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HP1 Proteins Form Distinct Complexes and Mediate Heterochromatic Gene Silencing by Nonoverlapping Mechanisms

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SUMMARY

HP1 proteins are a highly conserved family of eukaryotic proteins that bind to methylated histone H3 lysine 9 (H3K9) and are required for heterochromatic gene silencing. In fission yeast, two HP1 homologs, Swi6 and Chp2, function in heterochromatic gene silencing, but their relative contribution to silencing remains unknown. Here we show that Swi6 and Chp2 exist in nonoverlapping complexes and make distinct contributions to silencing. Chp2 associates with the SHREC histone deacetylase complex (SHREC2), is required for histone H3 lysine 14 (H3K14) deacetylation, and mediates transcriptional repression by limiting RNA polymerase II access to heterochromatin. In contrast, Swi6 associates with a different set of nuclear proteins and with noncoding centromeric transcripts and is required for efficient RNAi-dependent processing of these transcripts. Our findings reveal an unexpected role for Swi6 in RNAi-mediated gene silencing and suggest that different HP1 proteins ensure full heterochromatic gene silencing through largely nonoverlapping inhibitory mechanisms.

INTRODUCTION

Silent or heterochromatic DNA domains play important roles in maintenance of chromosome integrity and regulation of gene expression in eukaryotes ranging from fission yeast to human. A core characteristic of heterochromatin is its association with heterochromatin protein 1 (HP1) proteins, a highly conserved family of chromosomal proteins that bind to di- and trimethylated H3K9 via a conserved N-terminal domain called the chromodomain (CD) (Grewal and Moazed, 2003; Jacobs and Khorasanizadeh, 2002; Jacobs et al., 2001; Lomberk et al., 2006; Maison and Almouzni, 2004; Richards and Elgin, 2002). HP1 proteins contain a C-terminal chromo shadow domain (CSD) that is thought to mediate protein-protein interactions, including self-association (Brasher et al., 2000; Lomberk et al., 2006). The fission yeast *Schizosaccharomyces pombe* contains two HP1 homologs, Swi6 and Chp2, and a third CD protein, Chp1, all of which bind to methylated H3K9 and are involved in heterochromatic gene silencing (Bannister et al., 2001; Halverson et al., 2000; Partridge et al., 2002; Thon and Verhein-Hansen, 2000). Chp1 is associated with Tas3, Ago1, and repeat-associated short interfering RNAs (siRNAs) in the RITS complex and links heterochromatin to RNA interference (RNAi) (Motamedi et al., 2004; Verdel et al., 2004). However, despite their conservation and importance, the precise role(s) of Swi6 and Chp2 in heterochromatin assembly and function has remained unclear.

HP1 proteins specifically bind to methylated H3K9 (Jacobs and Khorasanizadeh, 2002; Jacobs et al., 2001) and are believed to recruit the H3K9 methyltransferase, KMT1 (Clr4 in *S. pombe*, Su[var]3-9 in *Drosophila*, and Suv39H in mammals), directly or indirectly to chromatin (Aagaard et al., 1999; Lachner et al., 2001; Rea et al., 2000; Schotta et al., 2002; Stewart et al., 2005). Sequential cycles of HP1/Swi6 binding and KMT1 recruitment have been proposed to mediate the spreading of H3K9 methylation along the chromatin fiber (Bannister et al., 2001; Grewal and Moazed, 2003; Nakayama et al., 2001). However, recent studies indicate that in fission yeast neither Swi6 nor Chp2 is required for the spreading of H3K9 methylation at centromeres (Sadaie et al., 2004). Instead, spreading requires the RITS-associated Chp1 protein and the RNAi machinery (Verdel et al., 2004; Volpe et al., 2002). Swi6 and Chp2 act downstream of H3K9 methylation to promote heterochromatic gene silencing by mechanisms that appear to involve the recruitment of additional chromatin-modifying activities. In this regard, previous studies have suggested a role for H3K9 methylation and Swi6/HP1 in recruitment of the SHREC complex, containing Clr1, Clr2, the Clr3 histone deacetylase (HDAC), and the Mit1 chromatin-remodeling protein. SHREC mediates the deacetylation of H3K14 and promotes transcriptional gene silencing (Sugiyama et al., 2007).

Most eukaryotes contain multiple HP1 proteins. For example, *Drosophila*, mouse, and human cells possess three closely related HP1 proteins (referred to as HP1, HP1b, and HP1c in

Drosophila, and HP1α, HP1β, and HPγ in human and mouse). The *Drosophila* HP1 and HP1b (and mammalian HP1α and HP1β) localize predominantly to heterochromatin, whereas HP1c and HP1γ localize to euchromatin and function in gene-specific silencing. In addition to methylated H3K9 and KMT1, HP1 proteins interact with diverse groups of nonhistone chromosomal proteins (Lomberk et al., 2006). The *Drosophila* HP1 associates with the nuclear lamin B receptor (LBR), subunits of the Origin Recognition Complex, and chromatin-remodeling proteins (Pak et al., 1997; Shareef et al., 2001; Ye and Worman, 1996). In human, HP1α interacts with Ku70 DNA repair protein (Song et al., 2001), HP1β associates with Dnmt1 and Dnmt3a DNA methyltransferses (Fuks et al., 2003), and HP1 and the human KMT1 (SUV39H1) associate with Rb and mediate cell-cycle-dependent repression of Rb targets in euchromatin (Nielsen et al., 2001). In addition to the interaction between the fission yeast Swi6/HP1 and the Clr3 HDAC (Yamada et al., 2005), interactions between the mouse HP1α class II HDACs have been reported (Zhang et al., 2002).

HP1 proteins also localize to actively transcribed genes in flies and mammals (Cryderman et al., 2005; Vakoc et al., 2005). The significance of this localization is not understood but has been proposed to reflect an HP1 affinity for RNA and possible involvement in RNA processing during transcription elongation. In fact, HP1 binds to RNA and DNA in vitro, with preference for the single-stranded form in the case of DNA (Muchardt et al., 2002). The latter binding activity may contribute to the telomere-capping function of HP1 in *Drosophila*, which is independent of its ability to bind to methylated H3K9 via its CD (Perrini et al., 2004). Although no RNA-binding activity has been described for the fission yeast HP1 proteins, Swi6 is required for efficient accumulation of centromeric siRNAs, suggesting that it may interact directly with the RNAi machinery (Buhler et al., 2006; Motamedi et al., 2004).

It has been unclear why heterochromatic gene silencing requires multiple HP1 proteins and whether these proteins play distinct or largely redundant roles within heterochromatin. Here we report studies aimed at understanding the relative contributions of each Swi6, Chp2, and RNAi to heterochromatic gene silencing in fission yeast. We perform biochemical purifications of Chp2 and Swi6 and use quantitative assays to determine the degree to which each of the above pathways contributes to silencing. We find that the Chp2 and Swi6 proteins associate with nonoverlapping sets of proteins. Chp2 is associated with the SHREC deacetylase complex (which we call SHREC-Chp2 or SHREC2), is required for deacetylation of H3K14, and mediates transcriptional gene silencing by limiting RNA polymerase II (Pol II) access to heterochromatin. On the other hand, Swi6 associates with a large number of chromosomal proteins, including transcription elongation proteins, chromatin-remodeling complexes, and DNA-binding and DNA replication proteins. Swi6 plays a minor role in limiting Pol II access to heterochromatin and a major role in processing of centromeric transcripts into siRNAs. Consistent with the latter result, Swi6 localizes to noncoding centromeric RNAs in a Clr4-dependent manner and is required for efficient localization of RNAi complexes (RITS and the RNA-dependent RNA polymerase complex, RDRC) to heterochromatic transcripts, suggesting that it may tether nascent transcripts to heterochromatin for the RNAi-dependent heterochromatic gene silencing. Overall, our results reveal the existence of two tiers of silencing mechanisms that act together to ensure that transcription within heterochromatic regions is properly regulated. In one tier, RNAi and an unknown RNAi-independent pathway act together to recruit Clr4 to mediate H3K9 methylation of heterochromatic nucleosomes. In the second tier, Chp2 and Swi6 act downstream of H3K9 methylation and ensure efficient silencing by limiting Pol II access and processing of heterochromatic transcripts into siRNAs, respectively.

RESULTS

Chp2 Associates with the SHREC Complex

The fission yeast HP1 homolog Chp2 is required for the transcriptional silencing of reporter genes inserted at all known *S. pombe* heterochromatic loci including the pericentromeric repetitive innermost (*imr*) and outer (*otr*) DNA elements, telomeres, rDNA, and the mating-type loci (Halverson et al., 2000; Thon and Verhein-Hansen, 2000). In order to gain insight into how Chp2 contributes to silencing, we constructed a strain in which a functional C-terminally TAP-tagged Chp2 protein was expressed from its endogenous promoter, and we purified this protein using a tandem affinity purification scheme (Motamedi et al., 2004; Rigaut et al., 1999). Polyacrylamide gel electrophoresis and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses revealed that Chp2 interacted with two previously characterized proteins, Clr1 and Mit1 (Figures 1A and 1E, and see Table S1 available online), both of which are components of the Snf2/Hdac-containing repressor complex (SHREC) (Figures 1B–1E and Sugiyama et al., 2007). Examination of the Clr1 peptide spectra from two independent Chp2-TAP purifications revealed a nonuniform distribution of peptides, mapping only to the N terminus of the Clr1 protein (Figure 1F), suggesting that only the N-terminal portion of Clr1 was present in the Chp2-TAP purifications. These data suggested that Chp2 was a component of the SHREC complex, interacting with Clr2 and Clr3 via the N terminus of Clr1.

In order to further analyze the composition of the SHREC complex, we constructed functional C-terminally TAP-tagged Clr3 and Clr1, and N-terminally TAP-tagged Clr2 expressed under the control of their endogenous promoters. Several tandem affinity purifications were performed, and the resulting purified proteins were subjected to LC-MS/MS analysis (Table S1). Results from three independent Clr3-TAP, two independent TAP-Clr2, and two independent Clr1-TAP purifications revealed a largely nonoverlapping Clr1 peptide spectra compared to those found in the two independent Chp2-TAP purifications (Figures 1A and 1F). In contrast to Chp2-TAP purifications in which only peptides from the N terminus of Clr1 were identified, in all Clr1-TAP, TAP-Clr2, and Clr3-TAP purifications (seven purifications in total), peptides only from the C terminus of the Clr1 protein were identified (Figure 1F). The only exception was a single peptide mapping to the middle of Clr1 protein, which was shared among the Chp2-TAP and TAP-Clr2 purifications. Western blot analysis of Clr1-TAP revealed a 160 kD protein compatible with the predicted full-length TAP-tagged protein, which is rapidly degraded after cell lysis, especially during purification

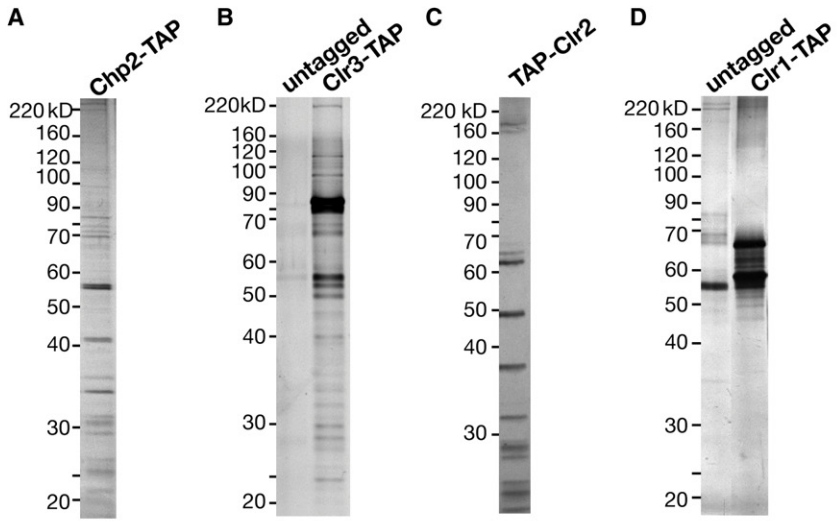


Figure 1. Purification of Chp2 and Identification of the SHREC2 Complex

(A–D) Representative silver-stained gels depicting the tandem affinity purifications of (A) Chp2-TAP, (B) Clr3-TAP, (C) TAP-Clr2, and (D) Clr1-TAP proteins.

(E) Proteins identified by tandem mass spectrometry sequencing of mixture of proteins (LC-MS/MS) for the indicated purification. The numbers in parentheses correspond to the number of unique peptides and protein coverage based on total number of amino acids.

(F) Clr1 peptide spectra in SHREC2 purifications. Red residues represent peptides only identified in Chp2-TAP purification (** in [E]). Blue residues represent peptides identified in Clr3-TAP, TAP-Clr2, or Clr1-TAP purifications (* in [E]). Yellow residues show peptides common to both Chp2-TAP and TAP-Clr2 purifications.

(G) SHREC2 complex is composed of Chp2 and Mit1, which interact with the N-terminal region of Clr1, and Clr2 and Clr3, which interact with the C-terminal region of Clr1.

E

LC-MS/MS of total mixtures

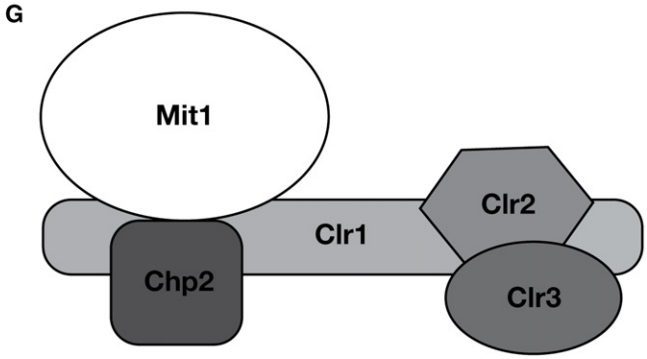
Protein (# unique peptides, %coverage)

Clr3-TAP	TAP-Clr2	Clr1-TAP	Chp2-TAP
Clr3 (33, 53)	Clr2 (16, 32)	Clr1 (12, 10)*	Chp2 (12, 37)
Ccq1 (13, 31)	Clr1 (25, 27)*	Clr2 (12, 31)	Clr1 (4, 5)**
Clr2 (10, 34)	Clr3 (18, 29)	Clr3 (13, 23)	Mit1 (22, 23)
Clr1 (16, 20)*			

F

MAEPDISSSE TLTELQQLRL LYFFCFYHAA PFNVKTLVHS LIPPGALSYL LSTYDILRPW LMALRVREGP VNDISTIVQL
YEEIVKTGFF INPPPFESYS QTLVARITTL GRPKLQVQQE AQSEVYQRAS TNTQQQVSNV SHGNFKPNSS VNTEPNTSIL
SNSKYAGIKP FDFQSSSQNP GLVCEQKTFY QHQFRPFSNL PSNKSSPVKH VSPNVKNNSK KTASSVNSNH SSIPSSITKS
NISSLDVYGS EKLISSGSQQ PGHGMVQTTS DKVNASASLY DRSPSKKDIT SSRNTSSYNL GSMRNPSTLK NAAHANPFEG
LRFQGSSAVL KEGLNSTVKK **TFFDNLNSEK** VCPSVSPFLT PDNIASSILY STASFSRSKP DRPRLNLSLE LKLMQNELNK
GQLKKQFKGD LR**NLADWNNL SLVSSK**FPSL PITNLRPDGS FLKHRRFNEE IAYNRQTLEK AIKQLDLSPD KVIQLREQNG
VAVNGRVCYP TRNK**HSEISA QSSSSLGVTK SLASEVYSSS TVDTISKLNT DKDNYLIK**SK KEPIQQK**SVS SETTLVKPSS**
TSSYIDTTNN VLKTNSSFKS SGLTSGPRNE KELLPEGIPT SHNNSETQAQ TADVSNIAAS ADGIYNSDQE KPPEKLDVTK
RAFGREIENS NEKELLTSTF LSPSAESQVC LAEIKTIRPG LVPKKQFSVD QNNVISDNTD CSLPKPSNSK **LSSISSDGDA**
SSNRMAVPDK **SPFVHAAPNS** **K**ALTKDSFST HISVSSLLHS DNEISPIDST RKDYFTSK**DS NLQTLK**EDAS STKQAKDSGT
NDFDKLISGN DVSKNNSGEE QSRSALKPLI SGKLSSCESI NLTKDISTVK RKEYFGIEST SSKQPFHDTG SIKIPAKR**SF**
DTIDKDFRSS NIPFADK**IKE DGGDKNVISS IHITTELPKS MPVEVPTNAG AQSDQSNVVD SESLNLRENI STSVADVSLS**
QAGNEAVLSK KACKPLVLID PFEEKVLKAF NMLSKGYAEY RCQWEGCLAN LHSLENFIKH VLLLHHPKSC SVVKCLWASC
DMVLPSEEFE MHLRGHLNNI RLNCEVSNCK KCFSNYEDMF KHLQHSHLPF KFTPESFIKI RNGNVKEEAR RTRNAYTQKS
GEVECFMETC TPIAKPAPAN WYPVPPPGFN SSLLSRLTQS NQSKDKIIAA LAKRNVYK**SF AGLYDSKGKN DNTGYDFDSN**
YARVGR**HGSF ILPVSKSVPT PSLLIEGSIV QR**KNIKIE

G



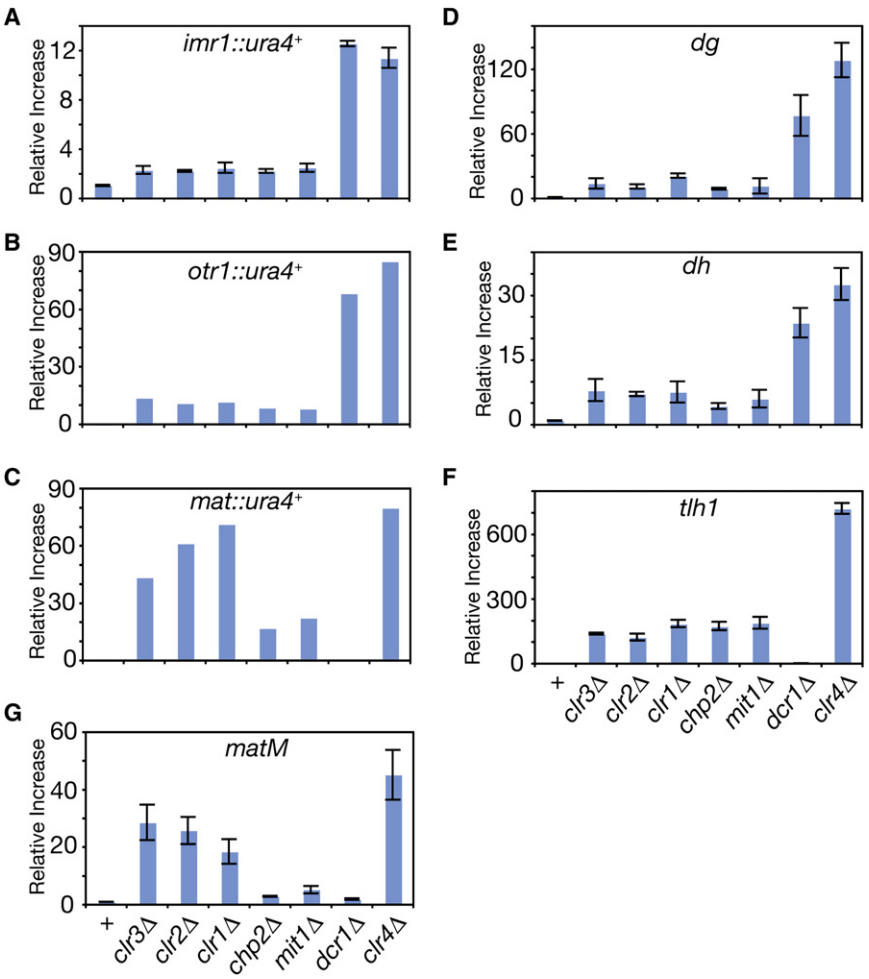


Figure 2. Contribution of SHREC2 to Heterochromatic Gene Silencing

Quantitative real-time RT-PCR depicting the steady-state levels of (A) *imr1::ura4*$^+$, (B) *otr1::ura4*$^+$, (C) *mat::ura4*$^+$, (D) *dg*, (E) *dh*, (F) *tlh1*$^+$, *and* (G) *matM* transcripts in *SHREC2* mutant cells compared to wild-type, *dcr1Δ*, and *clr4Δ* cells. Error bars represent the standard deviations from three independent biological experiments. All values were normalized to *act1*$^+$ transcript levels.

(Figures S1A–S1C). Based on these data, we conclude that Chp2, and probably Mit1, interacts with the N terminus, and Clr2 and Clr3 interact with the C terminus, of Clr1. This shows that Chp2 is a component of the SHREC complex, which we named SHREC associated with Chp2 complex, or SHREC2 complex (Figure 1G). The SHREC complex previously has been shown to mediate deacetylation of H3K14 (via Clr3) and chromatin remodeling (via Mit1) (Bjerling et al., 2002; Sugiyama et al., 2007). Our results suggest that these activities are recruited to heterochromatin via the association of the Chp2 subunit of SHREC2 with H3K9me.

The Requirement for SHREC2 in Heterochromatic Gene Silencing

Previous reports (Bjerling et al., 2004; Ekwall and Ruusala, 1994; Sugiyama et al., 2007; Thon and Verhein-Hansen, 2000) have shown that components of SHREC2 are required for the silencing of a reporter construct (e.g., *ura4*$^+$) inserted at various *S. pombe* heterochromatic loci, including centromeres, telomeres, mating-type region, and rDNA. In agreement with these findings, we found that deletion of any of the SHREC2 components led to the loss of silencing in strains carrying a *ura4*$^+$ reporter gene inserted at the innermost (*imr1::ura4*$^+$) or outer (*otr1::ura4*$^+$) pericentromeric DNA regions, the mating-type (*mat::ura4*$^+$) or the rDNA (*rDNA::ura4*$^+$) loci (Figures S2A–S2D). Even though these growth-based assays serve as an indicator for silencing defects, they are not quantitative and therefore do not reveal differences in expression levels above the minimum RNA level required to generate the growth phenotype.

In order to quantify the extent to which the components of SHREC2 contribute to heterochromatic gene silencing, we measured the steady-state levels of the inserted *ura4*$^+$ reporter gene (*imr1::ura4*$^+$, *otr1::ura4*$^+$, and *mat::ura4*$^+$), or endogenous heterochromatic transcripts originating from the *dg* and *dh* elements of centromeres, the *matM* gene of the mating-type locus, and *tlh1*$^+$ gene of the subtelomeres using reverse transcriptase real-time PCR. Compared to *clr4Δ* cells, in which heterochromatin is completely disrupted, we found that deletion of SHREC2 subunits resulted in only partial derepression of the centromeric *ura4*$^+$ reporter genes (Figures 2A and 2B), the endogenous centromeric *dg* and *dh*, and telomeric *tlh1*$^+$ transcripts (Figures 2D–2F). These results indicated that the contribution of SHREC2 to heterochromatic gene silencing at these loci is a fraction (10%–20%) of the full silencing levels observed in *clr4Δ* cells (Table S2). Similar to the 5-FOA silencing results (Figure S2C), the contribution of *clr1*, *clr2*, and *clr3* genes to silencing of *mat::ura4*$^+$ as well as *matM* (Figure 2C) was greater than that of *chp2* and *mit1*, suggesting a Chp2-Mit1-independent mechanism for Clr1, Clr2, and Clr3 recruitment to this locus. Overall, these results indicate a partial requirement for SHREC2 in heterochromatic gene silencing.

SHREC2 Couples H3K14 Deacetylation to H3K9me Binding via Chp2 and Limits Pol II Access to Heterochromatin

As mentioned above, the composition of the SHREC2 complex suggests that the Clr3-mediated H3K14 deacetylation activity is recruited to heterochromatin via Chp2 binding to H3K9me. In order to test this, we performed chromatin immunoprecipitation (ChIP) experiments using H3K14Ac antibody in cells lacking SHREC2 components. At the centromeric *dg* and *dh* repeats and

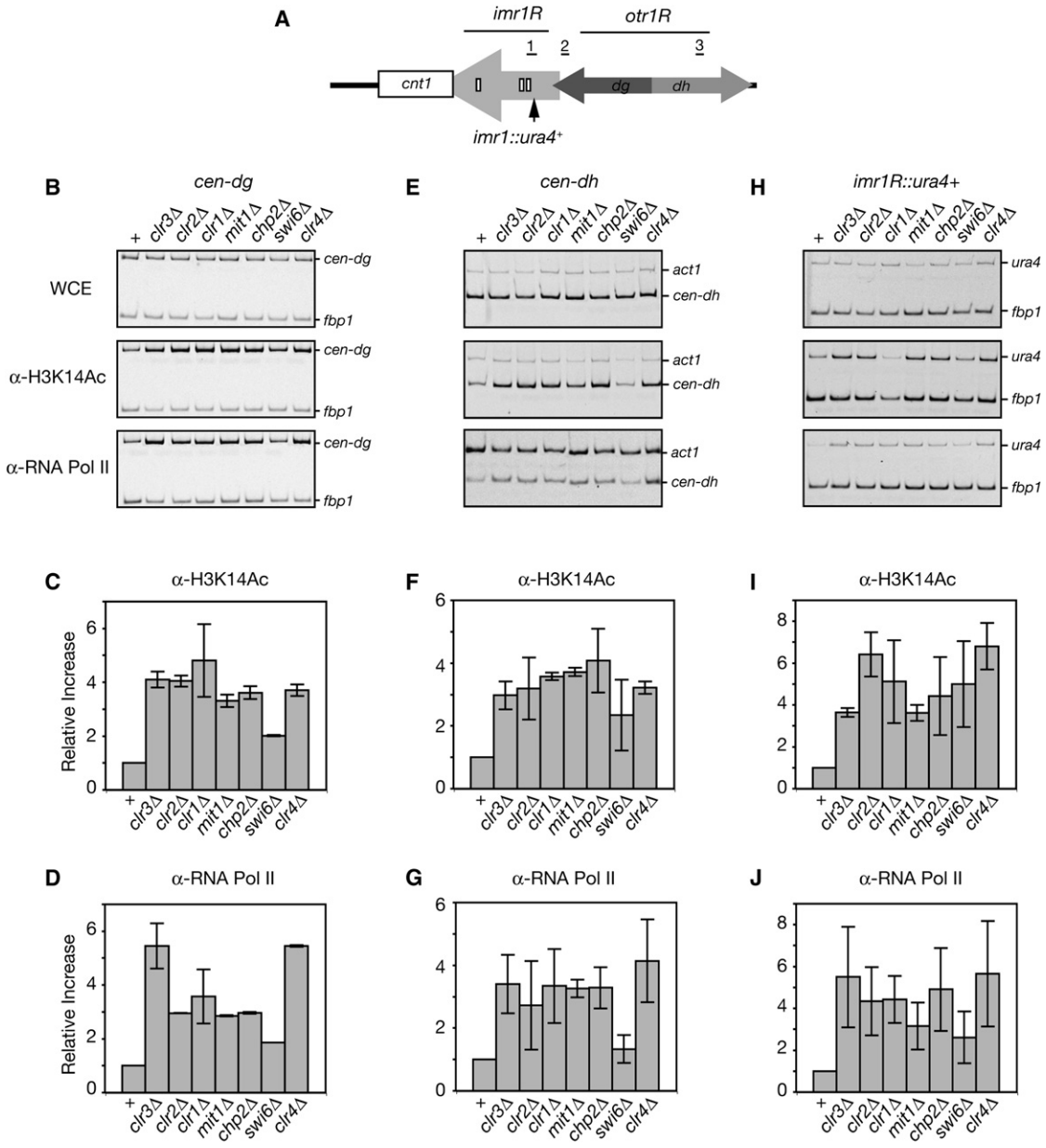


Figure 3. SHREC2 Is Required for H3K14 Deacetylation and Limits RNA Pol II Access to Heterochromatin

(A) Schematic diagram of the pericentromeric DNA repeats immediately to the right of the *S. pombe* centromere 1 central core region (*cnt1*, white box), including the *imr1R* (light gray arrow) and *otr1R* elements. The *dh* and *dg* elements of *otr1R* are shown as light gray and dark gray arrows, respectively. The site of the ectopic *ura4*$^+$ insert, three tRNA genes which act as barrier elements to heterochromatin spreading, and the DNA segments amplified in the ChIP experiments are shown as a black arrow, small white boxes, and underlined numbers 1 (corresponding to *imr1R::ura4*$^+$), 2 (corresponding to *cen-dg*), and 3 (corresponding to *cen-dh*), respectively. ChIP experiments showing that H3K14 deacetylation and Pol II occupancy increase in the absence of SHREC2 components at *cen-dg* (B–D), *cen-dh* (E–G), and *imr1R::ura4*$^+$ (H–J). In *swi6Δ* cells, the increase in H3K14 deacetylation and Pol II occupancy is smaller compared to SHREC2 mutant cells. Error bar represents variation from the mean from two independent biological experiments. *fbp1*$^+$ was used as an internal control for the ChIP experiments. WCE, whole-cell extract.

the *imr1R::ura4*$^+$ transgene (Figure 3A), we found that *SHREC2Δ* and *clr4Δ* mutants display a similar increase in H3K14Ac levels, compared to wild-type (Figures 3B–3I, lanes 1–6 and 8, respectively). In comparison, we observed a smaller increase in H3K14Ac in *swi6Δ* cells (Figures 3B–3I, lane 7), suggesting that the majority of Clr3-dependent H3K14 deacetylation occurs in a Swi6-independent but Chp2-dependent manner.

Previous work had shown that SHREC components limit the access of Pol II machinery to heterochromatin (Sugiyama et al., 2007). We performed ChIP experiments using an antibody

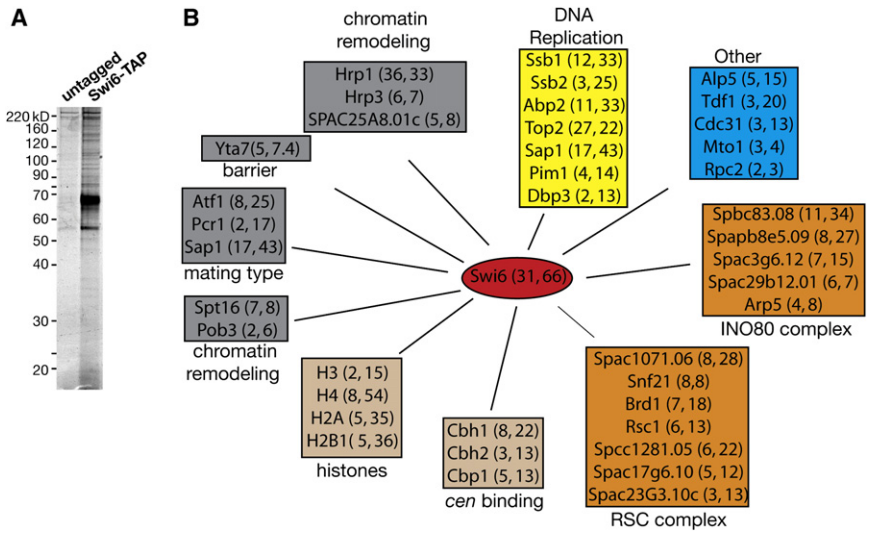


Figure 4. Swi6 Interacts with a Diverse Set of Nuclear Proteins Involved in a Variety of Nuclear Functions

(A) Silver-stained gel of Swi6-TAP and untagged control purifications.

(B) Schematic summary of the network of nuclear proteins that copurify with Swi6. Numbers in the parentheses next to each protein indicate the number of unique peptides and percent coverage based on total number of amino acids, respectively. Proteins are grouped in colored boxes based on previously defined roles in heterochromatin function, histone/centromere binding, chromatin remodeling, DNA replication, and other nuclear functions. Sap1 has been implicated in mating-type switching and DNA replication and is included in groups corresponding to both nuclear functions.

against Pol II to assess the role of SHREC2 components, including Chp2, in regulating Pol II access to centromeric heterochromatin. We found that *chp2Δ*, *clr4Δ* as well as *clr3Δ*, *clr2Δ*, *clr1Δ*, and *mit1Δ* mutants displayed a similar level of increase in Pol II occupancy compared to wild-type cells (Figures 3B, 3E, and 3H, lower panels, and Figures 3D, 3G, and 3J). In *swi6Δ* cells, however, the increase in Pol II occupancy (similar to H3K14Ac levels) was smaller than that in SHREC2 mutant cells (Figures 3B, 3E, and 3H, lower panels, and Figures 3D, 3G, and 3J). Because Clr4-dependent H3K9 methylation creates the binding substrate for Chp2 and Swi6, these data suggest that all H3K9me-dependent control of Pol II access to heterochromatin is SHREC2 mediated.

Even though we observed a comparable level of increase in Pol II occupancy in cells lacking Clr4 or any of the SHREC2 components, the increase in the accumulation of *dg*, *dh*, and *imr1R::ura4*$^{+}$ RNA levels in SHREC2 mutants (Figures 2A, 2D, and 2E) was only a fraction (10%–20%) of the total *clr4Δ* increase (Table S2). These results suggest that SHREC2-dependent transcriptional gene silencing contributes to only a portion of the total heterochromatic gene silencing that prevents the accumulation of endogenous and transgene centromeric RNAs. The rest appears to involve an RNAi-dependent posttranscriptional silencing mechanism that operates in *cis* (Buhler et al., 2006, 2007).

Swi6 Associates with a Distinct Set of Nuclear Proteins

Previous reports have shown that, similar to Chp2, Swi6 is required for silencing at all *S. pombe* heterochromatic loci (Allshire et al., 1995; Ekwall et al., 1995; Klar and Bonaduce, 1991). However, Swi6 also is required for the efficient processing of heterochromatic transcripts into siRNAs (Buhler et al., 2006; Motamedi et al., 2004), suggesting a role for Swi6 in the RNAi-dependent heterochromatic gene silencing pathway. In order to better understand the contribution of Swi6 to this pathway, we purified a C-terminally TAP-tagged version of Swi6 using the previously described tandem affinity purification scheme (Figure 4A) and analyzed the purified proteins using tandem mass spectrometry. The purifications yielded three main classes of nuclear proteins, none of which overlapped with any of the SHREC2 or other known RNAi complexes (Figure 4B, Table S1). DNA binding, chromatin remodeling/histone modifying, and DNA replication proteins represent the three main groups of proteins that copurified with Swi6. From among these, several proteins with previously characterized roles in silencing were identified. Three members of the chromo-ATP/helicase-DNA-binding (CHD) remodeling proteins, two of which, Hrp1 and Hrp3, previously have been shown to be involved in silencing (Jae Yoo et al., 2002; Walfridsson et al., 2005), copurified with Swi6. Chp2 also associates with another member of this family, Mit1, and mammalian HP1 has been previously shown to interact with the Brg1 subunit of the SWI/SNF chromatin-remodeling complex (Nielsen et al., 2002). Thus, interactions between HP1s and chromatin remodelers appear to be conserved.

Other Swi6-associated proteins with known heterochromatin silencing functions include the FACT chromatin-remodeling members Spt16 and Pob3 (Lejeune et al., 2007); ATF/CREB proteins Atf1/Pcr1, which are involved in silencing at the mating-type locus (Jia et al., 2004); and Sap1, a general replication factor (Krings and Bastia, 2005; Noguchi and Noguchi, 2007) also required for mating-type switching (Arcangioli and Klar, 1991). The *S. pombe* homolog of the *S. cerevisiae* Yta7 protein, which has been suggested to restrict spreading of silencing proteins at euchromatin-heterochromatin boundaries (Jambunathan et al., 2005), also copurified with Swi6. Furthermore, the three centromere-binding protein (CENP-B) homologs Cbh1, Cbh2, and Cbp1 with known functions in centromeric silencing (Nakagawa et al., 2002); the core histones H3, H4, H2A, and H2B1; and several protein involved in DNA replication copurified with Swi6 (Figure 4B and Table S1). These data suggest that Swi6 is at the center of a complex network of chromatin-associated proteins, whose activities contribute to a number of different chromosomal functions, including transcriptional silencing.

Contribution of Swi6 to Heterochromatic Gene Silencing

The current evidence on the requirement of Swi6 in heterochromatic gene silencing is largely based on the assessment of the

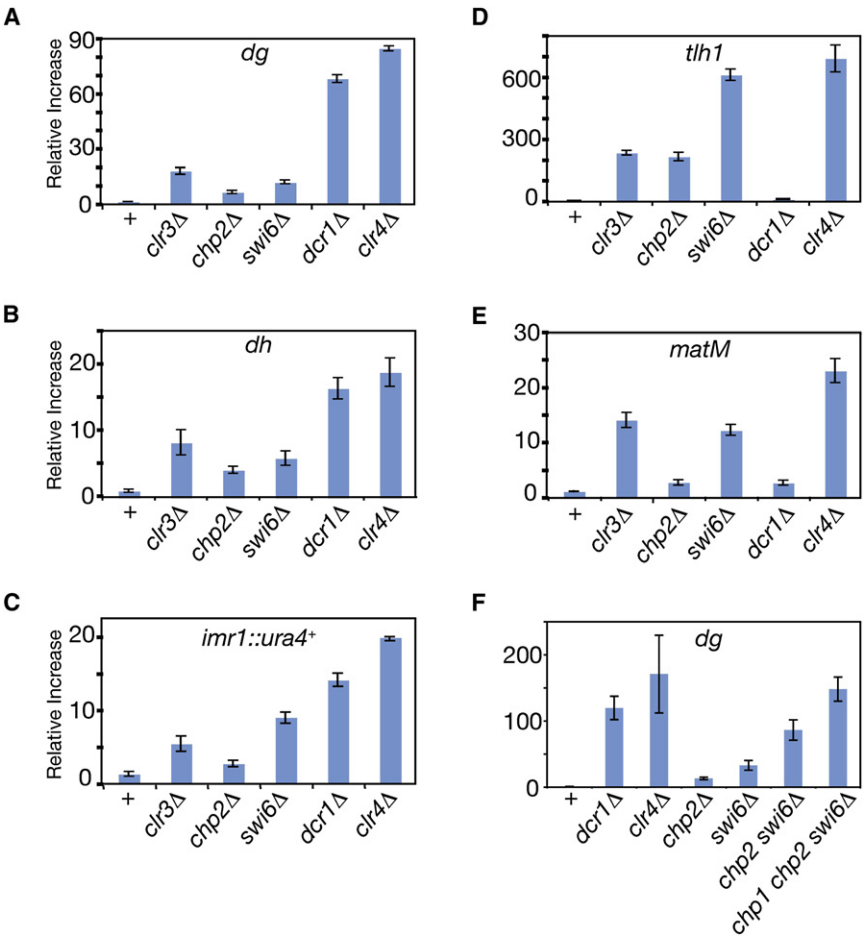


Figure 5. Swi6 and SHREC2 Components Make Nonredundant Contributions to Heterochromatic Gene Silencing

(A–E) Quantitative real-time RT-PCR showing the steady-state levels of (A) *dg*, (B) *dh*, (C) *imr1::ura4*$^+$, (D) *tlh1*$^+$, and (E) *matM* transcripts in *swi6Δ* cells compared to wild-type, *clr3Δ*, *chp2Δ*, *dcr1Δ*, and *clr4Δ* cells.

(F) The steady-state levels of the *dg* transcript in *chp2Δ swi6Δ* double and *chp1Δ chp2Δ swi6Δ* triple mutant cells were compared to single mutants. See Figure S4A for additional data, including *chp1Δ*, *chp1Δ chp2Δ*, and *chp1Δ swi6Δ* cells at *dg dh*, *imr1::ura4*$^+$, and *tlh1*$^+$. Error bars represent standard deviations for three independent biological experiments. All values were normalized to *act1*$^+$ transcript levels.

expression of transgenes inserted at various heterochromatic loci using growth-based assays (Allshire et al., 1995; Ekwall et al., 1995; Klar and Bonaduce, 1991). Although very sensitive, as shown above, these assays are not quantitative. Here, we quantified the relative contribution of Swi6 to heterochromatic gene silencing by using real-time PCR, measuring the accumulation of transgene (*imr1::ura4*$^+$) and endogenous heterochromatic RNAs originating from *dg* and *dh* of centromeric repeats, *matM* gene of the mating-type region, and *tlh1*$^+$ gene of subtelomeres. Because Clr4 is the sole H3K9 methyltransferase in *S. pombe*, we used RNA levels in *clr4Δ* cells as our reference (1.00) for complete loss of H3K9me-dependent silencing and present the RNA levels in *SHREC2* or *swi6* mutant cells relative to this maximal derepression in *clr4Δ* cells. Our analysis revealed that, similar to Chp2, Swi6 contributed to a fraction of the total H3K9me-mediated silencing at centromeres (Figures 5A, 5B, and 5E and Table S3), as the levels of centromeric *dg*, *dh*, and *imr1R::ura4*$^+$ transcripts in *swi6Δ* cells were 0.14, 0.32, and 0.44 compared to *clr4Δ*, respectively. Unlike Chp2, Swi6 was required for the majority of H3K9me-dependent silencing at the subtelomeric *tlh1*$^+$ gene (Figure 5D, Table S3). Also, at the mating-type gene *matM*, Swi6 contribution to silencing was greater than Chp2 and similar to the Clr3 contribution (Figure 5C and Table S3). These results show that centromeric gene silencing is only partially disrupted in *swi6Δ* cells.

The residual silencing in each *swi6Δ* or *chp2Δ* single mutant cell is likely to be mediated by the remaining HP1 protein (Chp2 or Swi6, respectively) or the Chp1-containing RITS complex. We therefore tested whether this residual silencing is lost in double mutant cells lacking both Swi6 and Chp2, or triple mutant cells lacking Swi6, Chp2, and Chp1. We used real-time RT-PCR to measure the accumulation of transgene (*imr1::ura4*$^+$) and endogenous (*dg*, *dh*, and *tlh1*$^+$) heterochromatic RNAs in double *chp2Δ swi6Δ* and triple *chp1Δ chp2Δ swi6Δ* mutant cells and compared these values to those obtained from *swi6Δ*, *chp2Δ*, and *clr4Δ* cells. We found that the double and triple mutant cells displayed a larger defect in silencing (see Figure 5F, Figure S4, and Table S3) than any of the single *swi6Δ*, *chp2Δ* mutants. In particular, deletion of all three CD proteins resulted in accumulation of heterochromatic transcripts to levels that were similar to that found in *clr4Δ* cells, which lack any H3K9 methylation. These data support a model in which Chp2 and Swi6 make distinct nonoverlapping contribution to heterochromatic gene silencing in *S. pombe*.

Swi6 Associates with Noncoding Centromeric Transcripts

The biochemical purifications of Chp2 and Swi6, their relative contributions to heterochromatic gene silencing, and their distinct roles in regulating Pol II access to heterochromatin show that these proteins contribute to silencing by nonredundant mechanisms. Previously, it has been shown that Swi6 is required for efficient accumulation of centromeric siRNAs (Buhler et al., 2007; Motamedi et al., 2004; also see Figure 6A, lane 8), whereas siRNA levels are slightly increased or unaffected in cells that lack the Clr3 component of SHREC2 (Figure 6A, lanes 1 and 2; Irvine et al., 2006; Sugiyama et al., 2007). Consistent with its association with Clr3 in the SHREC complex, we found that deletion of *chp2*$^+$ or other subunits

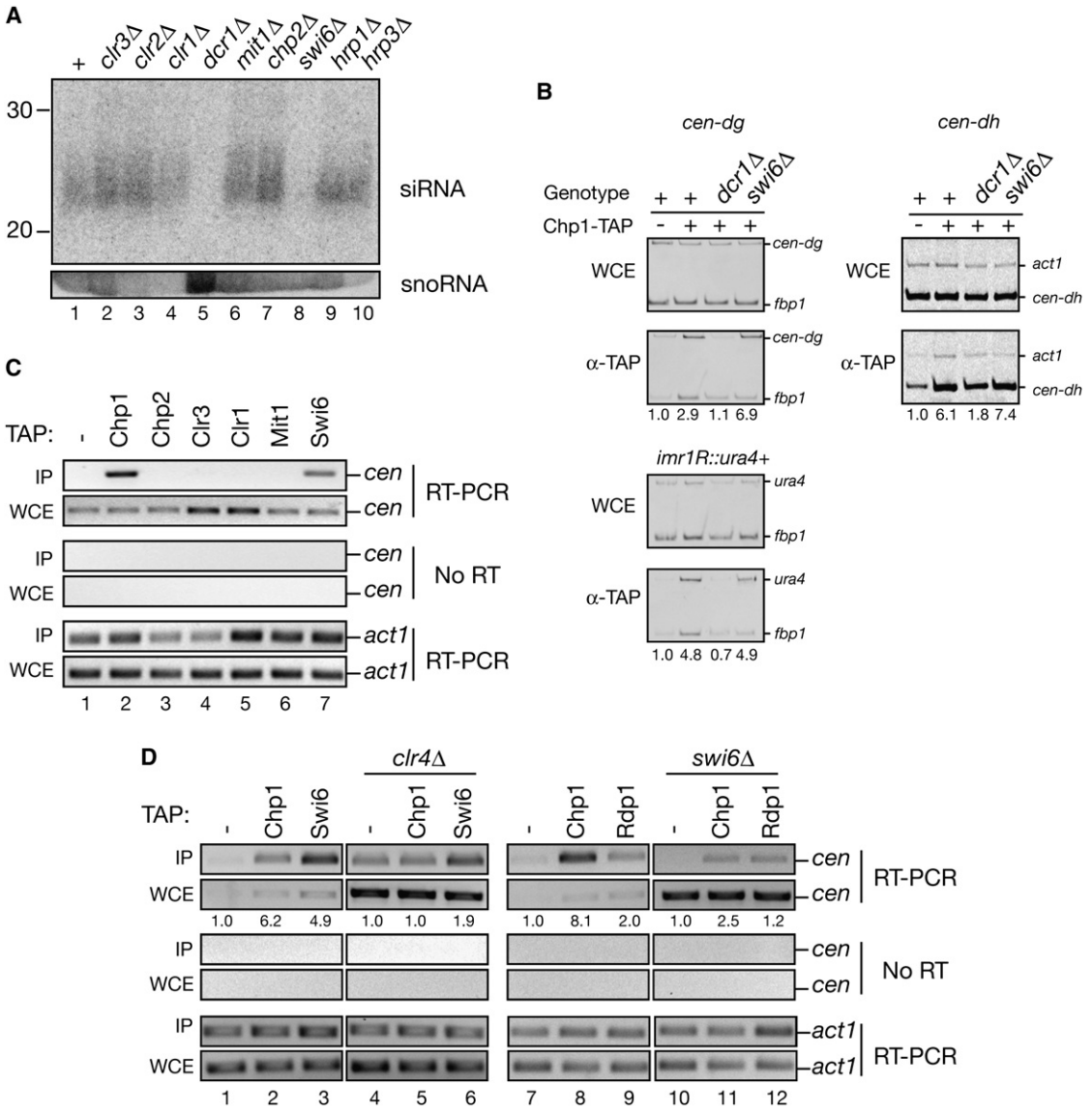


Figure 6. Swi6, but Not SHREC2, Associates with Noncoding *cen*RNAs and Is Required for Their Processing into siRNAs

(A) Northern blot showing that Swi6 is required for accumulation of centromeric siRNAs. Size-fractionated total small RNAs purified from the indicated cells were probed with end-labeled DNA oligos complementary to centromeric siRNAs. The membrane was then reprobed with snoR69, serving as a loading control. Unlike *swi6Δ*, SHREC2 mutant cells exhibited no defect in siRNA production.

(B) ChIP experiments showing that RITS recruitment to centromeric *dg*, *dh*, and *imr1::ura4*$^+$ is Swi6 independent. Immunoprecipitations were performed using wild-type, *dcr1Δ*, and *swi6Δ* cells carrying a *chp1-TAP* gene. Untagged cells were used as a control. Values below each lane represent enrichment compared to the untagged control. *fbp1*$^+$ and *act1*$^+$ were used as internal controls.

(C) RNA-IP experiments showing that centromeric transcripts (*cen*) associate specifically with Swi6, but not with SHREC2 components Chp2, Clr3, Mit1, or Clr1. Chp1 is used as a positive control. Actin mRNA (*act1*) was used as a control to show that the interaction is specific to heterochromatic transcripts.

(D) RNA-IP experiments showing that Chp1 and Swi6 associate with centromeric transcripts in a Clr4-dependent manner (lanes 1–6); efficient Chp1 and Rdp1 interaction with *cen* transcripts requires Swi6 (lanes 7–12). Actin mRNA (*act1*) and the untagged sample were used as specificity controls. The number shown below each lane is the average enrichment calculated by comparing the enrichment values to the untagged control in two independent experiments. WCE, whole-cell extract; IP, immunoprecipitation of the indicated TAP-tagged protein.

of the SHREC complex had little or no effect on the levels of centromeric siRNA (Figure 6A, compare lanes 1–4 and 6 and 7 with lanes 5 and 8). These results further support distinct roles for Swi6 and Chp2 in mediating RNAi-dependent RNA processing and transcriptional gene silencing, respectively. The modest increase in siRNA levels observed in SHREC2 mutant cells is likely to result from an increase in the transcription of siRNA precursors.

The significant reduction in siRNA levels in *swi6Δ* mutants (Figure 6A and Buhler et al., 2006; Motamedi et al., 2004) gives

rise to the possibility that Swi6 may be required for the recruitment of the RNAi effector complex RITS to centromeric heterochromatin. We used ChIP experiments to test this hypothesis and found that the Chp1 subunit of RITS associated with centromeric *dg* and *dh* repeats independently of Swi6 (Figure 6B), but, as shown previously (Verdel et al., 2004), this interaction required Dcr1-generated siRNAs (Figure 6B). Also, consistent with the MS/MS profile of proteins identified in Swi6 purifications, using coimmunoprecipitation assays, we did not detect an interaction between C-terminally tagged Rdp1, Tas3, Chp1, Cid14, Dcr1, or an N-terminally FLAG-tagged Ago1 with Swi6 (data not shown), suggesting that Swi6 did not interact with RDRC/Dcr1, RITS, TRAMP, or ARC complexes. The Swi6 contribution to RNAi-mediated processing of heterochromatic transcripts therefore is unlikely to be mediated by the recruitment of any of the above RNAi complexes to heterochromatin.

Prior work has shown that HP1 proteins are capable of binding to RNA in vivo and in vitro via their basic patch region, located in between the chromodomains and chormo shadow domains (Maison and Almouzni, 2004; Muchardt et al., 2002). We hypothesized that Swi6 may contribute to the RNAi-dependent pathway by binding to heterochromatic RNAs, transcribed within nucleosomal regions enriched for H3K9me, the binding substrate for Swi6. In our previous work, we used an RNA immunoprecipitation (RNA-IP) assay to show that RITS and RDRC complexes interact with noncoding centromeric transcripts in a Dcr1- and Clr4-dependent manner (Motamedi et al., 2004). Here we examined the ability of SHREC2 components, Chp2, Clr3, Clr1, and Mit1, and Swi6 to interact with *cen* transcripts. We found that, similar to the Chp1 subunit of the RITS complex (Motamedi et al., 2004), Swi6 specifically crosslinked to noncoding *cen* transcripts in a manner that was mostly Clr4 dependent (Figure 6C, lanes 2 and 7; Figure 6D, compare lanes 1–3 to lanes 4–6). In contrast, the Chp2, Clr1, Clr3, and Mit1 subunits of SHREC did not crosslink to *cen* transcripts (Figure 6C, lanes 3–6). Together with previous observations, these results suggest that Swi6 is a bifunctional protein that associates with both H3K9-methylated nucleosomes and noncoding transcripts within heterochromatic domains.

The association of Swi6 with *cen* transcripts and its requirement for siRNA generation suggest that Swi6 may contribute to the stable assembly of RNAi complexes on heterochromatic transcripts by tethering the transcripts to sites of RNA synthesis. To test this possibility, we used RNA-IP experiments to ask whether Swi6 was required for localization of Chp1 and Rdp1 (components of RITS and RDRC, respectively) to *cen* transcripts. As shown in Figure 6D, we observed a marked decrease in the association of Chp1 and a smaller decrease in the association of Rdp1 with *cen* transcripts in *swi6Δ* compared to the wild-type cells (compare lanes 7–9 with lanes 10–12), suggesting that Swi6 was required for efficient association of RITS and RDRC with centromeric transcripts. Together with the ChIP data in Figure 6B, these results reveal a role for Swi6 in promoting the association of RNAi complexes with heterochromatic transcripts by a mechanism that is independent of their association with H3K9-methylated chromatin.

DISCUSSION

HP1 proteins play a critical role in H3K9 methylation-dependent heterochromatic gene silencing in eukaryotes; however, the nature of their contribution to silencing has remained elusive. In this study, we show that the two *S. pombe* HP1 proteins contribute to heterochromatin-dependent gene silencing by nonredundant mechanisms. Chp2 associates with the SHREC complex and recruits the H3K14 deacetylase Clr3, whose activity is required for restricting RNA Pol II access to heterochromatin. Swi6, on the other hand, interacts with noncoding centromeric transcripts, similar to the RITS CD subunit Chp1, and contributes to silencing primarily through RNAi-dependent *cis* processing of heterochromatic transcripts (Figures 6D and 7). These findings demonstrate that efficient heterochromatic gene silencing requires the recruitment of distinct silencing mechanisms by different HP1 proteins.

SHREC2 Links H3K9me Binding to H3K14 Deacetylation

Previous purifications of Clr3 (Sugiyama et al., 2007), as well as our Clr3-TAP, TAP-Clr2, and Clr1-TAP purifications (Figures 1B–1E), failed to reveal SHREC interaction with Chp2. This is likely due to rapid degradation of Clr1 during purification (Figures S1A–S1C). Our LC-MS/MS analyses revealed that full-length Clr1 forms an N- and a C-terminal fragment, which interact with Chp2 and Mit1 or Clr2 and Clr3, respectively (Figures 1A–1F). The largely nonoverlapping peptide spectra of Clr1 in the Clr1-TAP, TAP-Clr2, and Clr3-TAP versus Chp2-TAP purifications (Figure 1F) demonstrate that Clr1 serves as a connector between Chp2 (and possibility Mit1) and Clr2 and Clr3 subunits of SHREC (Figure 1G).

The association of Chp2 with the SHREC complex demonstrates a coupling between two highly conserved posttranslational modifications of histone H3 tails (lysine 9 methylation and lysine 14 deacetylation), both of which are critical for heterochromatic gene silencing. What is the biological significance of this coupling? Previous work (Sadaie et al., 2004) and data present here (Figures S3A–S3G) show that Clr4-mediated H3K9 methylation of pericentromeric repeats occurs independently of Chp2 or Swi6. This, combined with the requirement of these proteins for silencing, suggests that these HP1 proteins associate with heterochromatin and recruit downstream activities that are critical for heterochromatin maturation. Consistent with this hypothesis, we found that the deacetylation of H3K14 by Clr3 at centromere is H3K9me and Chp2 dependent (Figure 3). Clr3-dependent H3K14 deacetylation regulates the transcriptional state of chromatin (Bjerling et al., 2002; Sugiyama et al., 2007), and the importance of H3K14 in transcriptional gene silencing has been demonstrated in a wide range of organisms including *S. cerevisiae* (Braunstein et al., 1996; Suka et al., 2001; Thompson et al., 1994) and *S. pombe* (Mellone et al., 2003; Yamada et al., 2005). Conversely, H3K14 acetylation is correlated with active transcription (Pokholok et al., 2005), and in human cells acetylated H3K14 is critical for the recruitment of TFIID to active promoters (Agalioti et al., 2002). Our results show that deacetylation of H3K14 by the SHREC complex occurs downstream of H3K9 methylation by Clr4 and is largely or entirely mediated by H3K9-binding HP1 protein, Chp2.

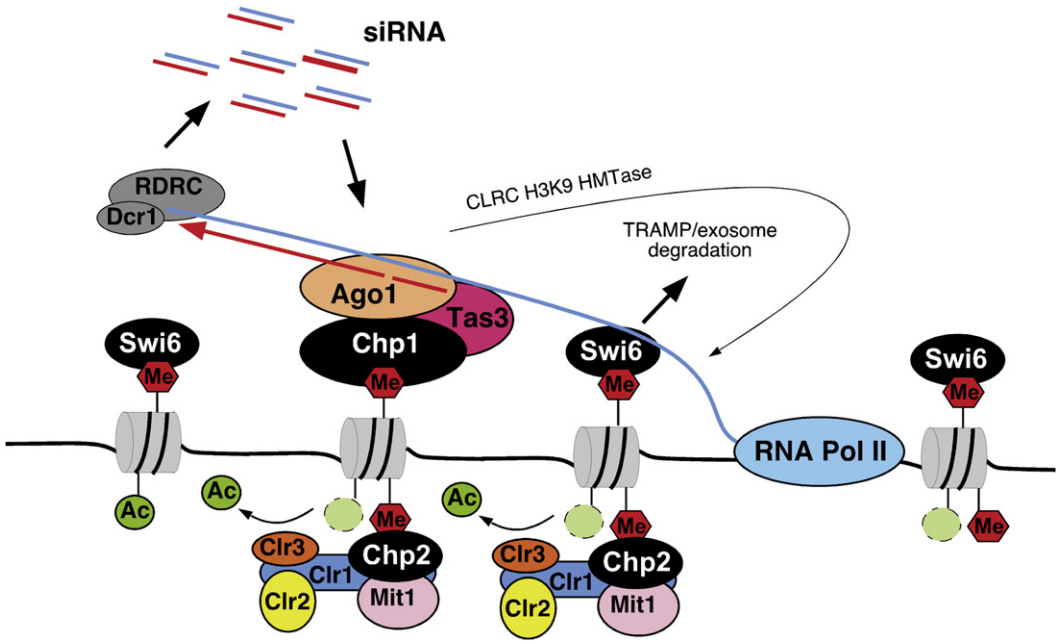


Figure 7. Model Highlighting the Contributions of HP1 Proteins to Heterochromatic Gene Silencing in *S. pombe*

Swi6 promotes the stable association of nascent heterochromatic transcripts to sites of RNA synthesis by its ability to bind to H3K9me and RNA. The association of Swi6 with the nascent transcript is required for the subsequent assembly of RNAi complexes (RITS and RDRC/Dcr1) on the RNA, probably by helping to tether the RNA to chromatin. Swi6-mediated tethering may also be required for targeting of heterochromatic transcripts for degradation by the TRAMP/exosome pathway. SHREC2 binds to H3K9me via its Chp2 component, targeting the H3K14 deacetylase Clr3 and CHD ATPase Mit1 to heterochromatin, which are required for limiting Pol II access to heterochromatin.

Conservation of HP1 proteins and their mode of association with heterochromatin, as well as histone hypoacetylation in heterochromatic gene silencing, raise the possibility that recruitment of homologous deacetylase complexes in other eukaryotes may be HP1 dependent.

Previous work has suggested that localization of Clr3 to a *ura4*$^+$ reporter gene inserted at the mating-type locus depends on both Swi6 and Chp2 (Yamada et al., 2005). We found that the silencing defect observed at *matM* or *mat::ura4*$^+$ transgene in *chp2Δ* or *mit1Δ* cells is 3- to 6-fold less severe compared to *clr1Δ*, *clr2Δ*, and *clr3Δ* cells (Figures 2C and 2G, and Figure 5E and Figure S2C; see Table S1 for quantification). Previous work has also shown that Atf1/Pcr1 proteins recruit Clr3 to its nucleation site at the *mat* locus within the *REIII* silencer element. We propose that in the absence of Mit1 and Chp2, Clr3 can be recruited to the *mat* region via the Atf1/Pcr1-dependent pathway, perhaps as a Clr1/2/3 subcomplex. In addition, the association of Swi6 with Clr3 (Yamada et al., 2005) and with Atf1/Pcr1 proteins (Figure 5C) (Jia et al., 2004) may provide a parallel, Chp2-independent, mechanism for the recruitment of Clr1/2/3 to the mating-type locus. Alternative mechanisms for the recruitment of SHREC have been reported for other loci. For example, at telomeres, the telomere-binding protein, Taz1, and the HEAT domain protein, Ccq1, cooperate with the RNAi machinery to recruit SHREC (Sugiyama et al., 2007). Overall, these results demonstrate the existence of distinct, partially redundant pathways for targeting of the SHREC complex to different heterochromatic domains.

Association of Swi6 with Noncoding Heterochromatic Transcripts

Recent studies suggest that chromatin and RNA silencing pathways intersect at several levels (Bernstein and Allis, 2005). Our analysis of the role of Swi6 in heterochromatic gene silencing suggests that this HP1 protein plays a central role in coordinating histone H3K9 methylation with RNA processing. Purification of Swi6 revealed a complex network of nuclear proteins involved in a wide range of cellular processes, including transcriptional repression and elongation, chromosome biogenesis, and chromatin remodeling. Remarkably, our purifications revealed no overlapping peptides in Chp2 and Swi6 purifications, suggesting that these closely related HP1 proteins make distinct nonoverlapping contributions to gene silencing. Consistent with the biochemical purifications, we found that Swi6 and Chp2 operate via different mechanisms to effect silencing within heterochromatic regions. While Chp2 functions to limit Pol II access to heterochromatin, and thereby mediate transcriptional gene silencing, the contribution of Swi6 to limiting Pol II access to heterochromatin is minimal (Figures 3D, 3G, and 3J, lanes 7), suggesting an unexpected silencing role for an HP1 protein in a step downstream of transcription initiation. Together with the requirement for Swi6 in siRNA accumulation (Buhler et al., 2006; Motamedi et al., 2004) (Figure 6A), our data suggest that the primary function of Swi6 in heterochromatin involves cotranscriptional processing of heterochromatic transcripts.

How does Swi6 contribute to the RNAi-mediated processing of heterochromatic transcripts? HP1 proteins have been shown

to bind to RNA in vitro and, in addition to heterochromatin, colocalize with transcriptional elongation complexes of highly transcribed genes in *Drosophila* and human (Cryderman et al., 2005; Vakoc et al., 2005), suggesting that they play a role in RNA processing. Our observation that Swi6 specifically interacts with noncoding *cen* transcripts suggests a possible model for the involvement of Swi6 in RNAi-mediated processing of heterochromatic transcripts. We propose that Swi6 is a bifunctional protein that tethers heterochromatic transcripts to the chromosome (Figure 7). In this model, the Swi6 CD binds to H3K9-methylated nucleosomes, and its basic hinge region, which in other HP1 proteins has been implicated in RNA binding, associates with nascent RNAs. This tethers nascent RNAs to heterochromatin and allows RNAi complexes to assemble on the transcript. In the absence of Swi6, centromeric siRNA levels are greatly diminished (~20-fold below *swi6*$^+$ cells), and RITS and RDRC localization to *cen* transcripts (Figure 6), and Rdp1 to *cen* DNA (Sugiyama et al., 2005), is reduced compared to wild-type, suggesting that Swi6 is required for the efficient association of RITS/RDRC/Dicer with their target centromeric transcripts. The actual Swi6-mediated decrease in the levels of heterochromatic transcripts may result from the conversion of the RNA to dsRNA and its degradation by Dicer or by the slicer activity of siRNA-programmed RITS complexes (Figure 7). Recent studies in *Drosophila* have uncovered a direct physical interaction between HP1 and Piwi, an Argonaute family protein that binds to small RNAs that originate from repetitive DNA elements and transposons (Brower-Toland et al., 2007). HP1 proteins therefore appear to play direct but distinct roles in distantly related chromatin-associated RNA silencing mechanisms. Finally, any model for the role of Swi6 in heterochromatic gene silencing must take into account the requirement for Swi6 in RNAi-independent gene silencing at the silent mating-type locus. In addition to Swi6 and H3K9 methylation, efficient silencing at the mating-type locus requires the TRAMP polyadenylation complex and exosome-mediated RNA degradation (Buhler et al., 2007). We propose that the RNA association activity of Swi6 serves a general function in retention of heterochromatic RNAs on chromatin that contributes to the processing of these RNAs by either the RNAi or the TRAMP/exosome pathways (Figure 7).

EXPERIMENTAL PROCEDURES

Strain Construction and Protein Purification

Strain construction was performed following standard procedures as described in the Supplemental Data (Bahler et al., 1998). *TAP-clr2*, *clr3-TAP* (10–20 g) and 80–110 g of *clr1-TAP* and *chp2-TAP* of logarithmically growing cells were harvested and frozen in liquid nitrogen for later use. All tandem affinity TAP purifications were preformed as described previously (Motamedi et al., 2004), except that all TEV cleavage steps were done at 4°C overnight. Proteins were identified as described previously (Verdel and Moazed, 2005).

Silencing Assays

All silencing assays were performed as described previously (Verdel et al., 2004). Briefly, cells were grown in 5 ml of yeast extract supplemented with adenine (YEA) to logarithmic phase, after which 5-fold dilutions of each culture were spotted on nonselective (N/S) YEA plates and YEA plates supplemented with 5-fluoro-oratic acid (5-FOA). 5-FOA is used as a counterselection against cells expressing *ura4*$^+$.

Chromatin Immunoprecipitation

ChIP was performed as described in detail previously (Huang and Moazed, 2003). Cells were grown to an OD600 of 1.5–2 and crosslinked for 15 min with 1% formaldehyde. Immunoprecipitation was performed with the following antibodies: 3 μl α-H3K14Ac (Upstate, 07-353), 3 μl α-H3K9Me2 (Abcam ab1220), and 4 μl α-RNA Pol II (Covance, 8WG16) per 400 μl reaction. PCR amplifications were performed to determine the linear range for each reaction, and a final nonradioactive PCR set was performed according to the linear range determination on all samples. PCR products were run on 6% acrylamide gels and stained with ethidium bromide (EtBr) at 2.5 mg/l. Image ReaderLAS-3000 was used to capture the image, and the PCR bands were quantified using ImageGauge V4.22 (Fuji Film Life Science, Stanford, CT).

RNA Analysis

RNA-IP experiments were performed as described previously (Motamedi et al., 2004). Cells were grown to an OD600 of 1.5–2, crosslinked with formaldehyde for 30 min, and frozen in liquid nitrogen. Whole-cell extracts were prepared from frozen pellets as described previously (Gilbert et al., 2004; Hurt et al., 2004) and treated with DNase I (Sigma, 700 units) for 1 hr at 30°C in the buffer supplemented with 25 mM $MgCl_2$ and 5 mM $CaCl_2$. RNA was precipitated and used to perform semiquantitative RT-PCR reactions (Motamedi et al., 2004).

RNA preparation, semiquantitative and real-time RT-PCR, and northern blot analysis were performed as described previously (Buhler et al., 2006, 2007; Leeds et al., 1991; Motamedi et al., 2004).

Western Blots

Western blots were performed as described previously (Motamedi et al., 2004). For detection of Clr1-TAP, cell lysis was performed in buffer containing 30% trichloroacetic acid as described in the Supplemental Data.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, four figures, five tables, and Supplemental References and can be found with this article online at http://www.molecule.org/supplemental/S1097-2765(08)00806-X.

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