, Dicer resides in a complex with a dsRNA-binding protein, such as RDE-4 in *C. elegans* (Tabara et al., 2002), R2D2 and Loquacious in *Drosophila* (Forstemann et al., 2005; Liu et al., 2003), and TRBP and PACT in human (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006b). In posttranscriptional gene silencing, Dicer and the dsRNA-binding protein are incorporated into RISC (Chendrimada et al., 2005; Gregory et al., 2005) and determine the polarity of siRNA loading based on thermodynamic stability rules (Tomari et al., 2004). To determine if fission yeast Dcr1 is in a similar complex, a fully functional N-terminally TAP-tagged Dcr1 expressed under the control of its endogenous promoter was purified. Mass-spectrometry analysis showed high coverage of peptides corresponding to Dcr1 and much lower coverage of other proteins (Figure 5A and data not shown), suggesting that Dcr1 is not in a stable complex with other proteins. Moreover, none of the other proteins consistently copurified with Dcr1, and their deletions did not exhibit a centromeric silencing defect (data not shown).

The heterochromatin-dependent mode of RNAi in fission yeast may have evolved to regulate Dcr1 activity in a manner distinct from that observed in posttranscriptional gene silencing. Such regulation may involve a different set of proteins that function at chromatin or nascent centromeric RNAs where RITS and RDRC complexes have previously been shown to localize (Motamedi et al., 2004). To determine if known RNAi components interact with Dcr1, TAP-tagged Dcr1 was analyzed for coimmunoprecipitation with other myc-tagged proteins. Dcr1 displayed a robust interaction with Rdp1 (Figure 5B, lanes 3 and 4). Treatment with various nucleases did not affect binding of Rdp1, suggesting direct protein-protein interaction (Figure 5C, compare lanes 3–6). We note that mass-spectrometry analysis of multiple TAP purifications of Dcr1 did not identify any of the known RNAi proteins in fission yeast, nor did TAP purifications of RITS and RDRC subunits reveal an interaction with Dcr1 (Motamedi et al., 2004; Verdel et al., 2004). Therefore, RDRC and Dcr1 may only interact transiently, but in a manner that is detectable in coimmunoprecipitation assays.

We next tested whether the Dcr1-Rdp1 interaction required other RDRC subunits or RNAi components. As shown in Figure 5D, Dcr1-Rdp1 interaction was dramatically reduced in *hrr1Δ*, *cid12Δ*, and *chp1Δ* cells, but not in *clr4Δ* cells (compare lane 2 with lanes 3–6). This result suggests that Dcr1 and Rdp1 preassemble in the absence of H3K9 methylation and heterochromatin assembly. Dcr1 also coimmunoprecipitated with the Cid12 and Hrr1

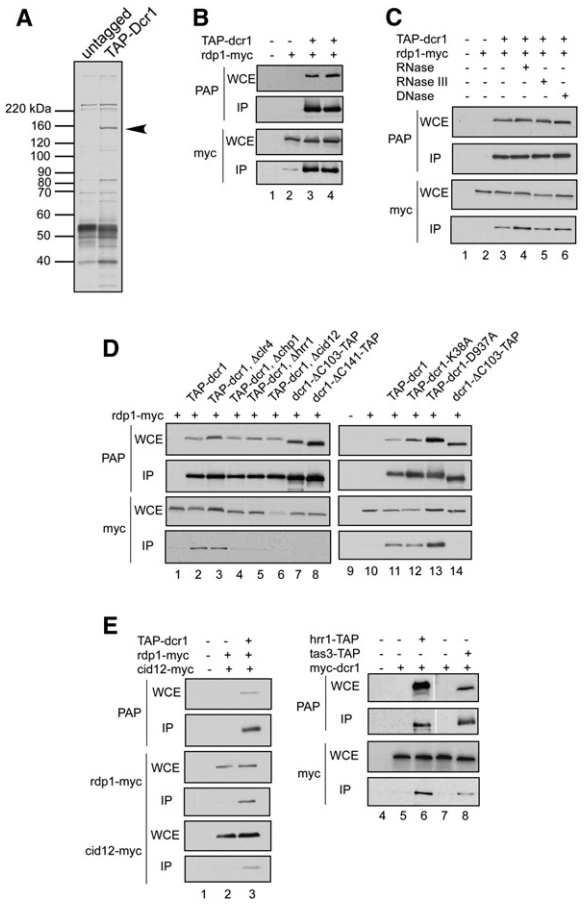

Figure 5. Dcr1 Interacts with RDRC and RITS

(A) Silver-stained polyacrylamide gel showing TCA-precipitated eluates from TAP purification of native Dcr1 and an untagged control strain.

(B) Western blot analysis of immunoprecipitated TAP-Dcr1 shows coprecipitation of myc-tagged Rdp1.

(C) Dcr1-Rdp1 interaction is resistant to RNase I, RNase III, or DNase I treatment.

(D) Dcr1-Rdp1 interaction in RNAi mutant cells.

(E) Dcr1 interacts with Rdp1, Cid12, Hrr1 subunits of RDRC, and the Tas3 subunit of RITS.

subunits of RDRC (Figure 5E, lanes 3 and 6). These results indicate that Dcr1 associates with the entire RDRC complex in a manner that requires the integrity of RDRC. Previously, RDRC has been shown to interact with RITS in an siRNA-dependent manner and is thought to be recruited to nascent centromeric RNAs by RITS. We also detected a weak interaction between Dcr1 and the Tas3 subunit of RITS, suggesting that Dcr1 is similarly recruited to nascent centromeric RNAs (Figure 5E, lane 8).

To determine if Dcr1-Rdp1 association requires a particular Dcr1 domain, we tested whether Rdp1 coimmunoprecipitated with various Dcr1 mutants. Both complete and partial truncations of the C-terminal region of Dcr1 (CΔ103 and CΔ141) abrogated Rdp1 interaction (Figure 5D, lanes 7, 8, and 14). The effect of the Dcr1 C-terminal truncations on Rdp1 binding is unlikely to be caused by disruption of Dcr1 structure because Dcr1-CΔ103 is competent for dsRNA cleavage and neither truncation caused a decrease in Dcr1 protein levels, suggesting that Dcr1 stability was not affected. Two distinct functions in the C terminus of Dcr1, dsRNA binding and RDRC association, are therefore likely to contribute to its in vivo function. In contrast, the K38A ATPase and D973A RNase IIIa mutants retained their ability to associate with Rdp1 (Figure 5D, compare lanes 11–13 with 14). These findings show that the interaction of RDRC with Dcr1 can persist even in the absence of Dcr1 cleavage activity and siRNAs.

The association of Dicer with RDRC suggests that Dicer mutants that retain their ability to associate with RDRC but are defective in siRNA generation may be able to act in a dominant-negative fashion by poisoning wild-type RDRC complexes. To test this hypothesis, wild-type and mutant *dcr1* expressed under the thiamine-repressible *nmt1* promoter were tested for their effects on centromeric silencing of wild-type cells. Overexpression of K38A and the RNase III mutants in cells that contained wild-type *dcr1*$^{+}$ strongly interfered with centromeric silencing, indicating that these mutants can compete with the wild-type protein for interactions within the RNAi pathway (Figure 6A). In contrast, overexpression of *dcr1* mutants lacking the C-terminal tail (Dcr1-CΔ103 and Dcr1-CΔ141), which cannot associate with RDRC or bind dsRNA, had no effect on silencing (Figure 6B). Northern blot analysis of these strains shows that overexpression of mutant Dcr1 severely reduced centromeric siRNA levels (Figure 6C). Interestingly, overexpression of wild-type Dcr1 resulted in generation of larger centromeric siRNAs, potentially due to the uncoupling of the overabundant Dcr1 from RDRC.

Coupling of Dicer and RDRC Activity

To determine the functional relationship between Dcr1 and Rdp1, we used purified proteins to examine possible effects of RDRC on Dcr1 activity and vice versa. Individual FLAG-tagged components of RDRC were overexpressed, purified from fission yeast, and shown to reconstitute RDRC activity. Addition of Rdp1, Hrr1, and Cid12 to a 500 nt single-stranded RNA corresponding to the centromeric *cen-dg* transcript generated radiolabeled products consistent with the size of the full-length template (Figure 7A, lane 2). Surprisingly, addition of the double RNase III mutant Dcr1 (R1R2), which lacks cleavage activity, stimulated synthesis of the double-stranded RDRC product (Figure 7A, compares lanes 2–4). This stimulation required the ability of Dcr1 to bind to RDRC, because catalytically inactive C-terminal truncation Dcr1 mutant, CΔ141, which is also defective for Rdp1 interaction, had no effect on dsRNA synthesis (Figure 7B). We also observed Dcr1 stimulation of Rdp1 activity independently of Hrr1 and Cid12 and to levels higher than that observed for RDRC reconstitution alone (data not shown). These data suggest a role for Dcr1 in increasing the

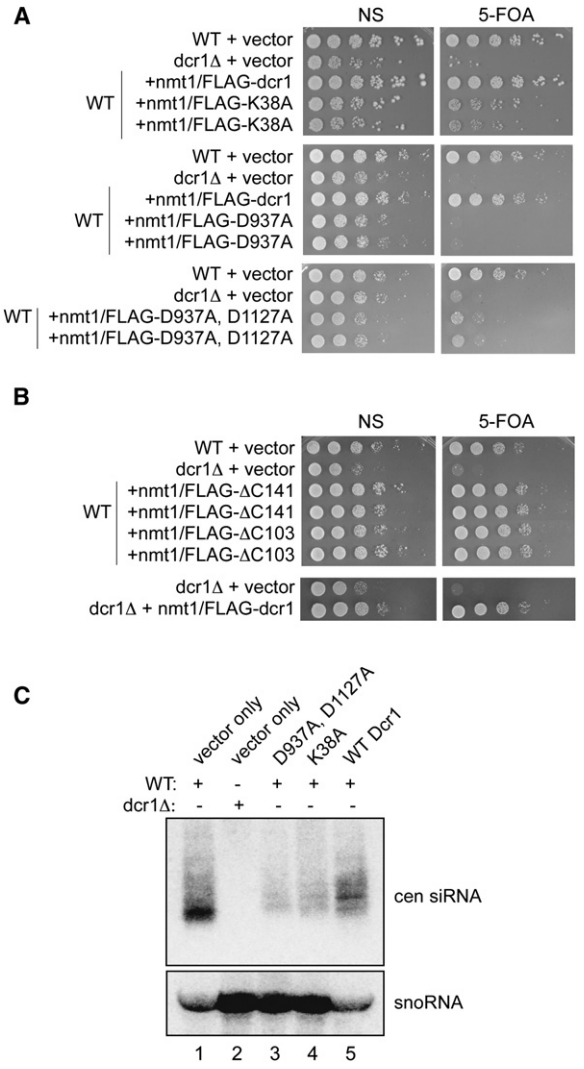

Figure 6. Dicer Mutants that Retain Rdp1 Interaction, but Are Defective for siRNA Generation, Are Dominant Negative for Centromeric Silencing

(A) Disruption of centromeric silencing by the Dcr1 helicase and RNase III mutants overexpressed from pREPN3XFLAG vectors in *dcr1*$^+$ cells.

(B) Overexpression of C-terminal tail truncations of Dcr1, which are unable to bind Rdp1, does not disrupt silencing in *dcr1*$^+$ cells.

(C) Centromeric siRNA levels are reduced in wild-type strains overexpressing the double RNase III and ATPase mutants of Dcr1 from the *nmt1* promoter (pREP-nmt1). Overexpression of wild-type Dcr1 increases the size range of centromeric siRNAs.

dsRNA synthesis activity of Rdp1 and further suggest that Dcr1 association with RDRC during dsRNA synthesis may function to target newly synthesized dsRNA for Dcr1 processing.

To verify that the products of RDRC/Dcr1-R1R2 reactions were double-stranded RNA, we employed a native gel assay that could resolve single- and double-stranded RNA size markers (Figure 7C, lanes 1 and 2). Native gel analysis of RDRC products indicated the presence of both single- and double-stranded RNA species (Figure 7C, lane 3). The single-stranded product may reflect either premature termination products that remained bound to the template or nucleotidyl addition to the template ends. Interestingly, Dcr1-R1R2 specifically stimulated the synthesis of the dsRNA product (Figure 7C, lane 4).

To recapitulate siRNA generation in a coupled RDRC-Dcr1 system, we examined siRNA production in reactions that contained RDRC and Dcr1 together with a single-stranded 500 nt RNA and ribonucleotides. Small RNAs consistent with the size of siRNAs were observed in the reactions containing RDRC and wild-type Dcr1 (Figure 7D, lanes 1–5), but not in reactions containing RDRC and R1R2 (Figure 7D, lanes 6–10). Dcr1 also produced siRNAs over a broad range of RNA template concentrations (Figure S3A). Together, these results demonstrate that Dcr1 could generate siRNAs directly from RDRC-synthesized dsRNAs.

To determine whether the coupling of Dcr1 and RDRC activities may be important for the efficient generation of siRNAs, we incubated Dcr1 with RNA purified from an RDRC/Dcr1-R1R2 reaction, which generates very high levels of dsRNA, and then examined siRNA production in the presence or absence of RDRC. The reactions were performed with ATP, but not other nucleotides, to prevent further RDRC dsRNA synthesis. As shown in Figure S3B, RDRC had no effect on siRNA accumulation by either Dcr1 (lanes 2 and 3) or Giardia Dicer (lanes 5 and 6). We also found that similar levels of siRNA were generated in reactions that contained Dcr1 during dsRNA synthesis by RDRC compared to reactions in which Dcr1 was added after RDRC/Dcr1-R1R2 was heat inactivated (Figure 7E, lanes 1–3, respectively). Similar results were obtained with Giardia Dicer (Figure 7E, lanes 4 and 5). Together our results indicate that the physical association of Dcr1 with RDRC stimulates dsRNA synthesis, but on preformed dsRNA templates, Dcr1 can generate similar levels of siRNA in the presence or absence of RDRC.

DISCUSSION

In this study, we have analyzed the mechanism of siRNA generation by the fission yeast Dicer enzyme. Our results suggest that the generation of heterochromatic siRNAs in fission yeast involves the coupling of dsRNA synthesis by RDRC with its processing into siRNA by Dcr1. We propose that Dcr1 and RDRC are targeted to single-stranded RNA templates as a complex or in an interdependent fashion. This hypothesis is supported by in vivo data showing that Dcr1 and RDRC physically interact and by in vitro activity assays demonstrating Dcr1-mediated stimulation of RDRC dsRNA synthesis. The importance of this coupled mechanism for RNAi-mediated heterochromatin assembly is supported by two observations. First, loss of Dcr1-Rdp1 interaction in C-terminal mutants of Dcr1 correlates with loss of centromeric siRNAs and centromeric gene silencing. Second, Dcr1 associates with both RITS and

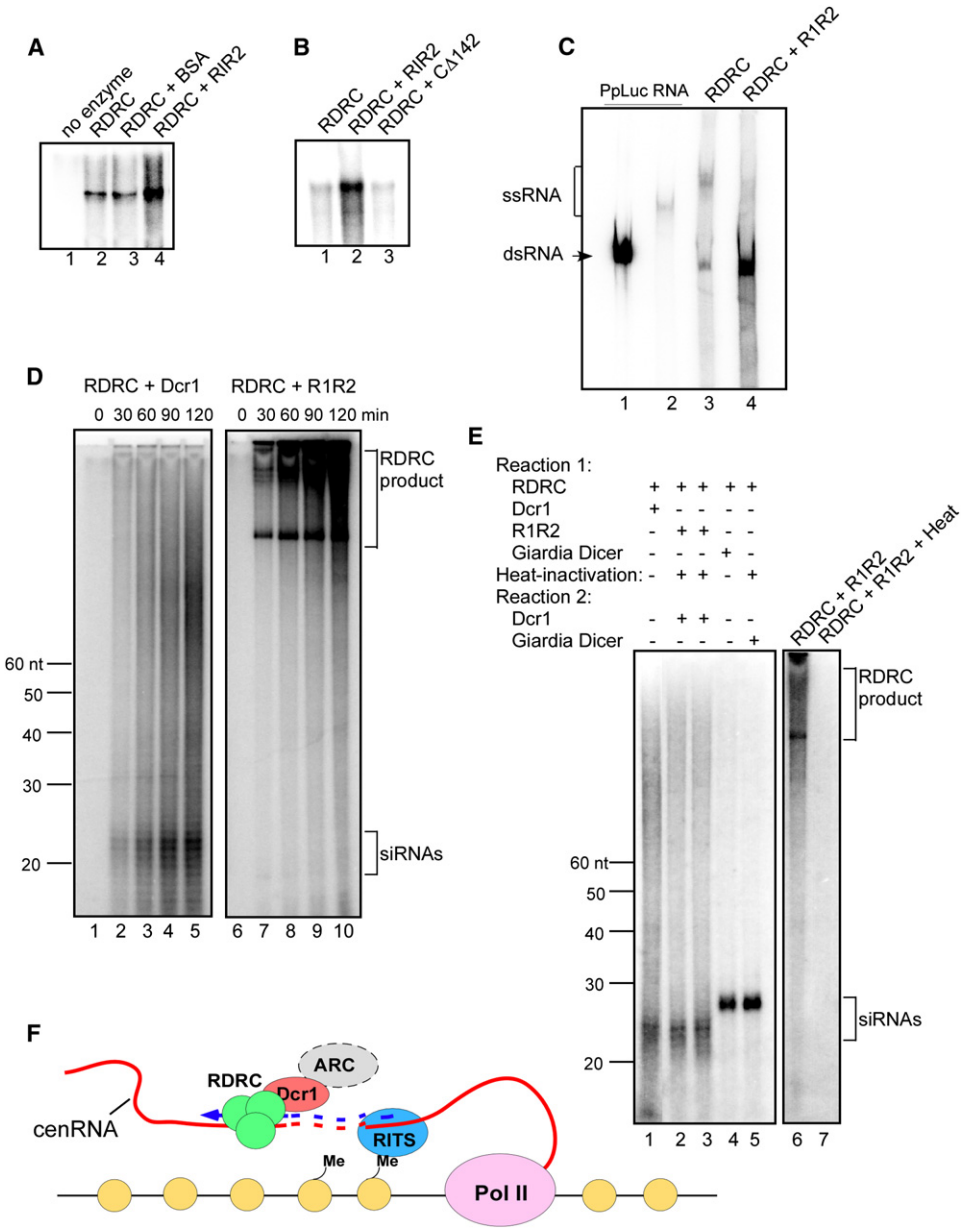

Figure 7. Reconstitution of siRNA Generation Using Purified RDRC, Dcr1, and a Single-Stranded Centromeric RNA Template

(A) RDRC activity is stimulated by addition of the catalytically inactive double RNase III mutant (R1R2), but not by BSA. RDRC activity was tested on a 500 nt template from the *cen-dg* transcript.

(B) Addition of an equal amount of a catalytically inactive C-terminal truncation mutant, CΔ141, fails to stimulate RDRC activity when compared to R1R2 mutant.

(C) Dcr1-R1R2 specifically stimulates dsRNA synthesis by RDRC. Reaction products were separated in a 5% native polyacrylamide gel and compared to migration of 500 nt double-stranded and single-stranded Pp luciferase RNA.

(D) Dcr1 generates siRNAs from dsRNA synthesized by RDRC; Dcr1-R1R2 is used as a negative control.

(E) Similar levels of siRNA are generated whether Dcr1 is present during dsRNA synthesis or added after RDRC heat inactivation. Giardia Dicer is used as an additional control.

(F) Coupling of siRNA generation and dsRNA synthesis at noncoding nascent centromeric RNAs. Dcr1 is recruited to single-stranded nascent centromeric RNA with RDRC and RITS, stimulates RDRC dsRNA synthesis, and converts the RDRC product into siRNAs. Another Ago1 complex (ARC) may participate in siRNA generation or stabilization of the Dcr1 product for transfer into the RITS complex.

RDRC and the Dcr1-RDRC interaction requires the integrity of both RITS and RDRC. Furthermore, our results demonstrate that the helicase domain of Dicer is required for ATP-dependent siRNA generation in vitro and for RNAi in vivo. The implications of coupled Dcr1-RDRC activities in RNAi-mediated heterochromatin assembly, as well as the roles of individual domains in Dcr1 function, are discussed below.

Functional Domains of Dcr1

Our investigation of Dcr1 activity has provided evidence for the requirement of the helicase, both RNase III domains, and C-terminal domains of Dcr1 in transcriptional gene silencing. We show that an active site mutation in a single RNase III domain disrupts RNAi in vivo (Figure 1C), which is consistent with previous results in *Drosophila* (Lee et al., 2004). Consistent with the idea that RNase III domains function independently of each other, Dcr1 retained in vitro cleavage activity unless both RNase III domains were mutated. Such single RNase III mutants may generate either single-stranded cuts or, in the absence of a PAZ domain, produce blunt ends due to rotation of the single catalytic site around the substrate. In either case, such products are predicted to lack the 2 nt 3′ overhangs characteristic of siRNAs, which may impede their transfer to Ago1 and lead to their degradation.

Our results indicate that Dcr1 belongs to an ATP-dependent class of Dicer proteins, which includes *C. elegans* DCR-1 (Hutvagner et al., 2001; Tabara et al., 2002) and *Drosophila* Dicer-2 (Bernstein et al., 2001; Liu et al., 2003), but not human Dicer (Provost et al., 2002a; Zhang et al., 2004) or *Drosophila* Dicer-1 (Jiang et al., 2005). ATP-dependent siRNA generation likely involves the N-terminal helicase domain, as a mutation of a known ATPase motif in this domain disrupts siRNA production in vitro and in vivo (Figure 3). This mutation (K38A) was based on the invariant lysine of the eIF-4a helicase motif AXXXXGKT, which is critical for ATP hydrolysis and helicase activity (Pause and Sonenberg, 1992). A mutation in the same motif also abrogated Dicer-2 activity in *Drosophila* (Lee et al., 2004), and missense mutations in the helicase domains of DCL1 and DCL4 disrupted function in *Arabidopsis* by altering the RNA-protein interface (Golden et al., 2002; Yoshikawa et al., 2005). The precise role of the helicase domain in dsRNA processing remains unclear, although the evidence presented in this study suggests that it regulates the loading of Dcr1 onto dsRNA ends in an ATP hydrolysis-dependent fashion. We speculate that ATP hydrolysis either induces the dissociation of Dcr1 from internal locations on dsRNA or promotes translocation along dsRNA until the enzyme finds the dsRNA end. With either scenario, the helicase domain itself or another domain of Dcr1 may perform an end-recognition function similar to that of the PAZ domain in other Dicers. Such a ''measuring'' mechanism must be relatively inefficient in Dcr1 and allows for a greater size range of siRNA products compared to PAZ-containing Dicers as shown here and in other studies (Cam et al., 2005; Reinhart and Bartel, 2002).

Finally, the C-terminal domain of Dcr1 contains a divergent dsRNA-binding domain that functions in substrate binding. The requirement of a C-terminal dsRNA-binding domain for RNA cleavage has previously been shown for human Dicer (Zhang et al., 2004) and for in vivo function in *Arabidopsis* DCL1 (Jacobsen et al., 1999). Analysis of the dsRNA-binding domain of bacterial RNase III indicates that it binds to the dsRNA substrate opposite to the RNA side that is presented to the RNase III catalytic domains (Gan et al., 2006). Recently, this domain has also been implicated in protein-protein interactions between DCL4 and its dsRNA-binding partner in *Arabidopsis* (Hiraguri et al., 2005). Our observation that this domain mediates Dcr1-RDRC association suggests that Dicer dsRNA-binding domains may have dual roles in RNAi.

Mechanism of Chromatin-Dependent siRNA Generation

The crystal structure of Giardia Dicer suggests that the PAZ domain acts to specify the size of siRNAs (Macrae et al., 2006). Binding of the 3′ overhang-binding pocket of the PAZ domain to dsRNA ends also ensures that Dicer-mediated cleavage proceeds from the end of the substrate. However, Dicers lacking an obvious PAZ domain are found in fungi and protozoans (Kadotani et al., 2004; Catalanotto et al., 2004; Mochizuki and Gorovsky, 2005), indicating that alternative mechanisms exist to provide size specificity for siRNAs. In fission yeast, siRNAs are predominantly 20–23 nt in size (Cam et al., 2005; Reinhart and Bartel, 2002; Verdel et al., 2004) and are generated by a single Dicer enzyme. Our in vitro studies show that purified Dcr1 is capable of generating siRNA-sized products despite the absence of an apparent PAZ domain. Instead, other structural features, particularly those of the N-terminal helicase domain, contribute to siRNA size determination. However, Dcr1 produces a broad range of RNA sizes from an end-labeled substrate that is indicative of random internal cleavage. In contrast, human and Giardia Dicers produce siRNAs of exclusive size as well as discretely sized intermediates that result from cleavage from the ends of the substrate (Macrae et al., 2006; Zhang et al., 2002) (Figures 2 and 4). Lack of a PAZ domain may therefore confer a certain level of flexibility in the Dcr1 structure to allow cleavage away from the 3′ overhangs of dsRNA ends and suggests a possible requirement for regulation by other factors.

The physical and functional interaction of RDRC with Dcr1 indicates that RDRC may target Dcr1 directly to newly synthesized dsRNA ends, thereby bypassing the requirement for PAZ domain-mediated loading. Based on these results and the interaction of Dcr1 with the Tas3 subunit of RITS, we propose a model where Dcr1 is recruited along with RDRC to sites of RITS assembly on the centromeric transcripts (Figure 7F). We have previously shown that the RITS complex is required for the recruitment of RDRC to nascent centromeric RNAs and

that RDRC exhibits primer-independent dsRNA synthesis that transcribes full-length products from the 3′ end of its template (Motamedi et al., 2004). Recently, the Ago1 slicer activity has been shown to be required for RNAi in fission yeast (Buker et al., 2007; Irvine et al., 2006), supporting the possibility that RITS-mediated cleavage of nascent RNAs may trigger RDRC activity through the formation of a free 3′ end. We hypothesize that Dcr1 interaction with RDRC provides an immediate and direct route from complementary strand synthesis to dsRNA processing. In this model, Dcr1 associates with RDRC throughout the dsRNA synthesis process (Figure 7F). This is supported by the recent finding that the *C. elegans* DCR-1 physically interacts with RRF-3 (Duchaine et al., 2006), an RNA-directed RNA polymerase that is required for the generation of endogenously derived siRNAs (Lee et al., 2006a).

Implications for the Mechanism of *cis*-Restriction and siRNA Strand Polarity

Our results suggest that the ability of Dicer to interact with RDRC is critical for siRNA generation in vivo. Deletions of the C-terminal domain of Dcr1, which disrupt its ability to interact with Rdp1, result in loss of siRNAs and abolish heterochromatin assembly. siRNA generation therefore appears to depend on both the catalytic activity of Dcr1 and its binding to RDRC. Coupling of dsRNA synthesis and siRNA generation may have evolved as part of selective pressure to ensure that RNAi effects on transcriptional or cotranscriptional silencing occur in a strictly *cis*-restricted manner. Consistent with this idea, siRNA-mediated heterochromatin formation in fission yeast is *cis*-restricted and negatively controlled by the Eri1 ribonuclease (Buhler et al., 2006). In this study, the partial codependence of Dicer-RDRC activity in in vitro activity assays, as well as the dependence of siRNA generation and heterochromatin assembly on the ability of these enzymes to interact, strongly suggests that this association plays an important physiological role in *cis*-restriction of RNAi in fission yeast (Figure 7F).

The physical associations described here may form the basis for siRNA strand-bias mechanisms. Endogenous siRNAs in *C. elegans* and *Tetrahymena*, and transgene siRNAs in fission yeast, display a striking strand bias in which the siRNA strand loaded onto Argonaute complexes is usually complementary to the sense RNA transcripts from the target locus (Buhler et al., 2007; Lee and Collins, 2006; Sijen et al., 2001). Such antisense strand preference suggests either direct or indirect interactions between RNA-dependent RNA polymerase and the loading apparatus. Based on our demonstration of Dcr1-RDRC and Dcr1-RITS association, as well as previous studies showing that Dicer directly binds Argonaute PIWI domain (Tahbaz et al., 2004) and loads siRNAs onto RISC (Liu et al., 2006), we propose that the physical association of Dicer with RdRP and Argonaute complexes provides a mechanism that can mediate the loading of siRNAs onto Argonaute in a directional manner, giving rise to antisense strand bias (Figure 7F).

EXPERIMENTAL PROCEDURES

Strains, Plasmids, Mutagenesis, and Silencing Assays

All strains used in this study are listed in Table S1 and described in the Supplemental Data. Plasmid construction and silencing assays were performed as described previously (Buker et al., 2007; Verdel et al., 2004).

siRNA Analysis and Protein Purification

Isolation of total RNA from whole-cell extracts and northern blot analysis were performed as described in Buhler et al. (2006). Dual-affinity purification was performed as outlined in Motamedi et al. (2004). FLAG purification of overexpressed proteins from fission yeast was also performed as described in Buker et al. (2007).

In Vitro RNA Synthesis, dsRNA Cleavage, and Binding Assays

Templates for T7 transcription were amplified by PCR from total yeast genomic DNA and fused to a minimal T7 RNA polymerase promoter to transcribe either forward or reverse strands. RNA was synthesized with T7 RNA polymerase, radiolabeled, and purified as described in the Supplemental Data. Dcr1 dsRNA cleavage assays were conducted on either end-labeled or internally labeled dsRNA for 1 hr at 32°C, and products were analyzed in denaturing polyacrylamide urea gels as described in detail in the Supplemental Data. Gel shift experiments were performed by incubation of Dcr1 with the same end-labeled dsRNA at 4°C for 30 min in the absence of Mg^{2+} and ATP, before separation in 5% nondenaturing PAGE.

ATPase Assay

Dcr1 dsRNA cleavage assays were performed as described except with 500 ng protein and 500 ng cold dsRNA, and with the addition of 20 nCi of γ-^{32}P ATP for 30 min at 32°C. Reactions were quenched with 25 mM EDTA and separated by PEI-cellulose thin-layer chromatography in 0.5 M LiCl, 1 M formic acid.

Domain Structure Prediction

Structure prediction was carried out as described in the Supplemental Data.

RDRC Activity and Immunoprecipitation Assays

These assays were performed as previously described in Motamedi et al. (2004) with the modifications outlined in the Supplemental Data.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, three figures, and one table and can be found with this article online at http://www.molecule.org/cgi/content/full/27/3/449/DC1/.

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