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Tethering RITS to a Nascent Transcript Initiates RNAi- and Heterochromatin-Dependent Gene Silencing

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SUMMARY

In the fission yeast *Schizosaccharomyces pombe,* the RNA-Induced Transcriptional Silencing (RITS) complex has been proposed to target the chromosome via siRNA-dependent base-pairing interactions to initiate heterochromatin formation. Here we show that tethering of the RITS subunit, Tas3, to the RNA transcript of the normally active *ura4*$^+$ gene silences *ura4*$^+$ expression. This silencing depends on a functional RNAi pathway, requires the heterochromatin proteins, Swi6/HP1, Clr4/Suv39h, and Sir2, and is accompanied by the generation of *ura4*$^+$ siRNAs, histone H3-lysine 9 methylation, and Swi6 binding. Furthermore, the ability of the newly generated *ura4*$^+$ siRNAs to silence a second *ura4*$^+$ allele in *trans* is strongly inhibited by the conserved siRNA nuclease, Eri1. Surprisingly, silencing of tethered *ura4*$^+$, or *ura4*$^+$ inserted within centromeric heterochromatin, or some of the endogenous centromeric repeat promoters, is not associated with changes in RNA polymerase II occupancy. These findings support a model in which targeting of nascent transcripts by RITS mediates chromatin modifications and suggest that cotranscriptional processing events play a primary role in the silencing mechanism.

INTRODUCTION

RNA-based silencing mechanisms are widespread in eukaryotes and act at multiple levels to regulate gene expression (Plasterk and Ketting, 2000; Hannon, 2002; Zamore, 2002; Bartel, 2004; Baulcombe, 2004; Meister and Tuschl, 2004; Mello and Conte, Jr., 2004). In fission yeast, *Tetrahymena*, plants, and *Drosophila*, RNA silencing mechanisms that share mechanistic similarity to the RNAi pathway mediate repressive histone modifications and heterochromatin assembly (Lippman and Martienssen, 2004). The assembly of heterochromatin involves an orchestrated array of chromatin modifications. In fission yeast, deacetylation of histone H3 amino termini by the class I and II histone deacetylases Clr3 and Clr6 as well as the class III NAD-dependent deacetylase Sir2 is followed by methylation of histone H3 at lysine 9 (K9) by the methyltransferase Clr4 to create a binding site for the Swi6 and Chp1 chromodomain proteins (Grewal et al., 1998; Partridge et al., 2000; Nakayama et al., 2001; Bjerling et al., 2002; Shankaranarayana et al., 2003). Histone H3-K9 methylation is a conserved hallmark of heterochromatin and appears to spread along the chromatin fiber through sequential cycles of methylation coupled to oligomerization of Swi6, a homolog of the *Drosophila* and mammalian HP1 proteins (Richards and Elgin, 2002; Grewal and Moazed, 2003). In addition to these histone binding proteins and histone-modifying enzymes, components of the RNAi pathway are required for heterochromatin assembly in *S. pombe* (Hall et al., 2002; Volpe et al., 2002).

RNAi and other RNA-silencing mechanisms are triggered by double stranded RNA (dsRNA) (Fire et al., 1998; Hannon, 2002; Bartel, 2004). The mechanism of silencing involves the generation of small RNA molecules of ~22 nucleotides from longer dsRNAs by an RNase III-like enzyme called Dicer (Hamilton and Baulcombe, 1999; Zamore et al., 2000; Bernstein et al., 2001; Elbashir et al., 2001). These small interfering RNAs (siRNAs) then load onto an effector complex called RISC (RNA-Induced Silencing Complex), which contains a conserved Argonaute family member and targets cognate mRNAs for degradation (Hammond et al., 2000; Caudy et al., 2003). In a related process, small RNAs, called miRNAs, are produced from hairpin RNA transcripts by Dicer enzymes and program RISC for translational repression or degradation of target mRNAs (Hannon, 2002; Pillai, 2005). In some organisms, the RNAi response also requires an RNA-directed RNA polymerase (RdRP) that may be involved in amplifying dsRNA using siRNAs as primers (Dalmay et al., 2000; Sijen et al., 2001). The *S. pombe* genome codes for a single homolog of each of the above key RNAi enzymes, called Dcr1, Ago1, and Rdp1, all of which are required for

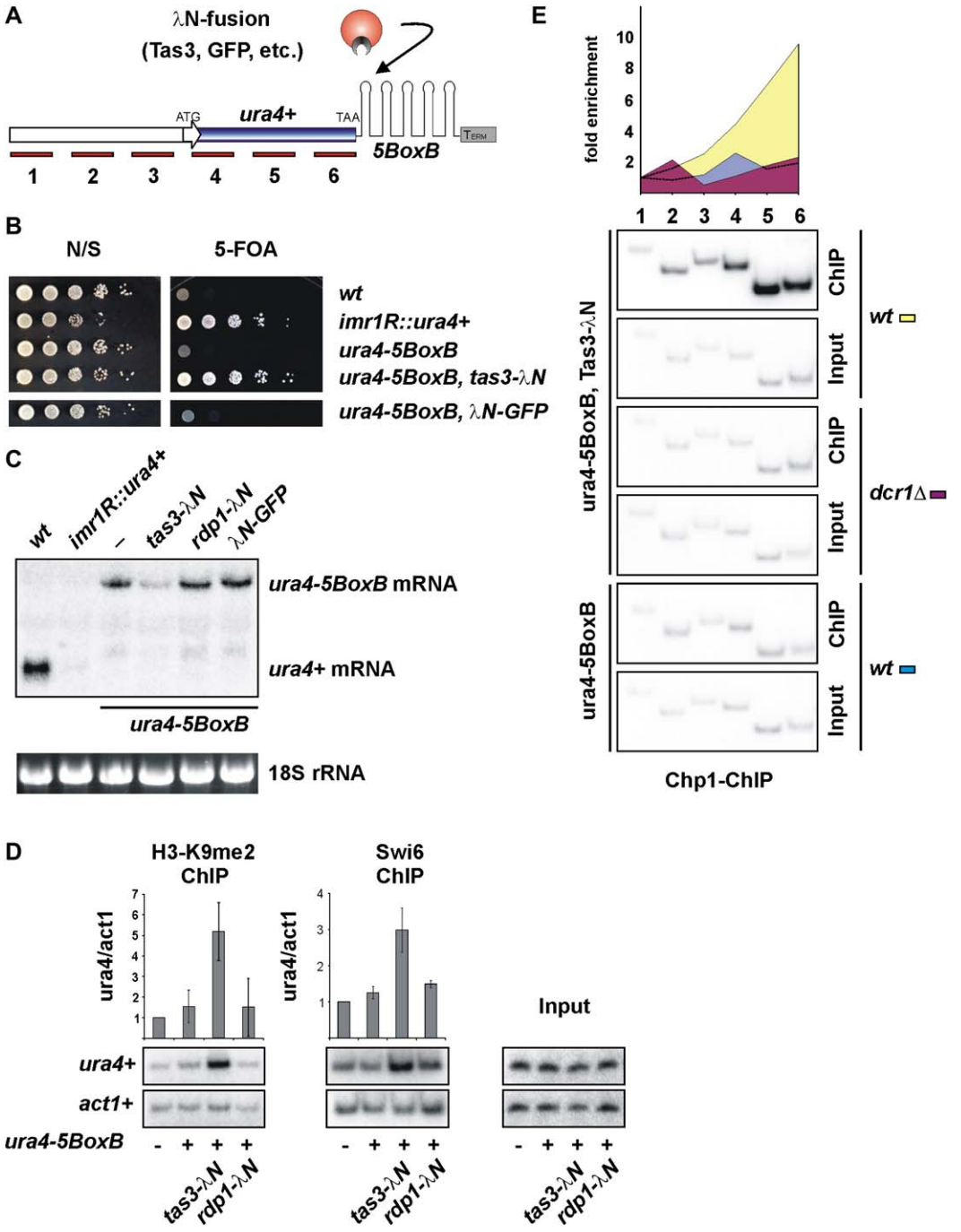

Figure 1. Tethering Tas3 to the *ura4*$^+$ Transcript Initiates *ura4*$^+$ Silencing

(A) Scheme representing the *ura4*$^+$ mRNA reporter, containing five 19nt BoxB hairpins, which bind to λN-fusion proteins, in the 3′UTR, at the endogenous *ura4*$^+$ locus. Red bars represent the amplified DNA fragments as shown in (E). The fragments are spaced by ~100 nt. ATG, start codon; TAA, stop codon; TERM, terminator sequence.

(B) Tethering Tas3 to the *ura4-5BoxB* reporter induces silencing of the *ura4*$^+$ gene as indicated by growth on 5-FOA medium. Silencing of a centromeric *imr1R::ura4*$^+$ reporter gene is shown for comparison. Neither insertion of 5BoxB sites alone nor tethering of GFP confers 5-FOA resistance. N/S, nonselective medium; wt, wild-type *S. pombe*.

(C) Northern blot was performed with total RNA isolated from the indicated strains, using a probe specific for *ura4*$^+$/*ura4-5BoxB* mRNA. The 18S rRNA band from the ethidium bromide-stained gel before blotting is shown in the lower panel as a loading control.

(D) ChIP experiments showing that Tas3-tethering induces H3-K9 dimethylation (left panel) and recruitment of Swi6 to the *ura4-5BoxB* locus (middle panel). Average fold-enrichment values from three experiments, normalized to *act1*$^+$, are presented as histograms above the corresponding gels. Error bars indicate standard deviations. The amplified *ura4*$^+$ DNA corresponds to fragment 5 shown in (A).

heterochromatin formation at centromeric DNA regions (Volpe et al., 2002). Moreover, components of the RNAi pathway are required for the formation of centromeric heterochromatin in *Drosophila* (Pal-Bhadra et al., 2004), DNA elimination in somatic macronuclei of Tetrahymena, and for siRNA-mediated DNA or histone H3-K9 methylation in plant and mammalian cells (Mochizuki et al., 2002; Taverna et al., 2002; Baulcombe, 2004; Morris et al., 2004).

In *S. pombe*, two effector complexes have been identified which are essential for the assembly and maintenance of heterochromatin at centromeric DNA repeats by an RNA silencing mechanism that uses the components of the RNAi pathway. Ago1, together with siRNAs that match the sequence of repetitive DNA at the outer centromeric repeats, Tas3, and the chromodomain protein Chp1 assemble into the RNA-Induced Transcriptional Silencing (RITS) complex (Verdel et al., 2004). The RNA-directed RNA polymerase Rdp1 is associated with two conserved proteins, Hrr1, an RNA helicase, and Cid12, a member of the polyA polymerase family, in a complex termed RNA-Directed RNA polymerase Complex (RDRC) that has RNA-directed RNA polymerase activity (Motamedi et al., 2004; Sugiyama et al., 2005). The RITS and RDRC complexes physically interact in a Dicer and Clr4-dependent fashion, suggesting that both siRNAs and chromatin recruitment are required for their interaction. Furthermore, crosslinking experiments have shown that subunits of both complexes associate with chromatin as well as centromeric transcripts in a Dcr1-dependent manner (Volpe et al., 2002; Motamedi et al., 2004). Intriguingly, two different subunits of RNA polymerase II (RNApII) have recently been shown to be essential for RNAi-mediated heterochromatin assembly in *S. pombe* (Djupedal et al., 2005; Kato et al., 2005). Based on these data, a model has emerged in which the association of the RITS complex with chromatin and the initiation of chromatin modifications is proposed to involve siRNA-pre-mRNA base pairing as RNApII synthesizes the RNA transcript. 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.

According to the above nascent transcript model, artificial tethering of RITS to a nascent RNA may be expected to result in heterochromatin assembly and silencing of the cognate gene. In this study, we show that tethering the Tas3 subunit of RITS to a modified *ura4*$^+$ RNA, using the site-specific RNA binding protein λN, results in histone H3-K9 methylation, Swi6 recruitment, and silencing of the *ura4*$^+$ gene. This silencing is accompanied by the generation of *ura4*$^+$ siRNAs in an RNAi- and chromatin-dependent manner and requires both RNAi and heterochromatin components. The *ura4*$^+$ siRNA-programmed RITS is unable to silence the expression of a second allele of *ura4*$^+$ in *trans* unless the conserved RNAi inhibitor *eri1*$^+$ is deleted. This *cis*-restriction of siRNA-mediated silencing in *eri1*$^+$ cells suggests that *S. pombe* siRNAs are normally restricted to their site of synthesis by Eri1 but can act in *trans* to initiate Swi6 recruitment and gene-specific silencing once Eri1 inhibition is removed. Furthermore, the inability of *ura4*$^+$ siRNAs to act in *trans* to silence the expression of a second *ura4*$^+$ allele in *eri1*$^+$ cells strongly sugges that Tas3-λN acts locally on nascent, rather than mature, transcripts to initiate RNAi-dependent chromatin modifications. Finally, we found that similar levels of RNA polymerase II (RNApII) are associated with *ura4*$^+$ genes that were active or silenced by either Tas3 tethering or insertion within centromeric heterochromatin. Our findings suggest that, in addition to transcriptional gene silencing (TGS), cotranscriptional gene silencing (CTGS) is a major feature of RNAi- and heterochromatin-mediated silencing mechanisms.

RESULTS

Tethering of Tas3 to a *ura4*$^+$ Transcript Initiates Heterochromatin Formation and *ura4*$^+$ Silencing

In order to test whether the association of RNAi factors with an RNA could initiate silencing of the gene from which the RNA is transcribed, we set out to individually tether subunits of RITS or RDRC to *ura4*$^+$ transcripts, taking advantage of the high-affinity binding of the λN-peptide to its cognate RNA binding site, termed BoxB (Baron-Benhamou et al., 2004). We generated a strain containing five BoxB sites in the 3′UTR of the endogenous *ura4*$^+$ gene (*ura4-5BoxB*, Figure 1A). As shown in Figure 1B, this strain was not able to grow on counter selective 5-FOA medium, which indicates that insertion of the five BoxB sites did not significantly interfere with *ura4*$^+$ expression. Subsequently, we generated cells expressing various λN-fusion proteins and tested them for *ura4*$^+$ silencing on 5-FOA medium. While λN-GFP, λN-Ago1, Chp1-λN, Cid12-λN, Hrr1-λN, or Rdp1-λN showed only weak or no silencing (data not shown), Tas3-λN conferred resistance to 5-FOA. We replated the FOA-resistant cells and further propagated them in nonselective medium (Figure S1A). After this propagation step, *ura4-5BoxB*/Tas3-λN cells maintained silencing of the *ura4*$^+$ locus with a similar efficiency to silencing observed for a *ura4*$^+$ reporter gene inserted at centromeric DNA repeats (compare *ura4-5BoxB*, Tas3-λN and *imr1R::ura4*$^+$, Figure 1B). However, silencing was lost upon replacement of Tas3-λN with Tas3, indicating that continued tethering was required to efficiently maintain *ura4-5BoxB* in the silent state (Figure S1B). Consistent with the 5-FOA silencing assay, Northern blot analyses revealed that Tas3-λN, but neither Rdp1-λN nor λN-GFP reduced *ura4-5BoxB* mRNA levels (Figure 1C).

(E) Chp1 binding to the entire *ura4-5BoxB* gene was mapped by ChIP, demonstrating that the Chp1 subunit of the RITS complex spreads across the entire *ura4-5BoxB* locus over a distance of about 1.5 kb away from the *5BoxB* sites in a Tas3-tethered and Dcr1-dependent manner. Fold-enrichment values from one typical experiment, normalized to input, are represented in the diagram. Location of the amplified DNA fragments is indicated in (A).

While the results described above could be attributed to either transcriptional or posttranscriptional gene silencing (TGS or PTGS, respectively), chromatin-immunoprecipitation (ChIP) experiments revealed that tethering Tas3 to *ura4*$^+$ transcripts induced di-methylation of histone H3 lysine 9 (H3-K9me2) and Swi6 binding (Figure 1D), demonstrating that the observed *ura4-5BoxB* silencing is accompanied by the recruitment of heterochromatin markers. Moreover, the Chp1 subunit of the RITS complex spread across the entire *ura4-5BoxB* locus over a distance of about 1.5 kb away from the *5BoxB* sites (Figure 1E). In *dcr1*Δ cells, the association of Chp1 with *ura4-5BoxB* was reduced to background levels observed in cells that lacked Tas3-λN. Finally, the association of RITS with *5BoxB* sites required transcription of *ura4-5BoxB* as replacement of the promoter and open reading frames of *ura4-5BoxB* with an antibiotic resistance marker abolished the association of Chp1 with *5BoxB* DNA (Figure S1D). This result ruled out the possibility that the Tas3-λN fusion protein in our experiments bound directly to the 5BoxB DNA sites rather than the *ura4-5BoxB* RNA transcript.

ura4$^+$ Silencing Requires the RNAi Machinery and Heterochromatin Proteins

RNAi components are required to initiate and maintain heterochromatin at centromeres, but they cooperate with DNA binding transcription factors to nucleate heterochromatin assembly at the mating type (*mat*) locus (Hall et al., 2002; Volpe et al., 2002). In order to test whether the RNAi pathway is required to maintain the Tas3-λN-induced silencing of the *ura4*$^+$ gene, we examined the effect of deleting several classes of factors involved in RNAi-mediated heterochromatin assembly on *ura4-5BoxB* silencing. Deletion of the genes encoding the other two subunits of RITS (*chp1*$^+$, *ago1*$^+$) or any subunits of the RDRC (*rdp1*$^+$, *hrr1*$^+$, *cid12*$^+$) disrupted *ura4-5BoxB* silencing (Figure 2A). These results show that the RNAi pathway is required to maintain Tas3-λN-induced silencing of the *ura4-5BoxB* locus, as it is the case for maintenance of heterochromatin at centromeric DNA repeats (Volpe et al., 2002). In addition, disruption of the Clr4 methyltransferase and the Sir2 histone deacetylase (HDAC) resulted in loss of *ura4-5BoxB* silencing, and disruption of Swi6, which binds to methylated histone H3, resulted in greatly reduced levels of silencing (Figure 2A). Tethered *ura4*$^+$ silencing was also sensitive to the deacetylase inhibitor trichostatin A but could be fully reestablished ten generations after removal of the inhibitor (Figure S1C), which suggests a requirement for NAD-independent deacetylases such as Clr3 and Clr6. These findings support a role for the assembly of a repressive chromatin structure in Tas3-λN-dependent silencing of *ura4-5BoxB*.

Tethering of Tas3 to *ura4*$^+$ RNA Induces *ura4*$^+$ siRNA Generation

The finding that RNAi is required for Tas3-λN-dependent silencing of the *ura4-5BoxB* locus is intriguing since tethering RITS directly to *ura4*$^+$ transcripts might circumvent the requirement for siRNA-directed association of RITS with nascent transcripts. The fact that Dcr1 is essential for the association of Chp1 with *ura4-5BoxB* (Figure 1E) and Tas3-λN induced silencing (Figure 2A) argues against this possibility and suggests that tethering RITS to RNA induces de novo siRNA production, which is essential for robust silencing. Indeed, we observed that Tas3-λN induced the generation of small RNAs which matched the *ura4*$^+$ open reading frame (ORF) and were similar in size to that observed for centromeric siRNAs (Figure 2B). To verify that *ura4*$^+$ siRNAs also loaded onto RITS, we used TAP-tagged Chp1 to affinity purify RITS from cells expressing *ura4-5BoxB* with either Tas3-λN or unmodified Tas3. While RITS complexes from both strains contained centromeric siRNAs, only RITS purified from cells carrying Tas3-λN contained *ura4*$^+$ siRNAs (Figure 2C). Interestingly, *ura4*$^+$ siRNA generation was abolished in the RNAi mutants as well as in strains lacking Clr4, Sir2, or Swi6 (Figure 2D). However, while deletion of *sir2*$^+$ and *swi6*$^+$ reduced the levels of centromeric siRNAs, *ura4*$^+$ siRNAs, induced by tethering Tas3-λN to *ura4*$^+$ RNA, were not detectable in these mutant backgrounds (Figure 2D). *ura4*$^+$ siRNAs may be less abundant than centromeric siRNAs in wild-type cells. We therefore cannot rule out the possibility that *ura4*$^+$ siRNAs are present at low levels in *sir2Δ* and *swi6Δ* cells, which might account for the incomplete loss of silencing observed for *swi6Δ* cells (Figure 2A). Thus, the general requirements for the generation of tethering-induced *ura4*$^+$ siRNAs mirror the requirements for the generation of centromeric siRNAs.

siRNA-Programmed RITS Is *cis*-Restricted

Similar to what has been observed in multicellular eukaryotes (Hannon and Rossi, 2004), the expression of dsRNA from a hairpin construct can induce PTGS in fission yeast by a mechanism that requires the RNAi components Ago1, Dcr1, and Rdp1 as well as the Clr4 H3-K9 methyltransferase (Sigova et al., 2004). Whether newly generated siRNAs can also silence homologous sequences in an RNAi- and heterochromatin-dependent manner in *trans* is unknown. Since our tethering approach results in loading of RITS with *ura4*$^+$ siRNAs and efficient silencing of the *ura4-5BoxB* gene, we asked whether this newly programmed RITS is able to silence a second *ura4*$^+$ allele in *trans*. To test this possibility, we generated cells containing an additional *ura4*$^+$ allele at the *leu1*$^+$ locus on chromosome 2 (*ura4*$^+$*-2*). In contrast to the first allele at the endogenous locus, the second allele does not contain the five BoxB sites but has an intron in the middle of the ORF (Figure 3A). This intron allowed us to distinguish between the two ORFs at the DNA level. If siRNAs that are generated by silencing of the *ura4-5BoxB* allele can act in *trans*, we would expect these siRNAs to promote the silencing of the *ura4*$^+$*-2* allele. As expected from the results presented in Figures 1 and 2, the *ura4-5BoxB* allele was efficiently silenced upon Tas3-λN tethering (Figure 3B). However, cells containing both alleles were unable to grow on medium containing 5-FOA, suggesting that the *ura4*$^+$*-2* allele was

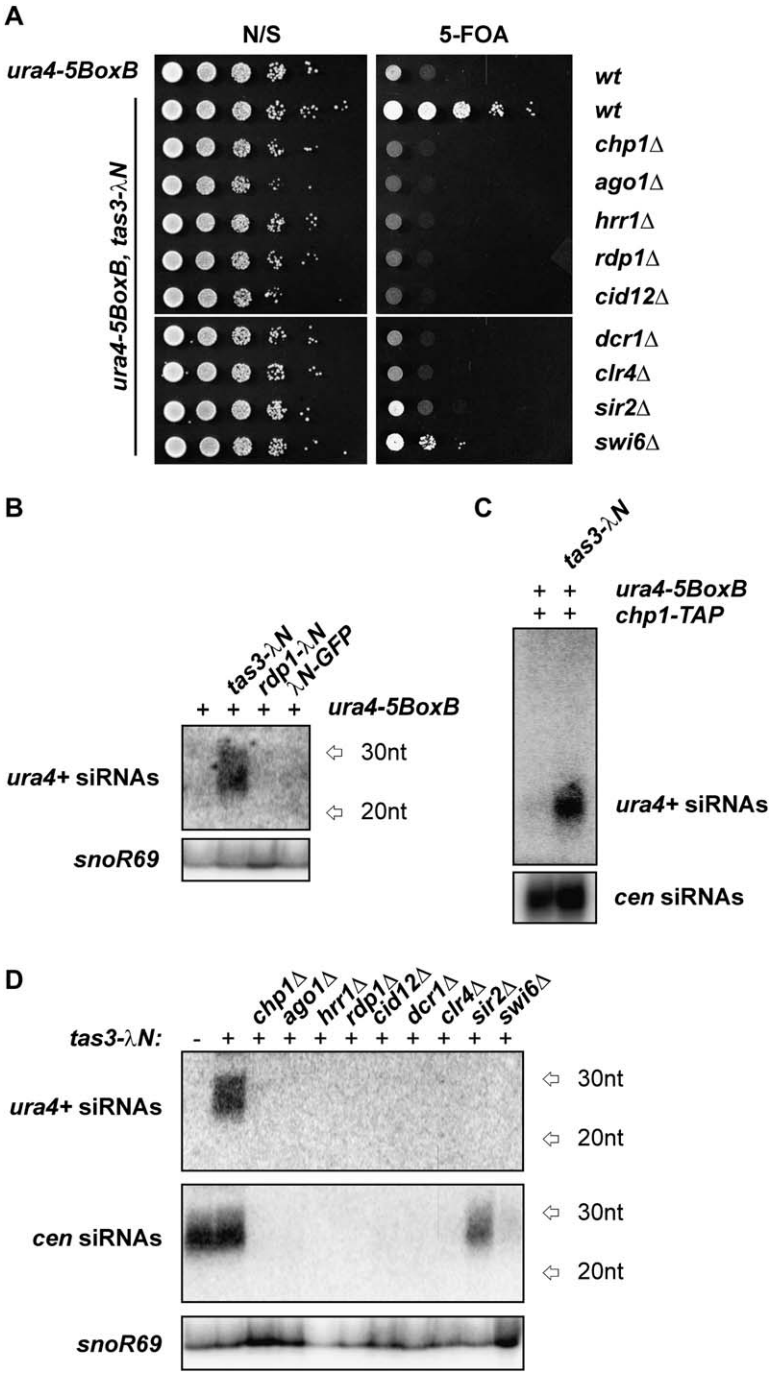

Figure 2. *ura4-5BoxB* Silencing and *ura4*$^+$ siRNA Production Depend on the RNAi Pathway, Histone-Modifying Enzymes, and Swi6

(A) Silencing assays showing that RNAi components, the histone methyltransferase Clr4, the histone deacetylase Sir2, and the heterochromatin protein Swi6 are required for Tas3-λN-dependent *ura4-5BoxB* silencing. Ten-fold serial dilutions of cells with the indicated genotype were plated on nonselective (N/S) or 5-FOA-containing medium.

(B) Northern blot showing that Tas3 tethering to *ura4-5BoxB* mRNA induces generation of siRNAs matching the *ura4*$^+$ open reading frame. End-labeled DNA oligos were used to probe for siRNAs. The snoRNA snoR69 serves as a loading control. 20nt and 30nt long DNA oligos were run as marker.

(C) Northern blot showing that *ura4*$^+$ siRNAs are associated with RITS. Parallel RITS purifications were performed from *chp1-TAP* cells carrying *ura4-5BoxB* with either Tas3-λN or unmodified Tas3. The membrane was probed with end-labeled DNA oligos specific for *ura4*$^+$ or centromeric siRNAs (cen siRNAs).

(D) Northern blot performed with total RNA preparations from the same strains as in (A). The membrane was probed with end-labeled DNA oligos specific for *ura4*$^+$ siRNAs, centromeric siRNAs (*cen* siRNAs), and the loading control snoR69. 20nt and 30nt long DNA oligos complementary to the end-labeled DNA oligos were run as size markers.

active (Figure 3B). To determine whether in cells containing both *ura4-5BoxB and ura4*$^+$*-2* alleles each allele displayed a distinct chromatin state, we examined the chromatin structure of each allele using ChIP experiments. As shown in Figure 3C, the Ago1 and Chp1 subunits of RITS localized to the *ura4-5BoxB* allele but not to the *ura4*$^+$*-2* allele. Moreover, we detected both H3-K9 methylation and Swi6 binding at the *ura4-5BoxB* but not the *ura4*$^+$*-2* locus. These results indicate that in *S. pombe* homologous sequences targeted by RNAi can coexist in the *on* and *off* states.

The Ability of siRNAs to Act in *trans* Is under Negative Control by the siRNA Ribonuclease Eri1

In *C. elegans*, RNAi is negatively regulated by Eri-1, an exoribonuclease that degrades double stranded siRNAs (Kennedy et al., 2004). Eri-1 is conserved in most eukaryotes and has a single *S. pombe* ortholog (Kennedy et al.,

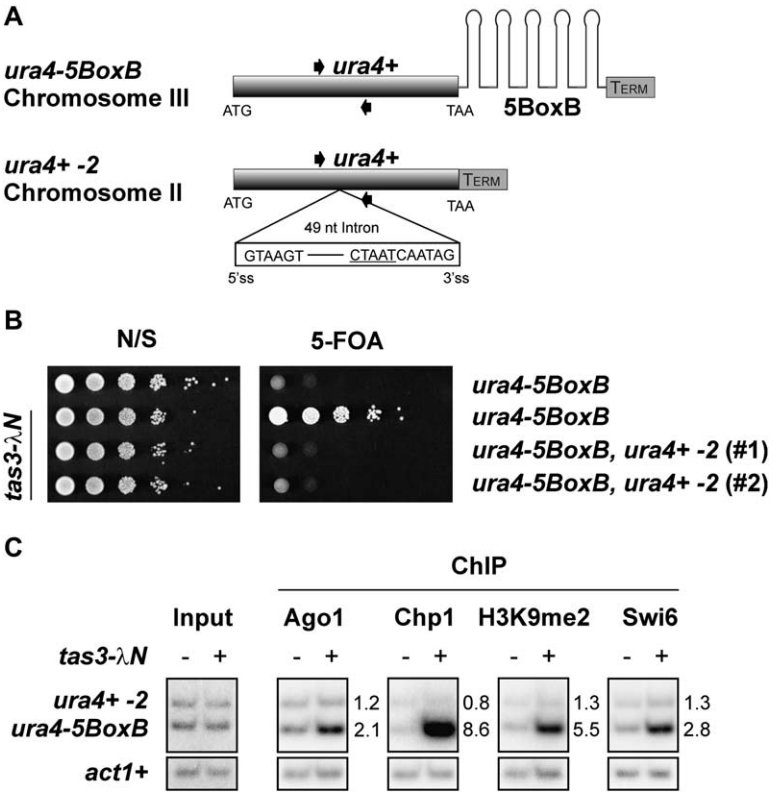

Figure 3. siRNA-Programmed RITS Acts in *cis* to Initiate Heterochromatin Formation and Is Unable to Target Homologous Sequences in *trans*

(A) Scheme representing the two *ura4*$^+$ alleles. *ura4-5BoxB* is located on chromosome III at its endogenous locus and contains five 19nt BoxB hairpins in the 3′UTR (see also Figure 1A). *ura4*$^+$*-2* is located at the *leu1*$^+$ locus on chromosome II. This allele does not contain the five BoxB sites but has an intron in the middle of the ORF. Splice site consensus and branchpoint (underlined) sequences are shown. Arrows indicate the location of primers used for ChIP in (C). ATG, start codon; TAA, stop codon; TERM, terminator sequence; 5′ss, 5′ splice site; 3′ss, 3′ splice site.

(B) Silencing assays showing that the *ura4-5BoxB* allele is efficiently silenced upon Tas3-λN tethering. Cells containing both *ura4-5BoxB* and *ura4*$^+$*-2* alleles are unable to grow on 5-FOA medium, suggesting that the *ura4*$^+$*-2* allele is active. Ten-fold serial dilutions were plated on the indicated medium.

(C) ChIP experiments showing that cells containing both *ura4-5BoxB* and *ura4*$^+$*-2* alleles display distinct chromatin states. Tas3-λN tethering induces H3-K9 di-methylation and recruits Ago1, Chp1, and Swi6 to the *ura4-5BoxB* allele, but not to the *ura4*$^+$*-2* allele. Enrichment of *ura4*$^+$ with the indicated proteins in Tas3-λN tethered cells is compared with *ura4*$^+$ from untethered cells and is shown next to the panels.

2004), which negatively regulates RNAi-mediated heterochromatin formation and reduces the levels of heterochromatic siRNAs (T. Iida and J. Nakayama, personal communication). We reasoned that Eri1 might inhibit *ura4*$^+$ siRNAs from acting in *trans*, perhaps by reducing *ura4*$^+$ siRNA levels. To test this hypothesis, we deleted *eri1*$^+$ in cells that contained both the *ura4-5BoxB* and *ura4*$^+$*-2* alleles as well as Tas3-λN. Most such *eri1*Δ cells were unable to grow on 5-FOA medium, indicating that the *ura4*$^+$*-2* allele was active (Figure 4A). However, *eri1*Δ *ura4-5BoxB ura4*$^+$*-2* cells consistently gave rise to 5-FOA resistant colonies at a low frequency, which grew at higher efficiency on 5-FOA medium upon replating (Figure 4A, bottom row). These results suggested that silencing at *ura4*$^+$*-2* was established at a low frequency in *eri1*Δ cells, but could be maintained relatively efficiently after this establishment event. To determine whether silencing of *ura4*$^+$*-2* was accompanied by changes in its chromatin structure, we used ChIP to examine the localization of the Chp1 subunit of RITS and Swi6 with both *ura4*$^+$ alleles in *ura4-5BoxB ura4*$^+$*-2* cells, which were either *eri1*$^+$ or *eri1*Δ. As shown in Figure 4B, deletion of *eri1*$^+$ resulted in an association of Chp1 and Swi6 with the *ura4*$^+$*-2* allele (~12- and ~2.5-fold enrichment relative to *eri1*$^+$ cells). Consistent with the role of Eri1 as a general inhibitor of RNAi-mediated heterochromatin formation, we also observed a ~2-fold increase in the association of both Chp1 and Swi6 with the *ura4-5BoxB* allele (Figure 4B). These results show that in *S. pombe* newly generated siRNAs can recruit heterochromatin proteins and initiate de novo silencing in *trans*, but that this *trans* silencing is strongly inhibited by Eri1.

TGS versus PTGS

In *S. pombe,* RNAi-mediated gene silencing is associated with histone H3-K9 methylation and heterochromatin assembly, suggesting that it inhibits gene expression, at least in part, by a chromatin-dependent transcriptional gene silencing (TGS) mechanism (Volpe et al., 2002; Noma et al., 2004; Verdel et al., 2004). Similarly, tethered silencing requires heterochromatin components and is associated with recruitment of RITS, histone H3-K9 methylation, and Swi6/HP1 binding, suggesting that it functions at the chromatin level (Figures 1–3). According to classical TGS models, silencing should result in a reduction in the rate of transcription initiation and reduced RNApII occupancy at the silenced gene (Richards and Elgin, 2002). To determine whether tethered Tas3-λN silencing inhibits transcriptional initiation that is correlated with reduced levels of RNApII occupancy, we used ChIP to examine the association of RNApII with *ura4-5BoxB* in the presence or absence of Tas3-λN. As shown in Figure 5B, RNApII associated with DNA fragments spanning the *ura4-5BoxB* locus, from −100 bp upstream of the promoter to just before the *5BoxB* sites (fragments 3–6, left panels) with a similar efficiency in the presence or absence

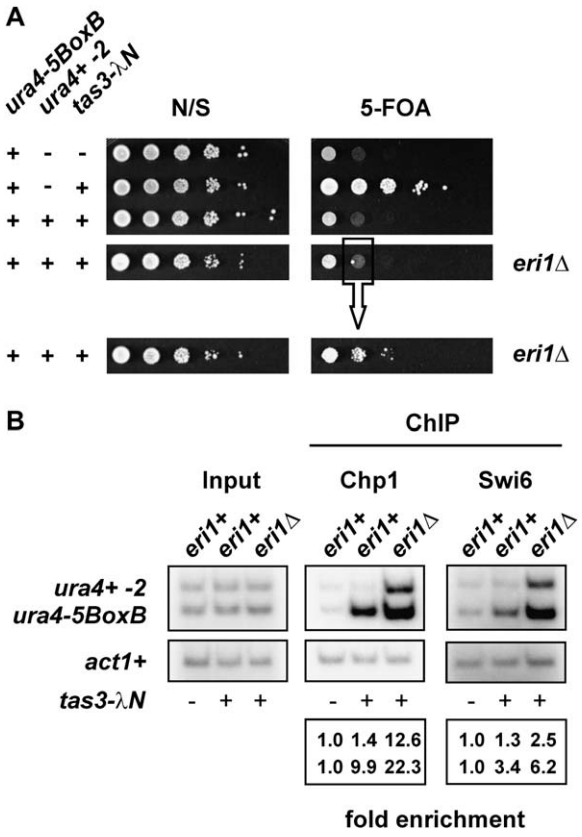

Figure 4. In *eri1*Δ Cells, siRNA-Programmed RITS Targets Homologous Sequences in *trans*

(A) Silencing assays showing that the *ura4-5BoxB* allele is efficiently silenced upon Tas3-λN tethering. While *eri1*$^+$ cells containing both *ura4-5BoxB* and *ura4*$^+$*-2* alleles (see also Figure 3A) are unable to grow on 5-FOA medium, *eri1*Δ cells consistently gave rise to single FOA-resistant colonies. These 5-FOA resistant *eri1*Δ cells (arrow) were isolated and grown for ten generations in nonselective medium before another silencing assay was performed (see lower panel). The same cells were used for ChIP experiments shown in (B). Ten-fold serial dilutions of cultures were plated on the indicated medium.

(B) ChIP experiments showing that Chp1 and Swi6 localize to the *ura4*$^+$*-2* allele in *eri1*Δ cells but not in *eri1*$^+$ cells (compare middle lanes with far right lanes; see also Figure 3C). The *eri1*Δ cells used for this experiment originate from a 5-FOA resistant colony as shown in (A). Quantification of fold-enrichments is shown in boxes below each ChIP panel.

of Tas3-λN (Figure 5B). In addition, we observed no change in RNAPII occupancy at Tas3-λN tethered *ura4-5BoxB* locus in *dcr1*Δ cells (Figure 5B), in which the locus was fully active (Figure 2A). Thus, *ura4-5BoxB tas3-λN* silencing, which requires RNAi and heterochromatin components, is not mediated by changes in RNAPII occupancy, suggesting that silencing occurs at a step after transcriptional initiation. Consistent with the ChIP results, transcription run-on experiments showed that Tas3-λN mediated silencing of *ura4-5BoxB* was not associated with a reduction in transcription rates (Figure S2D).

In the above experiments, we used the 8WG16 antibody, which recognizes the C-terminal heptapeptide repeat present on the largest subunit of RNAPII and has been used in previous ChIP studies to investigate changes in RNAPII occupancy (e.g., Keogh et al., 2003). To ensure that we could observe changes in RNAPII occupancy using this antibody, we deleted the 5′ half of the *ura4-5BoxB* gene to create a *promoter*Δ allele. *Promoter*Δ*-ura4-5BoxB* was nonfunctional as cells harbouring this allele grew efficiently on 5-FOA medium (Figure 5C, bottom row), and as expected, the association of RNAPII with *promoter*Δ*-ura4-5BoxB* was reduced (Figure 5D).

The genetic requirements for tethered *ura4-5BoxB* silencing are identical to the requirements for the silencing of unmodified *ura4*$^+$ reporter genes inserted within the *S. pombe* centromeric repeats (Allshire et al., 1994; Allshire et al., 1995; Volpe et al., 2002) (Figure 5A). We were therefore interested in testing whether disruption of *ura4*$^+$ silencing within the centromeric repeat regions in RNAi and heterochromatin mutants was associated with increased RNAPII occupancy. For these studies, we used a well-characterized strain in which the *ura4*$^+$ gene is inserted at the innermost centromeric repeats of chromosome 1 (*imr1R::ura4*$^+$) (Allshire et al., 1994). We carried out ChIP experiments with the same set of primers used to examine the association of RNAPII with *ura4-5BoxB* across the entire *ura4*$^+$ promoter and coding regions (Figure 5A), comparing wild-type, *dcr1*Δ, and *clr4*Δ mutant cells. Consistent with previous observations, deletion of either *dcr1*$^+$ or *clr4*$^+$ abolished *imr1R::ura4*$^+$ silencing (Volpe et al., 2002; Verdel et al., 2004) (data not shown), and resulted in a striking increase in the levels of *ura4*$^+$ transcript (Figure 5E, upper panels). Surprisingly, loss of *imr1R::ura4*$^+$ silencing in *dcr1*Δ and *clr4*Δ cells did not correlate with any increase in RNAPII occupancy at the *ura4*$^+$ reporter gene (Figure 5F). We also tested the effect of deleting *dcr1*$^+$ and *clr4*$^+$ on RNAPII occupancy at a *ura4*$^+$ reporter gene inserted at the centromeric outer repeats (*otr1R::ura4*$^+$). Again, the results showed that loss of silencing in the mutant cells did not correlate with an increase in RNAPII occupancy at *otr1R::ura4*$^+$ (Figures S2A–S2C).

To determine whether these observations applied to endogenous promoters within the centromeric repeats, we examined RNAPII occupancy at the centromeric *dh* and *dg* repeats (*cen-dh* and *cen-dg*, respectively). These repeats have previously been shown to produce forward and reverse centromeric transcripts, which accumulate to high levels in RNAi and heterochromatin mutant cells (Volpe et al., 2002; Figure 5E, middle panels). *cen-dg* transcripts were approximately 10-fold less abundant than *cen-dh* transcripts, suggesting that they are transcribed from weaker promoters (Figure 5E, middle panels). Similar to the results for *imr1R::ura4*$^+$ (Figure 5F), we observed no increase in RNAPII levels associated with the *cen-dh* region in *dcr1Δ* or *clr4Δ* cells as compared to *wild-type* cells (Figure 5G, lower panels). In contrast, deletion of either *dcr1*$^+$ or *clr4*$^+$ resulted in a 4- to 6-fold increase in the association of RNAPII with the *cen-dg* region (Figure 5G, upper panels). Together, these results suggest that

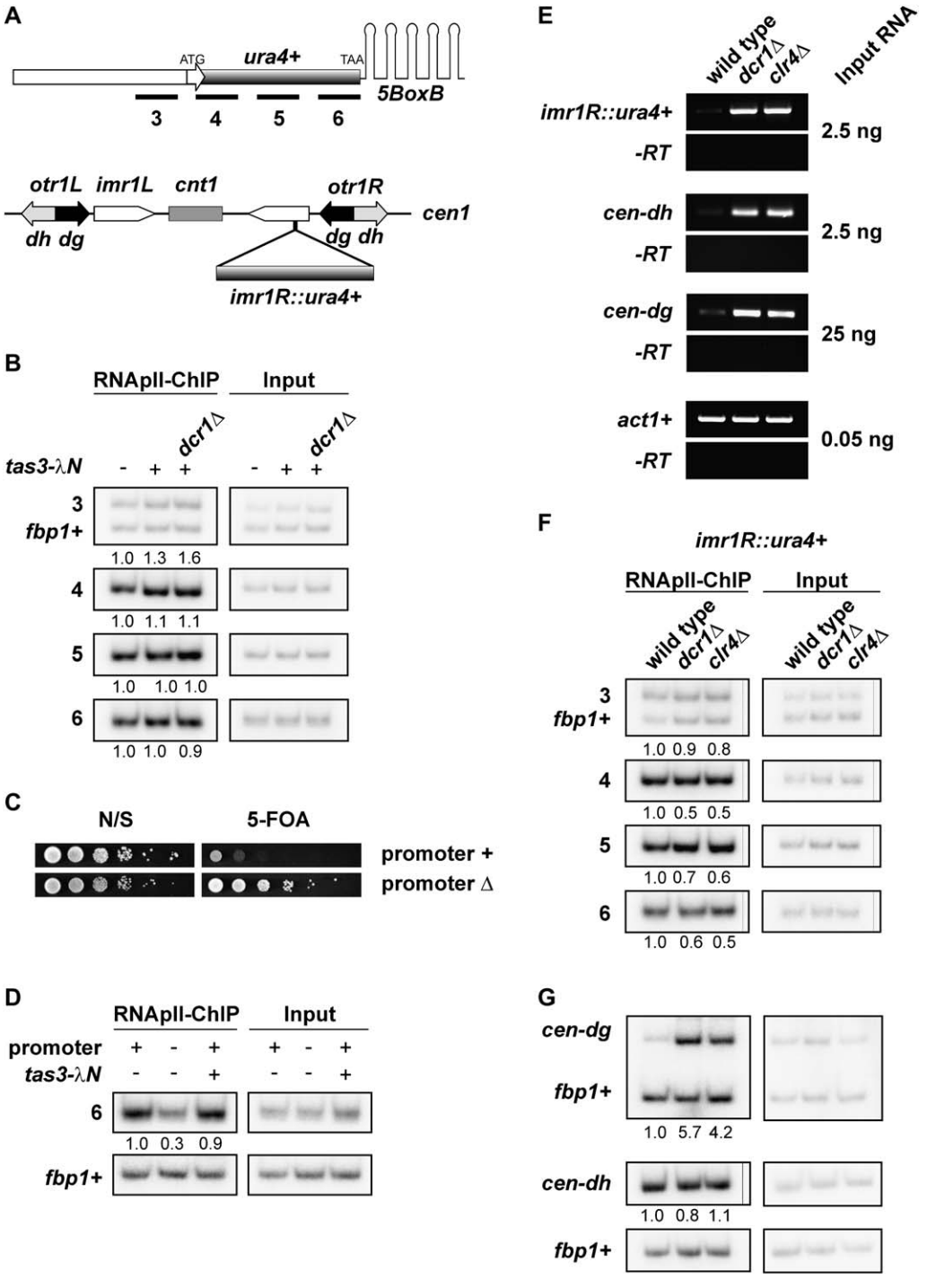

Figure 5. Silencing of *ura4*$^+$ by Either Tethering RITS to the Transcript or Insertion of the *ura4*$^+$ Gene within Centromeric Heterochromatin Is Not Associated with Changes in RNA Polymerase II Occupancy

(A) Schematic diagram representing the *ura4-5BoxB* locus (upper) and the DNA organization at the centromere of chromosome 1 (cen1, lower schematic). The central core domain (*cnt1*, gray) is juxtaposed by innermost (*imr1*) and outermost (*otr1*) repeats. *Otr1* DNA contains tandem dg and dh repeats (black and gray arrows, respectively). Position of the *ura4*$^+$ gene (gray bar) insertion at *imr1R* is indicated. Black bars represent the DNA fragments amplified in the ChIP experiments (B, D, and F; see also Figure 1A).

(B) ChIP experiments showing that Tas3-tethering does not change RNA polymerase II (RNAPII) occupancy at the *ura4-5BoxB* locus. Numbers to the left of the panels (3–6) indicate the location of amplified *ura4-5BoxB* region shown in (A). Fold-enrichment values for one typical experiment are presented below each panel; the value for wild-type cells was set to 1.0 and all values were normalized relative to *fbp1*$^+$.

heterochromatin assembly impedes transcription initiation at some but not all promoters within centromeric repeats.

DISCUSSION

We have developed a system that targets RNAi components to the transcript of a normally active euchromatic gene and initiates heterochromatin formation. Our findings highlight a central role for nascent pre-mRNA in heterochromatin assembly and demonstrate that siRNAs can initiate de novo silencing and chromatin modifications, although this capability is under strong negative control by the siRNA exoribonuclease Eri1. Furthermore, our analysis of the effect of RNAi- and heterochromatin mediated gene silencing on RNApII occupancy and transcription at a variety of targeted loci leads to the conclusion that the mechanism of silencing involves, at least in part, the cotranscriptional processing of nascent transcripts. Below we discuss the implications of these findings for the mechanism of siRNA generation and RNAi-mediated heterochromatin formation in *S. pombe*.

Tethered Tas3 Silencing and the Mechanism of siRNA Generation

Previous studies have shown that the generation of siRNAs and their loading onto the RITS complex requires RNAi components and several heterochromatin proteins. For example, in addition to Dicer, which processes long dsRNA into siRNA duplexes, Rdp1, Cid12, Hrr1, and components of the Clr4/Rik1 complex are required for siRNA generation (Motamedi et al., 2004; Hong et al., 2005; Li et al., 2005; Verdel and Moazed, 2005). These results, together with the Dicer- and Clr4- dependent physical association of the RITS and RDRC complexes, suggest that dsRNA synthesis in *S. pombe* is coupled to the recruitment of RDRC to the chromosome by RITS. In the present study, we found that the requirements for the generation of *ura4*$^+$ siRNA, mediated by tethering Tas3, are identical to the requirements for the generation of centromeric siRNAs (Figure 2). The simplest interpretation of these results is that dsRNA synthesis occurs on chromatin associated nascent transcripts. In this model, the dsRNA can still be released from its chromosomal site of synthesis and be processed elsewhere by Dicer into siRNA. However, restriction of *ura4*$^+$ siRNA-mediated silencing to the locus where they are generated suggests that Dicer also acts locally to produce siRNAs at the site of dsRNA synthesis. This *cis*-restriction (discussed below) may increase the fidelity of siRNA-mediated gene silencing.

We had previously suggested that RITS may recruit Rdp1 to specific RNA templates to initiate dsRNA synthesis (Motamedi et al., 2004). Our observation that the tethering of RITS to *ura4*$^+$ RNA promotes *ura4*$^+$ siRNA generation supports this hypothesis and further indicates that binding of RITS to a RNA transcript can initiate de novo dsRNA synthesis and siRNA generation. Because fission yeast cells are unlikely to contain any siRNAs that are complementary to the euchromatic *ura4-5BoxB*, the initial synthesis of *ura4*$^+$ dsRNA as a result of Tas3-λN binding must occur in the absence of a siRNA primer. Such primer-independent, but recruitment-dependent, dsRNA synthesis mechanism raises the possibility that association of RITS with heterochromatic domains may be coupled to dsRNA synthesis on transcripts that originate within these regions. This would then allow siRNA generation and RNAi-dependent silencing of any transcript within heterochromatin (Figure 6).

cis-Restriction and Role of Eri1 in Regulation of RNAi-Mediated Silencing

RNA silencing mechanisms in many systems act in *trans* to silence the expression of homologous sequences (Fire et al., 1998; Plasterk and Ketting, 2000; McManus and Sharp, 2002; Bartel, 2004). In particular, the ability of siRNAs to promote the degradation or translational inactivation of homologous sequences is a hallmark of RNAi-dependent PTGS mechanisms (Hannon, 2002; Zamore, 2002). The observation that in *S. pombe* siRNAs are unable to promote efficient silencing in *trans* is surprising and suggests that siRNA-mediated silencing may be under strong inhibitory control. In fact, we found that deletion of *eri1*$^+$, a homolog of the *C. elegans* enhancer of RNAi (eri-1) gene (Kennedy et al., 2004), allowed *ura4*$^+$ siRNAs to silence the expression of a second copy of *ura4*$^+$, which was located on another chromosome. These observations suggest that *ura4*$^+$ siRNAs are made near the *ura4-5BoxB* locus and have a limited range of action due to degradation by Eri1. Nonetheless, we note that even in *eri1*Δ cells the initial *trans* silencing of the second *ura4*$^+$ allele is inefficient and occurs at a frequency of 1 in 1000 to 1 in 10,000 (Figure 4). The ability of siRNAs to act in *trans* may therefore be limited by additional factors, such as position effects, subnuclear localization, or the presence of

(C) Growth assays confirming that deletion of the *ura4*$^+$ promoter results in growth on 5-FOA.
(D) ChIP experiment showing that, in contrast to the tethered *ura4-5BoxB*, RNA polymerase II (RNApII) occupancy at the *ura4*$^+$ gene is reduced when the *ura4*$^+$ promoter sequences are deleted.
(E) Semi-quantitative RT-PCR showing that *imr1R*::*ura4*$^+$, *cen-dh*, and *cen-dg* mRNA levels are substantially higher in *dcr1*Δ and *clr4*Δ cells compared to *wt* cells. *cen-dg* transcript levels are lower than *cen-dh* transcript levels; RT-PCR for *cen-dg* RNA was performed with 10-fold higher amounts of RNA in order to obtain a signal that is equivalent to *cen-dh*. *act1*$^+$ serves as a control. -RT, no reverse transcriptase.
(F) ChIP experiment showing that RNApII occupancy at the *ura4*$^+$ gene inserted at the *imr1R* centromeric region does not increase in *dcr1*Δ and *clr4*Δ cells. Numbers 3–6 to the left of each panel indicate the region of *ura4*$^+$ gene amplified (see A). Fold-enrichment values from one typical experiment, normalized to *fbp1*$^+$, are presented as numbers below each panel; the value for wild-type cells was set to 1.0.
(G) ChIP experiments showing that RNApII occupancy at centromeric *cen-dg*, but not cen-dh, repeats increases in *dcr1*Δ and *clr4*Δ cells. Fold-enrichment values from one typical experiment, normalized to *fbp1*$^+$, are presented as numbers below the panels.

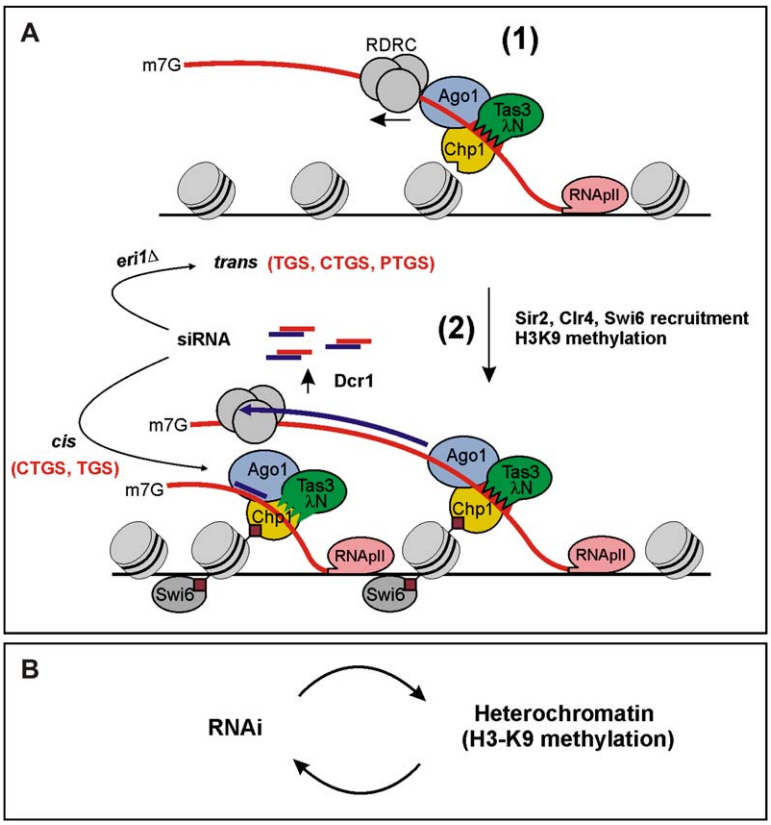

Figure 6. Model for RNAi- and Heterochromatin-Mediated Gene Silencing

(A) Tas3-λN tethering of the RITS complex to a 5BoxB-modified transcript (represented as a tooth saws in the Tas3 protein and the nascent transcript) mimics the association of RITS with the nascent transcript through siRNA-dependent base pairing (1). This leads to nucleation of heterochromatin assembly through the recruitment of chromatin modifying and binding proteins (Sir2, Clr4, Swi6), RDRC (RNA-Directed RNA polymerase Complex), and Dicer to generate siRNAs, which program and direct RITS complexes to nascent transcripts at the site of siRNA generation in *cis* (2). Once dimethylated by Clr4, H3-K9 serves as an anchor for Chp1, tethering the RITS complex to chromatin, which then mediates the degradation of the nascent transcript (CTGS, *cot*ranscriptional *g*ene *s*ilencing) and initiates chromatin modifications that can also mediate *t*ranscriptional *g*ene *s*ilencing (TGS). In *eri1*Δ cells, siRNAs are able to act in *trans* to silence the expression of homologous sequences by CTGS, TGS, and/or PTGS mechanisms. Red diamonds denote H3-K9 methylation.

(B) The ability of newly generated siRNAs to act in *trans* in *eri1*Δ cells shows that siRNAs can initiate de novo heterochromatin formation. Conversely, the ability of RITS to bind to H3-K9 methylated heterochromatin and to initiate heterochromatin-dependent, but primer-independent, dsRNA synthesis and siRNA generation suggests that heterochromatin can initiate RNAi to promote the degradation of nascent transcripts. See text for References and details.

methylated H3-K9. However, once *trans* silencing of *ura4*$^+$ is established, it is retained in a large fraction of cells (~10%) (Figure 4). Our observation that newly generated *ura4*$^+$ siRNAs load onto the RITS complex and mediate RITS association with and silencing of a second *ura4*$^+$ allele demonstrates that in *S. pombe* siRNAs can act to recruit heterochromatin components to a previously euchromatic gene. These results provide direct evidence that siRNAs are specificity factors that can initiate de novo chromatin modifications and gene silencing.

Our inability to detect siRNA-mediated silencing of *trans* loci in *eri1*$^+$ cells is consistent with previous observations that RNAi-mediated silencing of a GFP transgene inserted into the *S. pombe* genome occurs at the PTGS level and is not associated with transcriptional gene silencing (Sigova et al., 2004). In these experiments, GFP siRNAs were produced from a hairpin RNA, which was encoded by a plasmid borne inverted repeat corresponding to the GFP coding sequences. The expression of the GFP hairpin resulted in a 2- to 4-fold reduction in GFP levels. Although GFP silencing in this system required Clr4, it was independent of Chp1, Tas3 or Swi6, and did not correlate with reduced GFP transcription (Sigova et al., 2004), suggesting that GFP siRNAs cannot initiate heterochromatin assembly in *trans*, as is also the case with *ura4*$^+$ siRNAs in *eri1*$^+$ cells reported here. We note that PTGS may also affect *ura4-5BoxB* expression in our experiments. However, PTGS mechanisms act in *trans* and would be unable to discriminate between the products of the different *ura4*$^+$ alleles in our experiments. Therefore, classical PTGS can only be a minor component of Tas3-λN-dependent *ura4*$^+$ silencing.

Implications for the Mechanism of RNAi- and Heterochromatin-Dependent Gene Silencing

Centromeric repeat regions in *S. pombe* are packaged into a repressive chromatin structure that is widely believed to be an example of the type of heterochromatin that is associated with centromeres and other repeat sequences in metazoan model systems (Allshire et al., 1994; Grewal, 2000; Moazed, 2001). Like metazoan heterochromatin, *S. pombe* heterochromatin contains histone H3 that is K9 methylated, is coated with Swi6 (a homolog of *Drosophila* and mammalian HP1), and requires Clr4 (a homolog of the metazoan enzymes that carry out H3-K9 methylation) for its assembly. In addition, like metazoan systems, genes that are inserted within *S. pombe* heterochromatic DNA domains are silenced and this silencing is epigenetically inherited (Allshire et al., 1994). It is widely believed that such heterochromatic silencing occurs through the assembly of a compact chromatin structure that is inaccessible to transcription factors. On the

other hand, our findings and a number of other recent observations suggest that heterochromatin can be a relatively accessible structure and in some situations silencing occurs by a mechanism that does not prevent the association of RNApII with endogenous or transgene promoters within heterochromatic domains.

In fission yeast, centromeric *dg* and *dh* repeats are transcribed to produce both forward and reverse noncoding RNAs (Volpe et al., 2002). Consistent with our observations on the effect of heterochromatin assembly on RNApII occupancy at *cen-dg* repeats, transcription run-on experiments showed that heterochromatin assembly inhibits transcription of the cen-forward transcript, but has little or no effect on transcription of the cen-reverse transcript, although these transcripts are still less abundant in wild-type cells compared to RNAi mutant cells (Volpe et al., 2002). Similarly, a mutation in the Rpb2 subunit of RNApII that partially disrupts heterochromatin formation does not cause a significant increase in RNApII association with the centromeric *dh* repeats (Kato et al., 2005). Clearly, some promoters can be transcribed within heterochromatic domains, but the resulting RNA most likely is degraded by the RNAi machinery. Our results suggest that the mechanism of this degradation is distinct from RNAi-mediated posttranscriptional gene silencing (PTGS). Unlike classical PTGS, degradation of heterochromatic transcripts is a chromatin-dependent process that requires the RNAi pathway and the histone H3-K9 methyltransferase Clr4 (Figure 5E). Further distinguishing this process from classical PTGS is the fact that RNAi-mediated degradation of transcripts in wild-type *S. pombe* is *cis*-restricted (Figure 3). The simplest explanation for these observations is a cotranscriptional gene silencing (CTGS) model in which the targeting of nascent transcripts by the RITS complex can not only initiate chromatin modifications but also, directly or indirectly, mediates the degradation of nascent transcripts (Figure 6). A key feature of this model is that RNAi-mediated degradation of target transcripts is coupled to histone H3-K9 methylation, which we propose prevents RITS from readily acting in *trans* on mature or cytoplasmic transcripts and may also create a threshold for de novo siRNA-mediated heterochromatin assembly.

We note that the mechanism of RNAi- and heterochromatin-mediated gene silencing in *S. pombe* is versatile. While silencing of the *cen-dh* reverse and *ura4*$^+$ transgene promoters most likely occurs cotranscriptionally, silencing of *cen-dh* forward and *cen-dg* promoters occurs at the level of transcription (i.e., TGS) and in the case of *cen-dg* repeats is associated with decreased RNApII binding (Figure 5G). Additional work is required to determine the mechanism(s) responsible for these different types of silencing but one possibility is that repressive heterochromatin can more readily prevent the association of RNApII with weaker promoters.

Our results support a role for chromatin associated nascent transcripts as templates for the initial assembly of RITS and other RNAi components. Because nascent transcripts are physically associated with elongating or paused RNApII complexes, the presence of RNApII in centromeric heterochromatin is actually a direct prediction of the nascent transcript model. Furthermore, the ability of RNApII to associate with heterochromatin-like domains appears to be evolutionarily conserved. In *Drosophila*, Polycomb-mediated silencing of a heat shock reporter transgene impedes transcription at a step following the loading of RNApII pre-initiation complex (Dellino et al., 2004), and endogenous Polycomb-repressed genes are associated with general transcription factors (Breiling et al., 2001). Similarly, SIR-mediated silencing of heat shock reporter genes in budding yeast does not affect RNApII promoter occupancy (Pirrotta and Gross, 2005). In this regard, RNA processing events such as capping, splicing, and polyadenylation, as well as several chromatin modifying activities, are coupled to elongating RNApII complex, and the enzymes responsible for these activities are physically associated with RNApII (Maniatis and Reed, 2002; Hampsey and Reinberg, 2003). Structural features of RNApII that either affect its possible interaction with the RNAi machinery or limit its ability to transcribe in heterochromatin might therefore be expected to disrupt RNAi-mediated heterochromatin assembly. In fact, point mutations in two different RNApII subunits, Rpb2 and Rpb7 (*rpb2-m203* and *rpb7-G150D*, respectively), inhibit siRNA generation and heterochromatin assembly at centromeres (Djupedal et al., 2005; Kato et al., 2005). These results, together with our observation that similar levels of RNApII are associated with active and silenced centromeric repeats, suggest that RNApII and its associated nascent transcripts are structural components of heterochromatin.

EXPERIMENTAL PROCEDURES

Strain and Plasmid Construction

S. pombe strains used in this study are described in Table S1. All strains were constructed using a PCR-based gene targeting method (Bahler et al., 1998). Primer sequences, if not described, are available on request. Positive transformants were selected by growth in YEA medium containing 100–200 μg/ml antibiotic and confirmed by PCR.

A double-stranded DNA oligo encoding the λN sequence (5′-ATG GACGCACAAACACGACGACGTGAGCGTCGCGCTGAGAAACAAGC TCAATGGAAAGCTGCAAACtaa-3′) was inserted into PacI/AscI of pFA6a-3HA-kanMX6 (Bahler et al., 1998) to generate pFA6a-λN-kanMX6. The same sequence without the stop codon (taa) but followed by a ProProLeu linker was inserted into the BamHI/PacI site of pFA6a-GFP(S65T)-natMX6 (Bahler et al., 1998) to generate pFA6-λN-GFP-natMX6. pFA6a-λN-kanMX6 served as a template to amplify the λN-kan cassette with primers designed to fuse λN, separated by a ProProLeu linker, in frame to the N-term of the gene of interest. The λN-GFP-natMX6 cassette was amplified using pFA6-λN-GFP-natMX6 as a template and with primers designed to replace *leu1*$^+$.

A fragment encoding five BoxB sites (5′-GGGCCCTGAAG AAGGGCCC-3′) was PCR amplified from plasmid "β−globin5boxB" (Gehring et al., 2003) and inserted PacI/AscI into pFA6a-TAP-hphMX6 (Motamedi et al., 2004) to generate pFA6a-5BoxB-hphMX6. From this plasmid, the 5BoxB-hph cassette was amplified with primers designed to integrate the DNA fragment after the stop codon of the *ura4*$^+$ open reading frame.

The *ura4*$^+$ open reading frame was amplified from KS-ura4 (Bahler et al., 1998) and inserted into PacI/AscI of pFA6a-TAP-natMX6 (Motamedi et al., 2004) resulting in pFA6a-ura4-natMX6. The intronic sequence 5′-GTAAGTGGCTGATAAATTAGAAAAA GTTGGTTGGAATTC TAATCAATAG-3′ was then inserted into the StuI site (located in the middle of the *ura4*$^+$ open reading frame) to generate pFA6-3′ade6Intron-natMX6. The structure of the intron and the site of its insertion (StuI) were chosen based on previous work (Gatermann et al., 1989). To generate strains containing a second *ura4*$^+$ allele on chromosome 2, the ura4/intron-nat cassette was amplified from pFA6-3′ade6Intron-natMX6 with primers designed to replace *leu1*$^+$.

Silencing Assays

Silencing assays were conducted from overnight unsaturated cultures grown in 10 ml YEA (yeast extract supplemented with adenine). Ten-fold serial dilutions were made so that the highest density spot contained 1.2 × 10^5 cells. Cells were spotted on N/S plates (containing EMM supplemented with 226mg/l each adenine, leucine, and uracil) and 5-FOA plates (same as N/S plates with the addition of 1g/l 5-fluoro-orotic acid). The plates were then incubated at 32°C for 3 days.

Chromatin Immunoprecipitation

ChIP was performed as described previously (Huang and Moazed, 2003). Antibodies used were the following: Swi6 (abcam, #14898), dimethylated H3-K9 (Upstate, #07-212), Ago1 (abcam, #18190), Chp1 (abcam, #ab18191) or RNA Polymerase II 8WG16 Monoclonal (Covance, #MMS-126R). Primer sequences are shown in Table S2. In Figures 1D, 3C, and 4B, primer pairs used to amplify *ura4*$^+$ DNA were mb104/mb105 and mb193/mb194, respectively. The *ura4*$^+$ fragments 1 to 6 shown in Figures 1 and 5 were amplified using the primer pairs mb212/mb212, mb210/mb211, mb208/mb209, mb51/mb207, mb193/mb194 or mb21/mb134, respectively. DM566/DM567, mb263/mb264, DM554/DM555, mb5/mb6 and mb90/mb91 were used to amplify *cen-dg* DNA, *cen-dh* DNA, *fbp1*$^+$ DNA, BoxB DNA and *act1*$^+$ DNA, respectively. Serial dilutions of template DNA were used to ensure that the PCR reactions were in the linear range. ^{32}P-labeled PCR products were separated on 6% polyacrylamide gels. After drying, gels were exposed to a phosphor imager screen. All the ChIP experiments were completed at least twice.

RNA Analysis

Total RNA was isolated from logarithmically growing *S. pombe* (in YEA medium) using the hot phenol method (Leeds et al., 1991). For Northern blotting of *ura4*$^+$ mRNA, 10 μg of total RNA was electrophoresed on a 1.2% agarose gel containing 1× MOPS and 1% formaldehyde. RNA was transferred to positively charged nylon membranes (Roche) in 20× SSC by standard capillary blotting. Following UV crosslinking of the RNA to the nylon filter, prehybridization and hybridization were carried out at 35°C in UltraHyb-Oligo buffer (Ambion). For hybridization, 20 pmol DNA oligo mb134 (Table S2) was end-labeled with 20 pmol [$\gamma-^{32}$P]dATP (6000Ci/mmol) using T4 Polynucleotide Kinase (Roche). After overnight hybridization, membranes were washed three times in 2× SSC/0.5% SDS for 15 min at 35°C before exposure to a phosphor imager screen.

For detection of siRNAs, total RNA isolated as described above was subjected to RNeasy Midi columns (QIAGEN) following the "RNA cleanup protocol" provided by the manufacturer. The flow-through containing small RNAs (<200nt) was collected and precipitated with one volume isopropanol. Twenty-five μg small RNA was electrophoresed on a 17.5% polyacrylamide gel containing 7M urea. Subsequently, RNA was transferred to positively charged nylon membranes (Roche) in 1× TBE using a semi-dry transfer cell (Biorad; 3.3 mA/cm^2 for 30 min). The membrane was UV crosslinked and baked at 80°C for two hours. For hybridization, 20 pmol of DNA oligo was end-labeled with 20 pmol [$\gamma-^{32}$P]dATP (6000Ci/mmol) using T4 Polynucleotide Kinase (Roche). To detect centromeric siRNAs, a mix of oligos complementary to the siRNAs sequenced by Reinhart and Bartel was labeled. For *ura4*$^+$ siRNAs, a mix of 11 oligos distributed over the entire *ura4*$^+$ ORF was labeled (ura4A-K; Table S2). To detect the loading control snoR69 (SPSNORNA.19), the oligo mb151 served as a probe (Table S2). Prehybridization, hybridization, and washes were performed as described above.

To detect RITS loaded siRNAs, RITS was purified by tandem affinity purification of Chp1-TAP as described previously (Motamedi et al., 2004; Verdel et al., 2004). siRNAs were then recovered by phenol-chloroform extraction and isopropanol precipitation. The entire siRNA sample was electrophoresed on a 17.5% polyacrylamide gel containing 7M urea. Blotting and probing was performed as described above.

Transcription run-on assays were performed as described in Supplemental Data.

RT-PCR

For RT-PCR analysis, total RNA was cleaned up and DNase treated according to the "RNeasy Mini Protocol for RNA Cleanup" (QIAGEN). RT-PCR was performed using the one-step RT-PCR kit (QIAGEN). 30 Cycles of PCR after the RT-step were performed for all reactions. Serial dilutions of input RNA were used to ensure that the RT-PCR reactions were in the linear range. To detect *ura4*$^+$ mRNA and *cen-dh* transcripts, 2.5ng total RNA was used per reaction. To detect *cen-dg* transcripts and *act1*$^+$ mRNA, 25ng and 50pg total RNA was used, respectively. Primer pairs to detect *ura4*$^+$, *act1*$^+$, *cen-dg* and *cen-dh* transcripts were mb51/mb52, mb73/mb91, DM566/DM567 and mb265/mb266, respectively (Table S2).

Supplemental Data

Supplemental Data include two figures, two tables, Experimental Procedures, and References and can be found with this article online at http://www.cell.com/cgi/content/full/125/5/873/DC1/.

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