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RNAi-Dependent and -Independent RNA Turnover Mechanisms Contribute to Heterochromatic Gene Silencing

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SUMMARY

In fission yeast, the RNAi pathway is required for heterochromatin-dependent silencing of transgene insertions at centromeric repeats and acts together with other pathways to silence transgenes at the silent mating-type locus. Here, we show that transgene transcripts at centromeric repeats are processed into siRNAs and are therefore direct targets of RNAi. Furthermore, we show that Cid14, a member of the Trf4/5 family of poly(A) polymerases, has poly(A) polymerase activity that is required for heterochromatic gene silencing. Surprisingly, while siRNA levels in *cid14Δ* cells are dramatically reduced, the structural integrity of heterochromatin appears to be preserved. Cid14 resides in a complex similar to the TRAMP complex found in budding yeast, which is part of a nuclear surveillance mechanism that degrades aberrant transcripts. Our findings indicate that polyadenylation by a TRAMP-like complex contributes to robust silencing of heterochromatic genes in fission yeast via the recruitment of the exosome and/or the RNAi machinery.

INTRODUCTION

Heterochromatic DNA domains are required for stable chromosome transmission and regulation of gene expression in a variety of organisms ranging from yeast to human. In the fission yeast *Schizosaccharomyces pombe*, heterochromatin is associated with telomeres, the silent mating-type loci, and repetitive DNA elements surrounding centromeres. The assembly of heterochromatin at these loci involves a series of steps that ultimately lead to the association of specific histone modifications and structural proteins with extended DNA domains. One of the key steps is the methylation of histone H3 lysine 9 (H3K9) by the Clr4 methyltransferase, which creates a binding site for the Swi6, Chp1, and Chp2 chromodomain proteins (Bjerling et al., 2002; Nakayama et al., 2001; Partridge et al., 2000; Sadaie et al., 2004). Histone H3K9 methylation is a conserved hallmark of heterochromatin and has been proposed to spread along the chromatin fiber through sequential cycles of methylation that are coupled to oligomerization of Swi6, a homolog of the *Drosophila* and mammalian HP1 proteins (Grewal and Moazed, 2003; Richards and Elgin, 2002).

Heterochromatin assembly at fission yeast centromeres also requires components of the RNA interference (RNAi) pathway. RNAi is a conserved silencing mechanism that is triggered by double-stranded RNA (dsRNA) (Bartel, 2004; Hannon, 2002). RNAi-dependent posttranscriptional gene silencing (PTGS) involves the generation of small RNA molecules of ~22 nucleotides from the longer dsRNAs by an RNase III-like enzyme called Dicer (Bernstein et al., 2001). These small interfering RNAs (siRNAs) then load onto an effector complex called RISC (RNA-induced silencing complex). The RISC complex contains Argonaute, which is a member of the conserved Argonaute/PIWI family of proteins that are required for RNAi in a variety of systems (Caudy et al., 2002; Hammond et al., 2001; Hutvagner and Zamore, 2002; Mourelatos et al., 2002; Gregory et al., 2005). siRNA-programmed RISC acts in *trans* to target cognate mRNAs for degradation by an endonucleolytic cleavage event, also referred to as slicing, which is executed by Argonaute (Liu et al., 2004; Song et al., 2004).

S. pombe contains only a single gene for each of the main RNAi enzymes, Dicer, Argonaute, and RNA-directed RNA polymerase, called *dcr1*$^+$, *ago1*$^+$, and *rdp1*$^+$, respectively, all of which are required for heterochromatin formation (Volpe et al., 2002). Ago1, together with Chp1, Tas3, and siRNAs, forms a complex called RNA-induced transcriptional silencing (RITS) (Verdel et al., 2004), whereas Rdp1, together with the helicase Hrr1 and the putative poly(A) polymerase Cid12, forms a complex called RNA-directed RNA polymerase complex (RDRC) (Motamedi et al., 2004). Deletion of any of these genes results in defects in the spreading of H3K9 methylation and Swi6 localization (Volpe et al., 2002; Motamedi et al., 2004; Verdel et al., 2004; Jia et al., 2004). Recent results have led to a model in which the association of the RITS

complex with chromatin and the initiation and spreading of chromatin modifications are proposed to involve base-pairing between siRNAs and the nascent RNA polymerase II (RNApII) transcripts. Subsequently, RITS would recruit RDRC and histone-modifying enzymes to the targeted locus, leading to the generation of additional dsRNA, dsRNA processing into siRNA, and spreading of heterochromatin (Motamedi et al., 2004; Buhler et al., 2006; Kato et al., 2005; Djupedal et al., 2005; Noma et al., 2004).

In *S. pombe*, the insertion of reporter genes within or adjacent to heterochromatic regions results in clonally heritable gene silencing that exhibits the characteristic properties of classical position effect variegation (PEV) observed in multicellular eukaryotes (Allshire et al., 1994; Thon and Klar, 1992; Grewal and Klar, 1996). Unexpectedly, in wild-type cells and in cells defective in heterochromatin formation, similar levels of RNApII are associated with *ura4*$^+$ genes inserted within centromeric heterochromatin. In addition, tethering of the RITS complex to *ura4*$^+$ transcripts, which also results in the formation of heterochromatin, neither excludes RNApII from the *ura4*$^+$ gene nor reduces the rate of transcription (Buhler et al., 2006). Furthermore, heterochromatin has little or no effect on transcription of the reverse strand of centromeric repeats, even though these transcripts are less abundant in wild-type cells compared to RNAi mutant cells (Volpe et al., 2002). Finally, point mutations in subunits of RNApII that do not affect growth rate result in defects in RNAi-mediated heterochromatic gene silencing (Kato et al., 2005; Djupedal et al., 2005). These findings have challenged the paradigm that heterochromatin is transcriptionally inert. Instead, it seems that some promoters can be transcribed within heterochromatic domains, but the resulting RNA is rapidly degraded by the RNAi pathway. However, the mechanism of this degradation is distinct from RNAi-mediated PTGS. Unlike classical PTGS, RNAi-mediated degradation of heterochromatic transcripts is a chromatin-dependent process that requires the histone H3K9 methyltransferase Clr4 (Motamedi et al., 2004; Noma et al., 2004). The fact that RNAi-mediated degradation of transcripts in wild-type *S. pombe* is *cis*-restricted is consistent with the conclusion that degradation is chromosome-associated and further distinguishes this process from classical PTGS (Buhler et al., 2006). To explain these observations, we proposed a cotranscriptional gene silencing (CTGS) model, in which the targeting of nascent transcripts by the RITS complex not only mediates chromatin modifications (Motamedi et al., 2004) but also promotes the degradation of nascent transcripts (Buhler et al., 2006).

In this study we show that insertion of a *ura4*$^+$ gene into centromeric heterochromatin leads to the generation of siRNAs, which appear to have escaped previous detection due to their low abundance. Such transgene siRNAs could potentially mediate silencing through direct slicing of the *ura4*$^+$ transcript by Ago1. Furthermore, we show that a second RNA-processing pathway, involving the Cid14 poly(A) polymerase, is required for efficient silencing within centromeric DNA repeats. Surprisingly, in *cid14Δ* cells, siRNA levels are dramatically reduced, but the structural integrity of heterochromatin appears to be preserved. This observation indicates that Cid14-dependent siRNAs are largely dispensable for heterochromatin assembly and may be degradation products. Cid14 homologs in budding yeast are components of the TRAMP polyadenylation complex, which promotes the degradation of aberrant transcripts by the exosome (Wyers et al., 2005; Lacava et al., 2005; Vanacova et al., 2005). We show that point mutations in the catalytic core of Cid14 abolish its in vitro polyadenylation activity and disrupt its silencing function in vivo. Unlike other proteins required for RNAi, Cid14 is also required for silencing of a *ura4*$^+$ gene inserted at the silent mating-type locus, revealing the existence of an RNA-processing pathway that has a general role in heterochromatic gene silencing. Together, our results establish a role for the poly(A) polymerase subunit of the fission yeast TRAMP complex in heterochromatic gene silencing and suggest that this RNA processing pathway acts downstream of H3K9 methylation and Swi6 recruitment to promote robust silencing. We propose a model for CTGS in which assembly of DNA into heterochromatin interferes with normal RNA processing events, thus targeting transcripts for recognition by Cid14 and degradation by either the exosome or the RNAi pathway.

RESULTS

Detection of siRNAs from Reporter Genes Inserted into Centromeric Heterochromatin

The observation that similar levels of RNApII are associated with centromeric *ura4*$^+$ transgenes whether they are active or silenced suggests that silencing occurs primarily at the level of RNA degradation (Buhler et al., 2006). In order to determine whether RNAi is responsible for the degradation of heterochromatic transcripts, we set out to test whether the silencing of a *ura4*$^+$ gene inserted at different heterochromatic loci is accompanied by *ura4*$^+$ siRNA production (Figure 1A). In the strains we used for our analysis, the *ura4*$^+$ gene has been inserted at the innermost centromeric repeat region (*imr1R::ura4*$^+$), the outermost centromeric repeat region (*otr1R::ura4*$^+$), or the silent mating-type locus *mat3M* (*mat3M::ura4*$^+$). We and others were unable to detect siRNAs originating from various *ura4*$^+$ insertions in wild-type cells (Iida et al., 2006; Irvine et al., 2006). We suspected that this may be due to low levels of transgene siRNAs and therefore employed two different strategies to increase the sensitivity of our northern blot siRNA detection assay. First, we size selected the total RNA preparation (<200 nt) from cells that produce higher levels of siRNAs, such as cells lacking the exoribonuclease Eri1 (Iida et al., 2006) or cells that overexpress the RNA-dependent RNA polymerase Rdp1 (Figure 1B). Despite this, we were unable to detect *ura4*$^+$ siRNAs in either *eri1Δ* or Rdp1 overproducing cells (Figure 1B, middle panel).

To further increase sensitivity, we immunoprecipitated a functional FLAG-Ago1 protein (Buker et al., 2007) and isolated the associated RNA to obtain higher levels of siRNAs for northern blot assays. As shown in Figure 1B (upper panel, compare lane 5 with lanes 1–4), this enrichment method led to a great improvement of the siRNA signal. Isolation of siRNAs associated with FLAG-Ago1 from *eri1Δ* cells led to the detection of *ura4*$^+$ siRNAs originating from *otr1R::ura4*$^+$ (Figure 1C, lane 1). As a comparison for their abundance, we included a titration of FLAG-Ago1 purified siRNAs from strains where heterochromatic silencing of the endogenous *ura4*$^+$ gene was induced by tethering the RITS complex to the *ura4*$^+$ transcript (Buhler et al., 2006). Based on the intensity of the northern blot signals, the *otr1R::ura4*$^+$ siRNAs were ~40-fold less abundant than the *ura4-5BoxB* siRNAs (Figure 1C, compare lane 1 with lanes 3–6). *ura4*$^+$ siRNAs from an *imr1R::ura4*$^+$ insert were even less abundant compared to *otr1R::ura4*$^+$, and we could only detect them by loading 10-fold more FLAG-Ago1-associated siRNAs (Figure 1C, lane 2; Figure 1D, lane 3). Therefore, *imr1R::ura4*$^+$ siRNAs were also generated, but they were about 400-fold less abundant than *ura4-5BoxB* siRNAs. Finally, we were unable to detect *ura4*$^+$ siRNAs from an insertion at the silent mating-type locus (*mat3M::ura4*$^+$) by the same northern blot assay (Figure 1H).

Nonrandom siRNA Guide Strand Selection by Ago1

Double-stranded siRNAs are processed by the RNaseIII enzyme Dicer from long double-stranded RNA before loading onto Argonaute. Which strand of the siRNA duplex resides in Argonaute and which gets ejected has previously been shown to depend on the thermodynamic stability of either end of the siRNA duplex (Hutvagner, 2005). Because we do not know the exact sequences of the *ura4*$^+$ siRNAs, we could not predict which one of the two strands would be found in Ago1. Therefore, we probed northern blots with probes complementary to either the *ura4*$^+$ antisense or sense strand. Surprisingly, we observed that Ago1 has a preference for one of the strands over the other, depending on the locus from which the *ura4*$^+$ gene was expressed. While we mainly detected *ura4*$^+$ siRNAs of the sense orientation when assaying for *otr1R::ura4*$^+$ siRNAs (Figures 1A and 1C [lane 1]), only antisense *ura4*$^+$ siRNAs could be detected for *imr1R::ura4*$^+$ cells (Figure 1D). The antisense/sense ratio of *ura4*$^+$ siRNAs changed when the orientation of the *ura4*$^+$ gene at *otr1R* was flipped (oriA versus oriB, Figures 1A and 1D).

To further explore the basis of siRNA strand selection, we performed strand-specific northern blots with the more abundant Ago1-associated siRNAs purified from cells expressing the *ura4-5BoxB* allele. Two identical northern blots were prepared in parallel and hybridized with probes detecting either sense or antisense *ura4*$^+$ siRNAs. Quantification of the signals revealed that Ago1 contained about 3-fold more antisense than sense *ura4*$^+$ siRNAs. This ratio was not influenced by Eri1, although, as expected, *ura4*$^+$ siRNA levels increased in *eri1Δ* cells (Figures 1E–1G). In contrast, no difference in relative abundance of sense and antisense siRNAs was observed when we probed the same blot for centromeric siRNAs with strand-specific probes (Figure 1G). These observations suggest that the fission yeast RNAi machinery can somehow distinguish between the sense and antisense strands of transgene transcripts, a phenomenon that has been observed in other systems (Sijen et al., 2001). For centromeric siRNAs, the transcription of both strands in the forward and reverse directions may mask this strand specificity.

Cid14 Is Required for Heterochromatic Gene Silencing

The observation that *ura4*$^+$ siRNAs are generated and loaded onto Ago1 if the *ura4*$^+$ gene is situated within centromeric heterochromatin is consistent with the possibility that RNAi is responsible for the degradation of heterochromatic transcripts, via slicing of the nascent transcript by Ago1 or Dcr1-mediated degradation of double-stranded RNA synthesized by Rdp1. However, our failure to detect *ura4*$^+$ siRNAs from *mat3M::ura4*$^+$, the very low abundance of *imr1R::ura4*$^+$ siRNAs, and the observation that the siRNAs detected for *otr1R::ura4*$^+$ *oriA* were mostly of the sense orientation and therefore unable to target the *ura4*$^+$ mRNA, led us to test whether other nuclear degradation pathways might also contribute to the silencing of heterochromatic *ura4*$^+$ genes. A series of recent studies have uncovered a role for polyadenylation in stimulating RNA degradation in *Saccharomyces cerevisiae*. Two members of a broadly conserved family of noncanonical poly(A) polymerases, Trf4 and Trf5, share an essential function for this specific type of polyadenylation. Substrates for these poly(A) polymerases include aberrantly modified tRNAs, precursors of snoRNAs and rRNAs, and so-called *c*ryptic *u*nstable *t*ranscripts (CUTs) (Lacava et al., 2005; Wyers et al., 2005; Vanacova et al., 2005). Interestingly, a member of the *S. pombe* poly(A) polymerase family, Cid14, has recently been demonstrated to be the fission yeast Trf4/Trf5 functional homolog required for polyadenylation of rRNAs and proper chromosome segregation (Win et al., 2006). Therefore, we reasoned that Cid14 might be involved in targeting heterochromatic transcripts for degradation. We found that deletion of *cid14*$^+$ resulted in a complete loss of *ura4*$^+$ silencing at all the tested loci as assayed by growth on 5-FOA-containing medium (Figures 2A–2C). Importantly, deletion of *cid14*$^+$ had no effect on the 5-FOA sensitivity of a *ura4*Δ strain (not shown) and did not significantly affect the levels of *ura4*$^+$ mRNA transcribed from its euchromatic location (Figure S4B). Consistent with the 5-FOA silencing results, semiquantitative RT-PCR, as well as quantitative real-time RT-PCR, showed an increase in *ura4*$^+$ transcript levels upon deletion of *cid14*$^+$ (Figures 2D–2G). While *ura4*$^+$ transcript levels increased 2- to 3-fold for the centromeric insertions, an 18-fold increase was observed for the *ura4*$^+$ gene inserted at the silent mating-type locus. Finally, deletion of the other four members of the fission yeast Cid14/Trf4/5

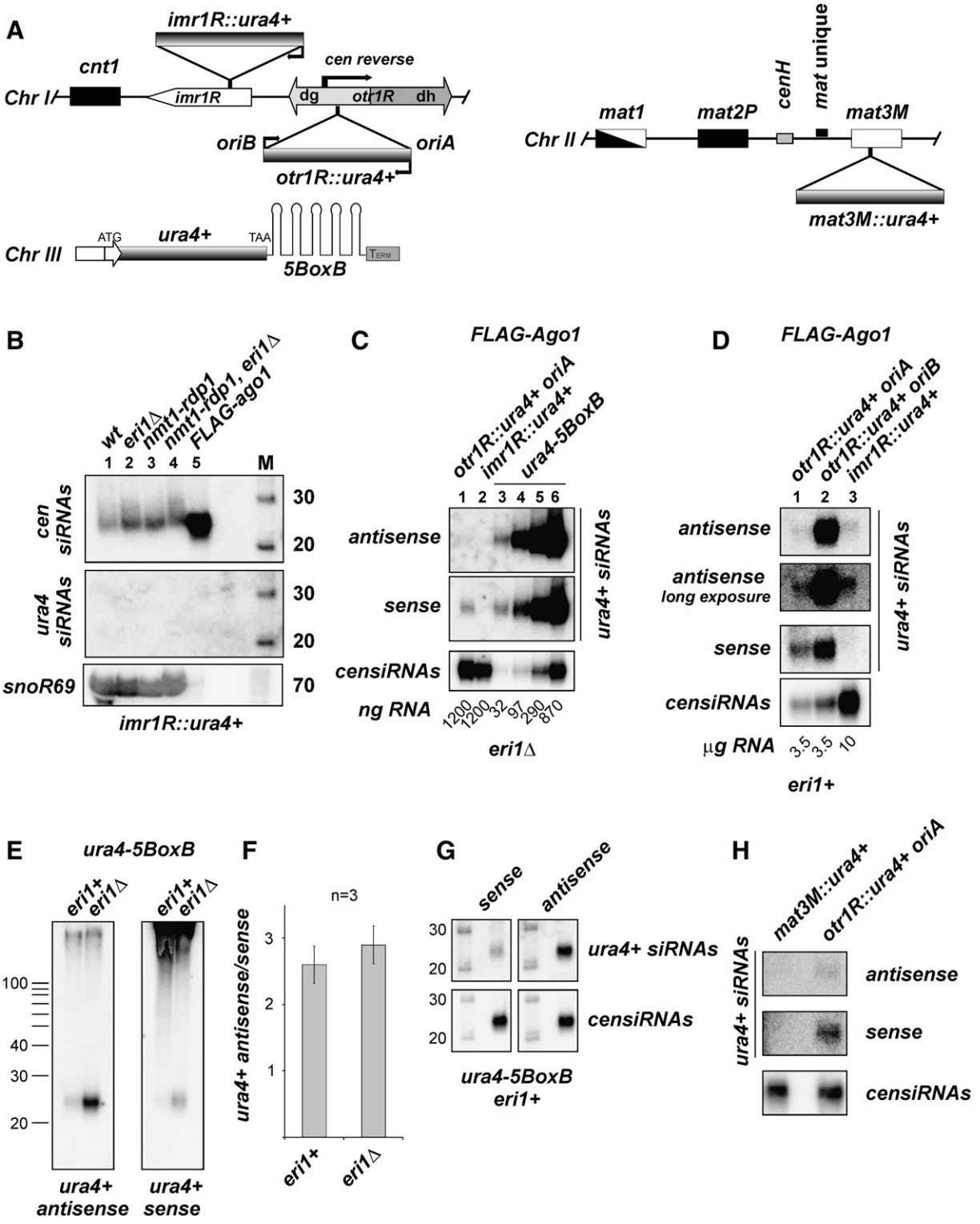

Figure 1. Insertion of Reporter Genes into Centromeric Heterochromatin Leads to the Generation of siRNAs

(A) Schematic diagram representing the DNA organization at the centromere of chromosome 1 (cen1, upper schematic), at the *ura4-5BoxB* locus (lower schematic, chromosome III), and at the mating-type locus (chromosome II). *cnt1*, central core; *imr1*, innermost repeats; *otr1*, outermost repeats; dg and dh, tandem repeats in otr; cenH, cen homology region; mat unique, mat-specific regions used for ChIP PCR in Figure 3C. Orientation of the *cen-reverse* promoter (Djupedal et al., 2005) is indicated by a large arrow.

poly(A) polymerase family did not affect silencing of an *imr1R::ura4*$^+$ reporter gene (Figure 2H). These results suggest that the Cid14 member of this family plays a specific role in promoting efficient heterochromatic gene silencing.

The Main Structural Features of Heterochromatin Are Preserved in *cid14*Δ Cells

Methylation of H3K9 and recruitment of Swi6 are thought to represent the final step in heterochromatin assembly (Nakayama et al., 2001). This conclusion is supported by the observation that the loss of these heterochromatic marks correlates with the loss of heterochromatic gene silencing in all mutants described so far (Grewal and Moazed, 2003). The loss of *ura4*$^+$ silencing in *cid14Δ* cells is unusual because, unlike RNAi components, Cid14 is required for silencing of *mat3M::ura4*$^+$ (Figures 2B and 2G). Therefore, we asked whether Cid14 might play a major role in the assembly of heterochromatin and tested whether deleting *cid14*$^+$ has a similar effect on the integrity of heterochromatin as deleting *clr4*$^+$. Surprisingly, in *cid14*Δ cells, neither Chp1 nor Swi6 binding was significantly reduced at several heterochromatic loci, including *mat3M::ura4*$^+$, *imr1R::ura4*$^+$, the subtelomeric *tlh1*$^+$ gene, and *cen-dg* and *cen-dh* repeats, as assayed by chromatin immunoprecipitation experiments (ChIP) (Figures 3A and 3B). In addition, while H3K9 methylation was lost in *clr4Δ* cells, only a slight reduction in H3K9 methylation was observed in *cid14*Δ cells (Figures 3A and 3B). Furthermore, consistent with a CTGS model for silencing of *mat3M::ura4*$^+$, none of the tested mutants affected RNApII occupancy at this locus (Figure 3C). These results suggest that Cid14 is unlikely to be involved in the formation of heterochromatin per se but rather plays a downstream role in heterochromatic gene silencing.

Cid14 Is Found in a Complex with Mtr4 and Air1

The finding that deletion of *cid14*$^+$ does not affect the recruitment of major structural components of heterochromatin but affects the degree of heterochromatic gene silencing is consistent with the idea that Cid14 is part of a signaling mechanism that leads to the degradation of RNAs that are transcribed from heterochromatic domains. Intriguingly, accumulation of cryptic transcripts in *Saccharomyces cerevisiae* has been observed in exosome-deficient or *trf4*Δ cells without a corresponding change in the levels of RNApII occupancy at these loci (Wyers et al., 2005). Both poly(A) polymerases Trf4 and Trf5 are found together with Air1/2 and Mtr4 in complexes termed TRAMP and TRAMP5, respectively (Wyers et al., 2005; Vanacova et al., 2005; Lacava et al., 2005). Mtr4 is a nuclear cofactor of the exosome that appears to be required for processing and degradation of diverse substrates and has been proposed to directly recognize exosome substrates (Mitchell and Tollervey, 2000). Air1 and Air2 are both predicted zinc-knuckle proteins and may be functionally redundant (Mitchell and Tollervey, 2000; Wyers et al., 2005). It has been proposed that unstable transcripts result from the transcription of cryptic promoters, producing RNAs that are targeted for TRAMP-mediated polyadenylation and rapid turnover by the exosome. We therefore hypothesized that heterochromatic transcripts might be similarly degraded by Cid14-mediated exosome activity in fission yeast.

To explore this possibility, we first asked whether Cid14 exists in a TRAMP-like complex in *S. pombe*. We constructed a strain that expressed a fully functional C-terminally TAP-tagged Cid14 (Cid14-TAP, Figure S1) and purified this protein as described (Buker et al., 2007). Analysis of the purification by polyacrylamide gel electrophoresis and mass spectrometry revealed that Cid14 copurified with two proteins that are homologs of Mtr4 and Air1 (Figures 4A and 4B). Thus, like Trf4 in *S. cerevisiae*, Cid14 is found in a complex together with Air1 and Mtr4, which we refer to as *sp*TRAMP (*S. pombe* TRAMP). Interestingly, several of the other proteins in Cid14-TAP purifications were ribosome synthesis factors, which would be consistent with the reported function of Cid14 in pre-rRNA polyadenylation (Win et al., 2006) (Table S4). We found that the deletion of *air1*$^+$ had no effect on heterochromatic gene silencing (Figure S2). However, we observed loss of silencing of *mat3M:::ura4*$^+$ in cells carrying a hypomorphic allele of *mtr4*$^+$ (*mtr4-1*, Figures 4F and 4H), suggesting the involvement of a TRAMP-like complex.

To directly test whether the exosome is responsible for the destruction of heterochromatic transcripts, we measured transcript levels in exosome mutants (*rrp6Δ* and *dis3-54)*. Consistent with a role for the exosome in degrading heterochromatic *ura4*$^+$ transcripts, we observed elevated *ura4*$^+$ transcript levels in *rrp6*Δ compared to wild-type cells (Figures 4C–4E). Moreover, we observed 7-fold and a 25-fold increases in *tlh1*$^+$ transcript levels in

(B) Northern blot probed with end-labeled DNA oligos specific for *ura4*$^+$ siRNAs, centromeric siRNAs (*cen* siRNAs), and the loading control snoR69 performed with 25 μg total RNA (lanes 1–4) or 1 μg Ago1-associated RNA (lane 5). M, RNA markers.

(C and D) Northern blot performed with Ago1-associated RNA from the indicated strains. The amount of RNA loaded is shown below each lane. Lanes 3–6 in (C) represent a 3-fold serial dilution of siRNAs from *ura4-5BoxB*/Tas3-λN cells. The membrane was sequentially probed with end-labeled DNA oligos specific for *ura4*$^+$ antisense siRNAs, *ura4*$^+$ sense siRNAs, and centromeric siRNAs (*cen* siRNAs).

(E) Northern blot was performed in duplicate with Ago1-associated RNA from *ura4-5BoxB*/Tas3-λN cells (*eri1*$^+$ or *eri1Δ* background). The membranes were probed with either antisense or sense *ura4*$^+$ specific end-labeled DNA oligos.

(F) The experiment shown in (E) was performed three times and quantified with Quantity One software. Error bars denote standard deviation.

(G) Northern blot performed as in (E) was stripped after probing for *ura4*$^+$ siRNAs and reprobed with end-labeled DNA oligos specific for centromeric siRNAs (*cen* siRNAs) of both orientations.

(H) Six micrograms or 1 μg Ago1-associated RNA from *mat3M::ura4+* or *otr1R::ura4+* cells, respectively, were loaded, and the membrane was sequentially probed with end-labeled DNA oligos specific for *ura4*$^+$ antisense siRNAs, *ura4*$^+$ sense siRNAs, and centromeric siRNAs (*cen* siRNAs).

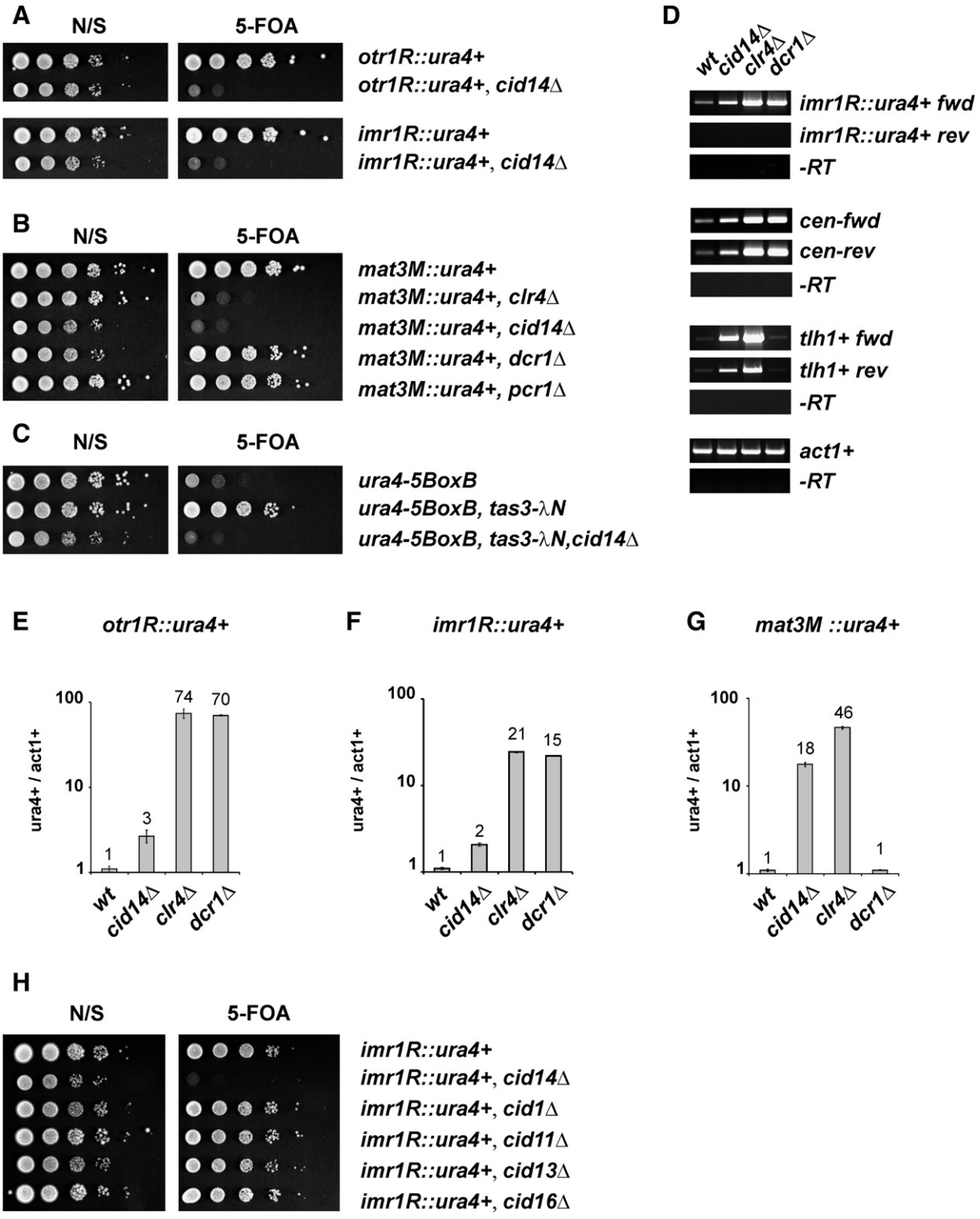

Figure 2. Cid14 Is Required for Heterochromatic Gene Silencing

(A–C) Silencing assays showing that cid14 is required for heterochromatic *ura4*$^+$ gene silencing

(D) Strand-specific, semiquantitative endpoint RT-PCR showing that *imr1R*::*ura4*$^+$ mRNA levels are higher in *cid14*Δ cells compared to *wt* cells. *cen-fwd/rev and tlh1*$^+$ represent endogenous transcripts from the centromere and the subtelomeric region, respectively. Both strands of the centromere and of the subtelomeric gene *tlh1*$^+$ are transcribed, and the RNA levels increase in *cid14Δ* cells. *act1*$^+$ serves as a control. −RT, no reverse transcriptase.

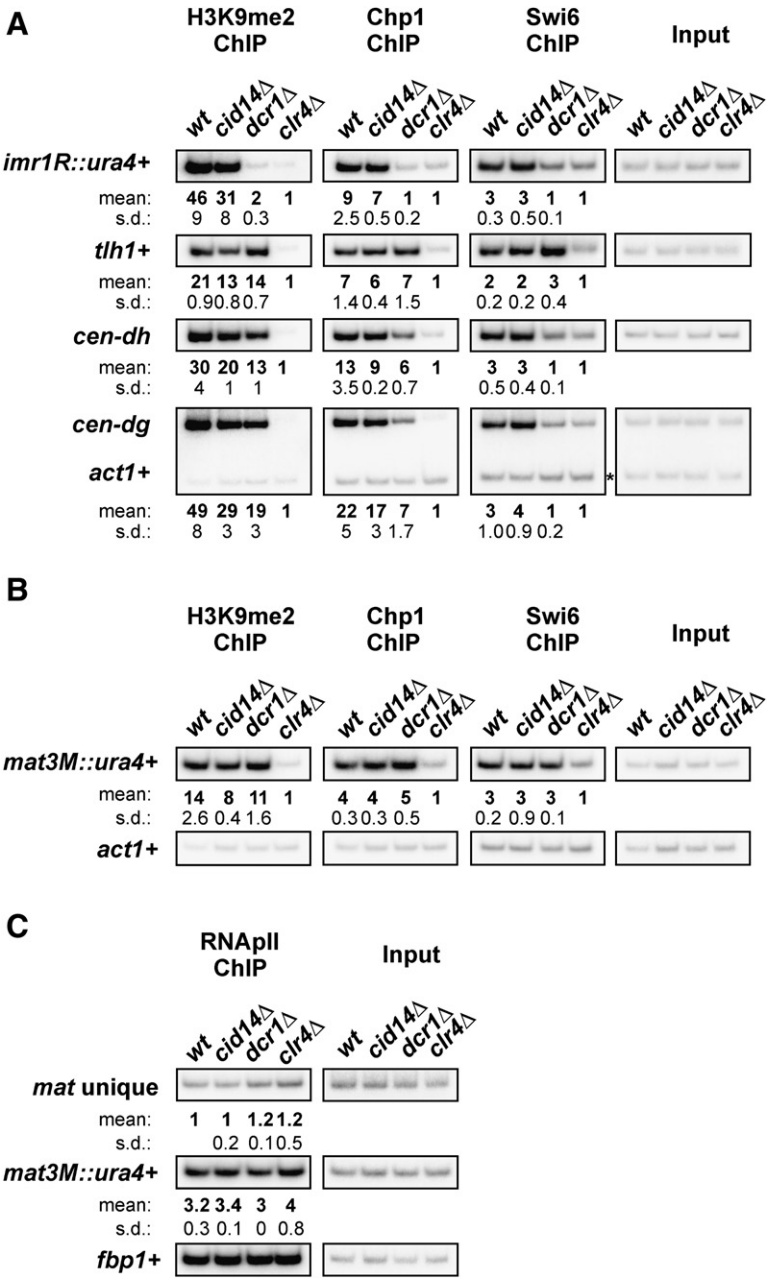

Figure 3. Cid14 Is Not Required for H3K9 Methylation and Swi6 Binding

(A and B) ChIP experiments showing that H3K9 dimethylation (left panel), recruitment of Chp1 (middle panel), and Swi6 (right panel) to the indicated loci is largely unaffected in *cid14*Δ cells.

(C) ChIP experiment showing that RNAPII occupancy at the *ura4*$^+$ gene inserted at the silent mating-type locus *mat3M* is 3- to 4-fold higher than the *mat* unique region but does not significantly increase in *dcr1*Δ, *clr4*Δ, and *cid14*Δ cells compared to *wt* at any of the tested loci. The region *mat* unique is located between the two silent mating-type loci *mat2P* and *mat3M* and has no known promoter elements (see Figure 1A). Standard deviation and average fold-enrichment values from three experiments are presented as numbers below each panel. Fold-enrichment in (A) and (B) was normalized to *act1*$^+$ (H3K9me2 and Chp1) or *fbp1*$^+$ (*, Swi6). The value for *clr4Δ* (A and B) and *mat* unique (C) was set to 1.0.

*rrp6*Δ and *dis3-54* cells, respectively, and 33- and 100-fold increases in *cid14*Δ and *clr4*Δ cells, respectively (Figures 4G and S3). *tlh1*$^+$, which shares sequence similarity with centromeric repeats, has previously been shown to give rise to siRNAs (Cam et al., 2005). Consistent with this observation, we observed a 3-fold increase in *tlh1*$^+$ RNA levels in *dcr1Δ* cells and a 10-fold increase in *mtr4-1* cells (Figures 4F and 4H), indicating that both RNAi and TRAMP contribute to the full silencing of this subtelomeric gene. Together, these results support a role for *sp*TRAMP-mediated degradation of heterochromatic transcripts by the exosome.

(E–G) Quantitative real-time RT-PCR showing that *ura4*$^+$ transcript levels increase in *cid14Δ* cells at the indicated loci. Mean values normalized to actin are shown, and error bars represent standard deviation (n = 3).

(H) Silencing assay showing that other members of the fission yeast Cid poly(A)-polymerase family are not required for heterochromatic silencing.

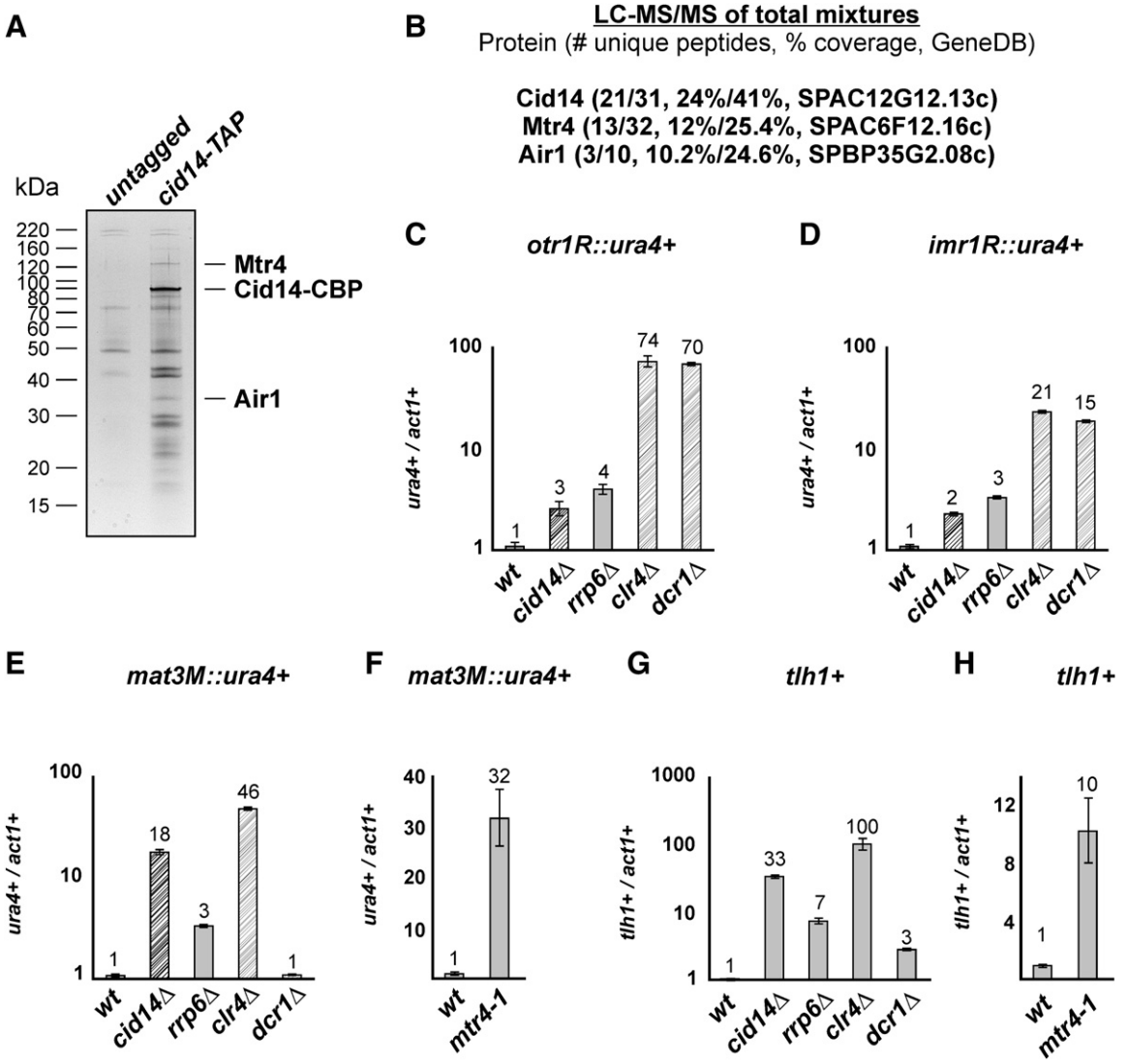

Figure 4. Purification of the Fission Yeast TRAMP Complex and Its Requirement for Heterochromatic Gene Silencing

(A) Silver-stained gel showing the purification of Cid14-TAP and a control purification from an untagged strain. The approximate position of *sp*TRAMP components identified by mass spectrometry of mixtures of proteins is indicated.

(B) Result of tandem mass spectrometry sequencing of mixtures of proteins (LC-MS/MS) from two independent Cid14-TAP purifications (purification #1/ purification #2) is shown. A complete list of proteins identified in both purifications is provided in Table S4.

(C–E) Quantitative real-time RT-PCR showing that *otr1R::ura4*$^+$, *imr1R::ura4*$^+$, *mat3M::ura4*$^+$ transcript levels increase in *rrp6Δ* cells.

(F–H) Quantitative real-time RT-PCR showing that *mat3M::ura4*$^+$ and *tlh1*$^+$ transcript levels increase in the *mtr4-1* and *rrp6Δ* mutant cells. Mean values are shown, and error bars represent standard deviation (n = 3). Hatched bars, values taken from Figure 2 and shown for comparison.

Point Mutations in the Catalytic Core of Cid14 Abolish Its In Vitro Polyadenylation Activity and Disrupt Its In Vivo Silencing Function

In order to directly determine whether Cid14 is a bona fide poly(A) polymerase, we assayed recombinant wild-type or mutant Cid14 (GST-Cid14wt or GST-Cid14DADA, respectively; Figure 5A) for polyadenylation activity in vitro and found that wild-type Cid14 was able to extend a synthetic oligo$(A)_{15}$ RNA but not an oligo$(dA)_{15}$ DNA substrate (Figures 5B, 5C, and 5E). Importantly, Cid14 activity was completely abolished in Cid14DADA, whose aspartate residues 298 and 230, which are known to be essential for the catalytic activity of this protein family, were mutated to alanine (Read et al., 2002; Win et al., 2006) (Figure 5B). Furthermore, while Cid14 activity was detected in the presence of purines (ATP or GTP), no activity was detected in the presence of pyrimidines (CTP or UTP) (Figure 5D). Cid14 was also able to add an oligoA tail to a larger ~300 nucleotide RNA template (Figure S6). These results demonstrate that Cid14 has polyadenylation activity, which strictly depends on a functional nucleotidyl transferase motif.

To determine whether the degradation of heterochromatic transcripts requires the polyadenylation activity of Cid14, we performed silencing assays with cells overexpressing either wild-type or mutant Cid14. Loss of *imr1R::ura4*$^+$ and *mat3M::ura4*$^+$ silencing in *cid14*Δ cells could be rescued by overexpressing Cid14wt (pRep-Cid14) but not by Cid14DADA (pRep-Cid14DADA) (Figure 5F). Consistent with the silencing assay, quantitative RT-PCR showed that pRep-Cid14 but not pRep-Cid14DADA rescued the derepression of *ura4*$^+$ transcripts in *cid14*Δ cells (data not shown). Similarly, pRep-Cid14 but not pRep-Cid14DADA rescued the derepression of *tlh1*$^+$ transcripts observed in *cid14*Δ cells (Figure 5G). Our results parallel previous observations for Trf4 in the *S. cerevisiae* TRAMP complex (Wyers et al., 2005; Vanacova et al., 2005) and suggest that the polyadenylation activity of the *sp*TRAMP complex is required for the degradation of heterochromatic transcripts.

siRNA Levels Are Greatly Reduced in *cid14*Δ Cells

The generation of centromeric siRNAs is required for the assembly of centromeric heterochromatin, since deletion of *dcr1*$^+$ results in loss of centromeric gene silencing and in greatly reduced levels of H3K9 methylation and Swi6 binding. Moreover, both of these heterochromatic marks are completely lost at centromeric *ura4*$^+$ insertions in *dcr1*Δ cells, indicating that RNAi is required for the spreading of H3K9 methylation and Swi6 binding (Sadaie et al., 2004; Volpe et al., 2002). Vice versa, a proper heterochromatin conformation is required for the generation of siRNAs by Dcr1, as centromeric siRNAs are lost or greatly reduced in *clr4*Δ or *swi6*Δ cells (Motamedi et al., 2004; Buhler et al., 2006; Noma et al., 2004). Based on these observations and data presented here showing that heterochromatin is not affected in *cid14Δ* cells (Figure 3), we expected to see no effect on centromeric siRNA levels upon deletion of *cid14*$^+$. However, centromeric siRNAs from *cid14Δ* were barely detectable on total RNA northern blots (Figure 6A). In order to increase sensitivity, we isolated RNA from Flag-purified Ago1. While we were not able to detect any centromeric siRNAs in *cid12Δ* cells, centromeric siRNAs were about 22-fold reduced in *cid14Δ* compared to wild-type cells (Figure 6B). We obtained the same result after stripping the blot and reprobing with a *ura4*$^+$ specific probe; *ura4*$^+$ siRNAs originating from the *otr1R::ura4*$^+$ locus were 5-fold less abundant in *cid14Δ* compared to wild-type cells (Figure 6C). The *ura4*$^+$ probe also hybridized to a RNA population of >100 nt in *cid14Δ* cells, which were barely detectable in wild-type cells (Figure 6C). We note that these larger RNAs were associated with Ago1 and therefore may represent some form of precursor RNA. These large RNAs seem to be single stranded, since they were only detectable with a probe hybridizing to sense *ura4*$^+$ RNA (data not shown), suggesting that Cid14 may be required for the conversion of single-stranded precursor RNAs to dsRNA.

As mentioned earlier, mutation of the conserved aspartate residues in Cid14 abolished polyadenylation activity in vitro and disrupted the silencing of *imr1R::ura4*$^+$ and *mat3M::ura4*$^+$. Like *cid14Δ*, Cid14 active site mutations had dramatically reduced centromeric siRNA levels (Figure 6D), suggesting that the polyadenylation activity of Cid14 is required for efficient silencing as well as siRNA generation. The polyadenylation activity of TRAMP activates the exosome to degrade RNA, but in some cases also promotes the processing of certain substrates. It is therefore possible that the exosome is activated by Cid14 in order to process precursor RNAs into a proper substrate for siRNA generation. However, we found that centromeric siRNA levels were not affected in *rrp6*Δ cells (Figure 6A). Together, these results indicate that Cid14-dependent polyadenylation of heterochromatic transcripts is required for accumulation of high levels of siRNAs. Furthermore, since heterochromatin appears to be largely preserved in *cid14Δ* cells (Figures 3A and 3B), we conclude that these Cid14-dependent siRNAs are degradation products that are not required for RITS recruitment or maintenance of H3K9 methylation and Swi6 binding.

DISCUSSION

In this study we describe a role for the Cid14 member of the Trf4/5 poly(A) polymerase family in heterochromatic gene silencing in fission yeast. Cid14 resides in a complex that resembles the previously defined TRAMP complex, which is part of a nuclear surveillance mechanism that degrades aberrant transcripts in budding yeast. There are three important features concerning the silencing functions of Cid14 that provide new insight into the mechanism of heterochromatin-mediated gene inactivation. First, the structural integrity of heterochromatin, as judged by the degree of H3K9 methylation and Swi6 localization, appears to be unaffected in *cid14Δ* cells. Remarkably, rather than a deficiency in heterochromatin assembly, defects in silencing are likely to result from a failure to degrade transcripts that emerge from heterochromatic domains. Second, deletion of *cid14*$^+$ results in a dramatic decrease in siRNA levels. The fact that this decrease in siRNA levels occurs without a significant defect in the recruitment of heterochromatin structural components indicates that the low levels of siRNAs observed in *cid14Δ* cells are sufficient for heterochromatin assembly. Therefore, the vast majority of siRNAs, which are produced by a Cid14-dependent pathway, are likely to be degradation products that result from the processing of heterochromatic transcripts. Third, Cid14 is required for efficient transgene silencing at both the centromeric repeats and the silent mating-type loci. At the centromeric repeats, silencing is RNAi-dependent, and the requirement for Cid14 is consistent with its role in siRNA generation. However, silencing at the silent mating-type loci does not require RNAi. Cid14 therefore defines an RNA-processing pathway that may be particularly important for efficient heterochromatic gene silencing in the absence of RNAi. Consistent

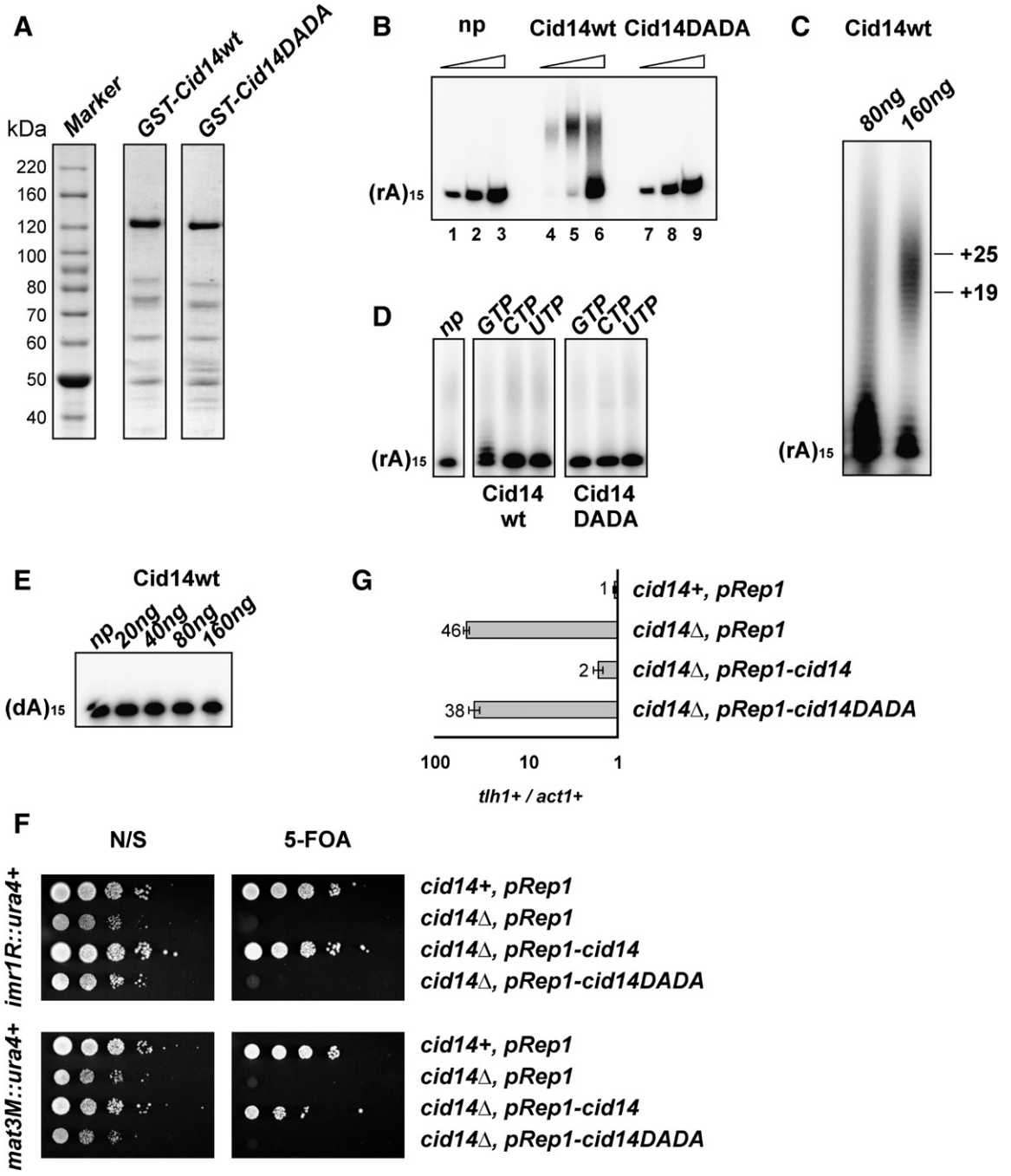

Figure 5. Mutations in the Nucleotidyl Transferase Motif of Cid14 Abolish Polyadenylation Activity In Vitro and Disrupt Heterochromatic Silencing In Vivo

(A) Colloidal Coomassie staining of purified wild-type and mutant recombinant GST-Cid14 proteins.

(B) Fifty, one hundred and fifty, and four houndred and fifty femtomoles 5′-end-labeled oligo$(A)_{15}$ was incubated for 30 min in the presence of ATP with 160 ng recombinant Cid14wt (lanes 4–6) or Cid14DADA (lanes 7–9) protein. np, no Cid14 protein (lanes 1–3). $(rA)_{15}$ indicates the migration position of the 5′-end-labeled oligo$(A)_{15}$.

(C) Fifty femtomoles of 5′-end-labeled oligo$(A)_{15}$ was incubated for 30 min in the presence of ATP with recombinant Cid14wt protein. Numbers indicate the number of adenosine monophosphates added.

(D) Polyadenylation assay with 80 ng of Cid14wt or Cid14DADA in the presence of different ribonucleotide triphosphates showing that Cid14 preferentially adds purines to the $(rA)_{15}$ substrate.

(E) Fifty femtomoles of 5′-end-labeled DNA oligo$(A)_{15}$ (dA_{15}) was incubated for 30 min in the presence of ATP with the indicated amounts of recombinant Cid14wt protein.

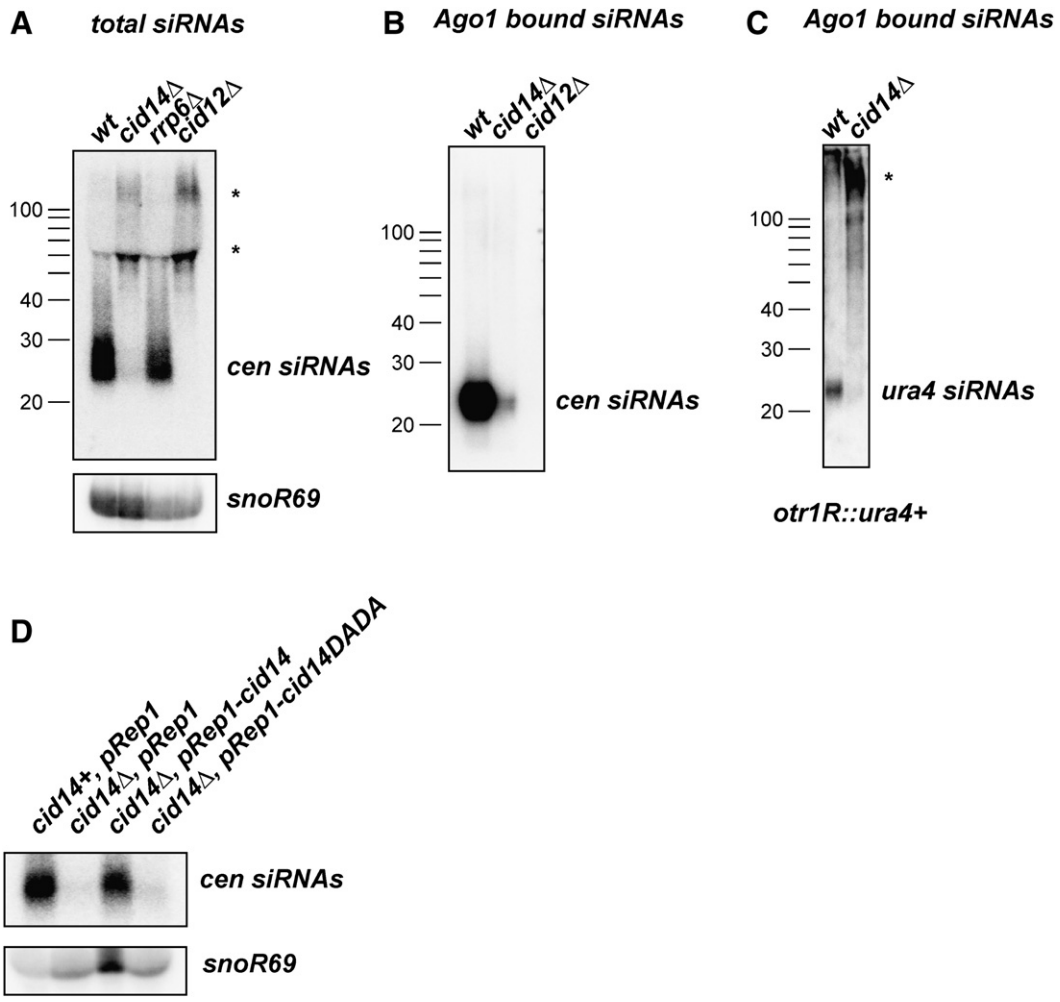

Figure 6. Cid14 with an Active Nucleotidyl Transferase Motif Is Required for siRNA Accumulation

(A and B) Northern blots performed with 25 μg total RNA (A) or 2 μg Ago1-associated RNA (B). The membranes were probed with end-labeled DNA oligos specific for centromeric siRNAs (*cen* siRNAs) and the loading control snoR69 in (A).

(C) The membrane shown in (B) was stripped and reprobed with end-labeled DNA oligos specific for *ura4*$^{+}$ sense siRNAs. Asterisks in (A) and (C) denote possible precursor RNAs.

(D) Northern blot performed with 25 μg total RNA from the indicated strains shows that Cid14 with active site mutations in the nucleotidyl transferase motif (*pRep1-cid14DADA*) fails to restore centromeric siRNA levels in *cid14Δ* cells. The membrane was probed with end-labeled DNA oligos specific for centromeric siRNAs (*cen* siRNAs) and the loading control snoR69.

with the latter possibility and with the previously described role of the Trf4/5 family of poly(A) polymerases, we show that the exosome is required for efficient heterochromatic gene silencing in fission yeast.

Role of RNA Degradation in Efficient Heterochromatic Gene Silencing

What is the nature of the degradation machinery that is recruited and activated by Cid14? One possibility is that degradation is directly mediated by the RNAi pathway through the slicer activity of Ago1. Degradation of target RNAs is initiated by Argonaute-mediated slicing during classical PTGS (Liu et al., 2004; Song et al., 2004; Orban and Izaurralde, 2005). Similarly, siRNAs might mediate the slicing of heterochromatic nascent transcripts by RITS or another Ago1-containing complex. Indeed, recombinant *S. pombe* Ago1 has recently been shown to have slicer activity in vitro, and putative slice products of

(F) Silencing assays showing that overexpression of Cid14 wild-type protein rescues the silencing loss phenotype of *cid14Δ* cells, whereas Cid14 with active site mutations in the nucleotidyl transferase motif does not (*pRep-cid14* and *pRep1-cid14DADA*, respectively).

(G) Quantitative real-time RT-PCR showing that overexpression of Cid14 wild-type protein rescues loss of *tlh1*$^{+}$ silencing in *cid14Δ* cells, whereas Cid14 with active site mutations in the nucleotidyl transferase motif does not (*pRep-cid14* and *pRep1-cid14DADA*, respectively). Mean values are shown, and error bars represent standard deviation (n = 3).

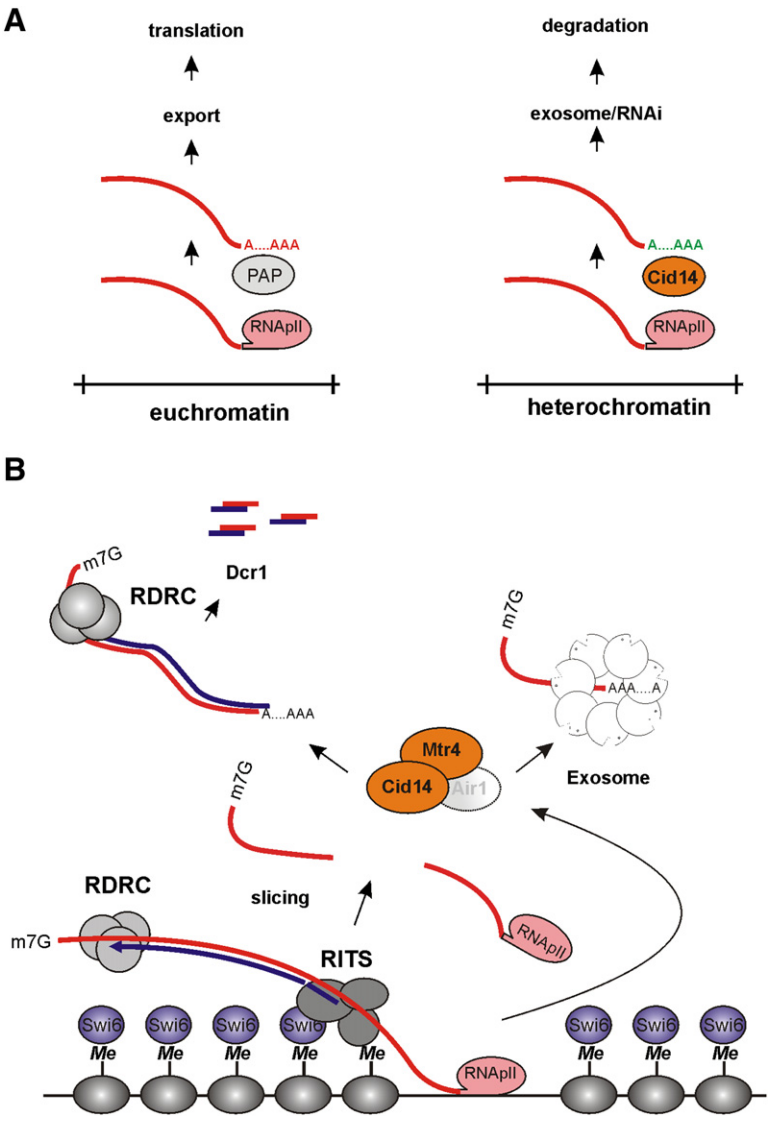

Figure 7. RNA Turnover Mechanisms Contribute to Heterochromatic Gene Silencing in Fission Yeast

(A) Both euchromatic and heterochromatic structures are transcribed by RNA polymerase II, but the fate of the resulting transcripts differs. Transcripts synthesized in euchromatic regions are properly processed, poly(A)-tailed by a canonical poly(A) polymerase (PAP), and exported to the cytoplasm for translation by ribosomes unless they lack processing signals. In contrast, transcripts emerging from heterochromatic domains are marked by a noncanonical poly(A) polymerase (Cid14) for degradation by the exosome or RNAi.

(B) Heterochromatic transcripts are targeted for degradation by RNAi-dependent and -independent RNA turnover mechanisms. The model proposes that heterochromatin interferes with normal RNA processing. This in turn targets transcripts for degradation, which is mediated by the polyadenylation activity of the *sp*TRAMP subunit Cid14. While the "marking" of the heterochromatic transcripts by Cid14 is most likely to occur at the site of transcription, degradation may occur either on or off chromatin. RNA degradation can be mediated by the RNAi machinery (RITS/RDRC/Dcr1), the exosome, or both. Cid14-dependent siRNAs, which constitute the vast majority of centromeric and transgene siRNAs, are largely dispensable for histone H3K9 methylation, Swi6, and RITS recruitment, and are proposed to be RDRC/Dcr1-generated degradation products.

centromeric transcripts have been cloned (Irvine et al., 2006). Similarly, *ura4*$^+$ siRNAs observed in this study may mediate the slicing and subsequent degradation of *ura4*$^+$ transcripts.

In *S. pombe,* plants, and *C. elegans*, the RNAi response requires an RNA-directed RNA polymerase (RdRP), which has been proposed to be involved in amplifying dsRNA using siRNAs as primers (Dalmay et al., 2000; Sijen et al., 2001; Sugiyama et al., 2005; Motamedi et al., 2004). The sole RdRP in *S. pombe*, Rdp1, is found in the RDRC complex, which physically interacts with the RITS complex (Motamedi et al., 2004). Although Rdp1 shows primer-independent RNA polymerase activity in vitro, RITS might act as a priming complex to recruit RDRC to its substrate in vivo (Buhler et al., 2006; Motamedi et al., 2004). Therefore, another possible mechanism may involve the recruitment of RDRC by siRNA-programmed RITS, which would also require Cid14. Subsequently, Dicer would mediate degradation of the transcript after its conversion to dsRNA. In this scenario, RITS would recruit RDRC but may not necessarily have to slice the target RNA. In this regard, in *cid14Δ* cells, the levels of centromeric siRNAs are dramatically reduced, yet we observe little or no defect in H3K9 methylation and Swi6 or Chp1 localization. This result suggests that very low levels of siRNAs are sufficient to maintain heterochromatin in *cis* and to spread histone H3K9 methylation into *cen::ura4*$^+$ inserts and gives rise to the possibility that the majority of siRNAs that we detect in *S. pombe* are the degradation products of an RDRC/Dicer pathway.

While RNAi might indeed be involved in the degradation of centromeric transcripts, it is unlikely to degrade heterochromatic transcripts from other loci in the fission yeast genome. Two lines of evidence support this view: (1) RNAi plays a redundant role with other pathways in silencing at the mating-type locus and the subtelomeric gene

tlh1$^+$ (Jia et al., 2004; Cam et al., 2005), and (2) we are unable to detect any *ura4*$^+$ siRNAs originating from a *ura4*$^+$ gene inserted at the silent mating-type locus *mat3M*. These observations suggest that Cid14 may directly mediate the degradation of these transcripts by the exosome. This possibility is supported by higher levels of *tlh1*$^+$ and *mat3M::ura4*$^+$ transcripts in *sp*TRAMP and exosome mutants.

CONCLUSIONS

Several lines of evidence support a role for chromatin structure in regulation of RNA-processing events. Chromatin remodeling factors have been shown to affect the proper 3′-end processing of a variety of ORFs in fission yeast as well as in budding yeast (Alen et al., 2002). In some cases, RNA processing seems to act in concert with transcriptional gene silencing to achieve high levels of silencing. For example, it was recently reported that total repression of meiotic genes during vegetative growth requires exosome-mediated RNA degradation, in addition to transcriptional repression, although it is unclear whether this pathway also requires a TRAMP-related complex (Harigaya et al., 2006). The role of the Trf4/5 and Cid14-related poly(A) polymerases in the regulation of the RNAi pathway, as well as of the exosome, appears to be highly conserved. rde-3, a *C. elegans* member of this family, is required for siRNA accumulation during the RNAi response (Chen et al., 2005). rde-3, which is more closely related to Cid14 and Trf4/5 than to the Cid12 subunit of RDRC, has been proposed to activate RdRP by polyadenylating RISC cleavage products or aberrant transcripts. In *S. pombe*, polyadenylation of heterochromatic transcripts by Cid14 may mark these transcripts to become substrates for either processing or degradation by the RNAi pathway or the exosome (Figure 7). The high degree of conservation of both the heterochromatin machinery and RNA-processing pathways suggests that similar mechanisms may couple epigenetic gene regulation to RNA processing in multicellular eukaryotes.

EXPERIMENTAL PROCEDURES

Fission Yeast Strains, Plasmids, and Silencing Assays

S. pombe strains used in this study are described in Table S1 and were grown at 30°C in YEA medium (yeast extract supplemented with adenine). All strains were constructed using a PCR-based gene targeting method (Bahler et al., 1998). Positive transformants were selected by growth in YEA medium containing 100–200 μg/ml antibiotic and confirmed by PCR. Plasmids pRep1-cid14 and pRep1-cid14DADA were kindly provided by S.W. Wang (Win et al., 2006). For overexpression of Ago1 and Rdp1, the corresponding open-reading frames were cloned into pREP1-NTAP where TAP was replaced by 3xFLAG (Buker et al., 2007). For expression in *E. coli*, cid14wt and cid14DADA open-reading frames were cloned BamH1-Not1 into pGex-6P-1 (Amersham Biosciences). Silencing assays were done as described (Buhler et al., 2006).

ChIP

ChIP was performed as described previously (Huang and Moazed, 2003; Buhler et al., 2006). Antibodies used were the following: Swi6 (abcam, #14898), dimethylated H3-K9 (abcam, #ab1220), Chp1 (abcam, #ab18191), or RNA polymerase II 8WG16 monoclonal (Covance, #MMS-126R). For primer information see Table S2 and Figure S5.

Protein Purification and Mass Spectrometry

Cid14-TAP and 3xFLAG-Ago1 was purified from 30 g and 600 mg of cells, respectively, as described previously (Buker et al., 2007). Proteins from duplicate Cid14-TAP purifications and untagged strain control purifications were precipitated with trichloroacetic acid. The pellets were subjected to LC-MS/MS as described previously (Haas et al., 2006; Buker et al., 2007). Recombinant *S. pombe* Cid14wt and Cid14DADA were expressed as GST fusions in *E.coli* BL21 Codon Plus (DE3) RIL (Stratagene) from pGEX-Cid14wt or pGEX-Cid14DADA plasmids. The expression and purification of GST fusions were performed as described (Buker et al., 2007).

Polyadenylation Assays

Standard polyadenylation assays were carried out in 20 μl reaction mixtures containing 80 ng of the recombinant protein, 50 fmol of end-labeled RNA (rA_{15} or mouse β-actin RNA transcribed from pTRI-actin-mouse (Ambion), 0.5 mM ATP, 0.05 mg/ml BSA, 20 mM Tris-HCl (pH 7.0), 0.5 mM MnCl2, 0.2 mM EDTA, 10% glycerol, and 40U RNAsin (Promega). Reactions were incubated at 37°C for 30 min. RNA was phenol/chloroform extracted and EtOH precipitated before loading on 6% denaturing polyacrylamide gel (8 M urea).

RNA Analysis

Total RNA was isolated from logarithmically growing *S. pombe* (in YEA medium) using the hot phenol method (Leeds et al., 1991). For detection of siRNAs, either total RNA was isolated and enriched for RNAs <200 nt, as described previously (Buhler et al., 2006) or Ago1-associated RNAs were recovered from Flag-purified FLAG-Ago1 protein by phenol-chloroform extraction and EtOH precipitation. Twenty-five micrograms small total RNA or 1–10 μg Ago1-associated RNA was used for northern blot analysis performed as described (Buhler et al., 2006). To detect centromeric siRNAs, a mix of oligos corresponding to the siRNAs sequenced by Reinhart and Bartel (Buhler et al., 2006) was labeled. For *ura4*$^+$ antisense and sense siRNAs, a mix of 11 oligos distributed over the entire *ura4*$^+$ ORF was labeled (ura4A-K and ura4A′-K′, respectively; Table S2). To detect the loading control snoR69 (SPSNORNA.19), the oligo mb151 served as a probe (Table S2).

RT-PCR

Semi-quantitative endpoint RT-PCR was performed as described previously (Buhler et al., 2006). For primers, see Table S2 and Figure S5. Quantitative real-time RT-PCR was performed on a LightCycler 1.5 Instrument (Roche) using Light Cycler RNA Master SYBR Green I (Roche #03064760001). Relative RNA levels were calculated from C_T values according to the ΔC_T method (Applied Biosystems) and normalized to *act1*$^+$ RNA levels. For reaction conditions see Table S3.

Supplemental Data

Supplemental Data include six figures, four tables, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/129/4/707/DC1/.

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