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SHREC, an Effector Complex for Heterochromatic Transcriptional Silencing

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SUMMARY

Transcriptional gene silencing (TGS) is the mechanism generally thought by which heterochromatin effects silencing. However, recent discovery in fission yeast of a *cis*-acting posttranscriptional gene-silencing (*cis*-PTGS) pathway operated by the RNAi machinery at heterochromatin challenges the role of TGS in heterochromatic silencing. Here, we describe a multienzyme effector complex (termed SHREC) that mediates heterochromatic TGS in fission yeast. SHREC consists of a core quartet of proteins—Clr1, Clr2, Clr3, and Mit1—which distribute throughout all major heterochromatin domains to effect TGS via distinct activities associated with the histone deacetylase Clr3 and the SNF2 chromatin-remodeling factor homolog Mit1. SHREC is also recruited to the telomeres by multiple independent mechanisms involving telomere binding protein Ccq1 cooperating with Taz1 and the RNAi machinery, and to euchromatic sites, via mechanism(s) distinct from its heterochromatin localization aided by Swi6/HP1. Our analyses suggest that SHREC regulates nucleosome positioning to assemble higher-order chromatin structures critical for heterochromatin functions.

INTRODUCTION

Eukaryotic chromosomes are composed of two general types of chromatin domains: euchromatin, gene-rich chromatin that is accessible to factors involved in different aspects of DNA transactions including transcription, and heterochromatin, which is typically highly condensed and forms highly ordered nucleosomal arrays (Grewal and Elgin, 2002; Huisinga et al., 2006). Heterochromatin has a characteristic histone-modification profile distinguished by hypoacetylation of histones and methylation of histone H3 at lysine 9 (H3K9me) (Grewal and Elgin, 2002; Jenuwein and Allis, 2001). Deacetylation of histones appears to be a universal prerequisite for establishing repressive heterochromatin structures (Jenuwein and Allis, 2001). Whereas heterochromatic islands are interspersed throughout the genomes, major targets of heterochromatin formation are chromosome regions that contain high density of repetitive elements such as transposons present at centromeres and telomeres (Hall and Grewal, 2003). Heterochromatin not only silences underlying DNA sequences but also prohibits recombination, which is essential for protecting genome integrity. Moreover, defects in heterochromatin disrupt chromosome segregation and developmentally controlled long-range chromatin interactions (Jia et al., 2004b; Pidoux and Allshire, 2004).

The fission yeast *Schizosaccharomyces pombe* contains large blocks of heterochromatin, and many factors involved in heterochromatin assembly in higher eukaryotes are also conserved in this system (Grewal and Elgin, 2002; Hall and Grewal, 2003). These factors include Clr4, a member of the mammalian SUV39 histone methyltransferases, and HP1 family proteins Chp1, Chp2, and Swi6, which all are preferentially enriched at heterochromatic loci (Cam et al., 2005; Jia et al., 2005; Nakayama et al., 2001; Sadaie et al., 2004; Thon and Verhein-Hansen, 2000). Clr4 exists in a cullin 4 (Cul4)-based E3 ubiquitin ligase complex that specifically methylates H3K9 (Hong et al., 2005; Horn et al., 2005; Jia et al., 2005). In addition, heterochromatic silencing requires multiple conserved histone deacetylases (HDACs) (Clr3, Clr6, and Sir2) (Ekwall, 2005; Freeman-Cook et al., 2005; Grewal et al., 1998; Shankaranarayana et al., 2003) and RNAi machinery (Matzke and Birchler, 2005). Specialized repeat (*dg* and *dh*) elements present at all major heterochromatic regions, including pericentromeric regions, subtelomeres, and the mating-type locus, have been shown to act as RNAi-mediated heterochromatin nucleation centers (Cam et al., 2005; Hall et al., 2002; Kanoh et al., 2005; Volpe et al., 2002). In addition, DNA binding proteins cooperate with Clr3 to nucleate heterochromatin independently of the RNAi machinery (Yamada et al., 2005).

Heterochromatic silencing in *S. pombe* occurs at both transcriptional and posttranscriptional levels (Noma et al., 2004). RNAi machinery localizes across heterochromatic domains to process nascent repeat transcripts into siRNAs (Buhler et al., 2006; Cam et al., 2005; Motamedi et al., 2004; Noma et al., 2004; Sugiyama et al., 2005; Verdel et al., 2004). This *cis*-acting posttranscriptional gene silencing (*cis*-PTGS) mechanism appears to couple siRNA generation to the establishment of H3K9me by Clr4 (Noma et al., 2004; Sugiyama et al., 2005). H3K9me provides binding sites for Swi6/HP1 (Bannister et al., 2001; Nakayama et al., 2001) and Chp1, a component of the RNA-induced initiation of transcriptional gene silencing (RITS) RNAi effector complex that includes Tas3 and Ago1 (Noma et al., 2004; Petrie et al., 2005; Verdel et al., 2004). RITS contains siRNAs corresponding to *dg* and *dh* repeats, which likely provide specificity for detection of repeat transcripts generated by RNA polymerase II (Pol II) (Cam et al., 2005; Djupedal et al., 2005; Kato et al., 2005; Verdel et al., 2004). RITS also interacts with RNA-directed RNA polymerase (RDRC) containing Rdrp1, whose catalytic activity is critical for siRNA generation and heterochromatin assembly (Motamedi et al., 2004; Sugiyama et al., 2005). These studies indicate that RITS recruits RDRC and perhaps other RNAi factors to heterochromatic loci and, together, these factors process repeat transcripts into siRNAs.

While recent studies have considerably illuminated the contribution of *cis*-PTGS to heterochromatic gene silencing, the mechanism of transcriptional gene silencing (TGS) and its relative contribution to silencing at heterochromatic loci in fission yeast is less understood. Our recent analysis revealed that the HDAC activity of Clr3 contributes to the silencing of a *dg*/*dh*-like element (*cenH*) located at the silent *mat* locus by regulating Pol II occupancy (Yamada et al., 2005). These results suggest that Clr3 operates at the level of transcription and that Clr3 may be a part of an apparatus that enforces heterochromatic transcriptional silencing.

In this study, we describe an effector complex involved in TGS in *S. pombe*. This complex termed SHREC (*S*nf2/*H*dac-containing *Re*pressor *C*omplex) contains at least two distinct enzymatic functions: Clr3 HDAC and a novel factor named Mit1 belonging to the SNF2 family of chromatin remodeling factors. Other components include silencing factors, Clr1 and Clr2, which together with Clr3 and Mit1 constitute a core quartet of SHREC present across all heterochromatin domains. SHREC interacts with Ccq1, a telomere binding protein that along with Taz1 (a homolog of mammalian TRF1/2) acts in a parallel mechanism to the RNAi pathway to facilitate SHREC recruitment to the telomere ends. Whereas factors such as Swi6/HP1 provide a platform for stable binding and spreading of SHREC across heterochromatic domains, the core SHREC components also localize to euchromatic loci independent of Swi6. Our analyses suggest that the enzymatic activities associated with Clr3 and Mit1 are critical for proper positioning of nucleosomes at heterochromatic loci and for TGS function of SHREC and that SHREC acts to restrict Pol II occupancy at heterochromatic repeats.

RESULTS

Purification of Clr3 and Identification of Its Interaction Proteins

To gain insights into the function of Clr3 in TGS, we purified Clr3 from a strain expressing Clr3 tagged at its carboxyl terminus with triple FLAG epitopes (Clr3-FLAG). Clr3-FLAG is fully functional (Figures 1A and 1B). Analysis of the Clr3-purified fraction revealed several polypeptides, ranging in sizes from 65 to 170 kDa, which were present in immunoaffinity-purified fraction of Clr3-FLAG but not in that of the control (Figure 1C). Mass spectrometry analyses identified the 80 kDa polypeptide as Clr3-FLAG, and the 140 and 65 kDa polypeptides respectively as Clr1, a C2H2-type zinc finger protein, and Clr2, a protein with no obvious conserved domain (Figures 1C and S1) (Bjerling et al., 2004). Clr1, Clr2, and Clr3 were identified in screens for factors required for heterochromatic silencing at the *mat* locus (Ekwall and Ruusala, 1994; Thon et al., 1994; Thon and Klar, 1992), and previous genetic evidence indicates that Clr1 and Clr3 may function jointly in the same pathway (Grewal et al., 1998). We identified the 90 and 170 kDa bands as Ccq1 and SPBP35G2.10, respectively. Ccq1 contains a HEAT repeat and a coiled-coil domain and has been implicated in linking telomeres to the spindle-pole body (Flory et al., 2004). We named SPBP35G2.10 as Mit1 (*Mi*2-like protein *i*nteracting with Clr *t*hree *1*) due to its high similarity to Mi-2/CHD3 proteins, in possessing PHD finger, SNF2_N helicase and helicase C-terminal domains commonly found in SNF2 family chromatin remodeling factors. However, unlike its orthologs, Mit1 lacks a chromodomain (Figure 1).

Clr1, Clr2, Ccq1, and Mit1 Stably Associate with Clr3 In Vivo

To confirm that the proteins identified in the Clr3-purified fraction stably associate with Clr3, we constructed strains expressing epitope-tagged versions of these proteins. In strains carrying Clr1-myc, V5-Clr2, Mit1-myc, or Ccq1-GFP, we observed the expected size for each protein, and silencing assay showed that none of these tagged proteins displayed defect in centromeric or telomeric silencing (Figure S2), indicating that the tagged proteins are functional. We performed immunoprecipitation experiments and found that Clr3 coimmunoprecipitated with Clr1, Clr2, Mit1, and Ccq1 (Figure 1D). These analyses indicate that factors mentioned above form a complex with Clr3 in vivo.

SHREC Core Quartet Localizes to All Major Heterochromatic Loci and Euchromatic Sites

Clr3 is known to localize to centromeres and the *mat* locus (Wiren et al., 2005; Yamada et al., 2005). In addition, genome-wide studies implicate a global transcriptional regulatory role for Clr3 (Hansen et al., 2005; Wiren et al.,

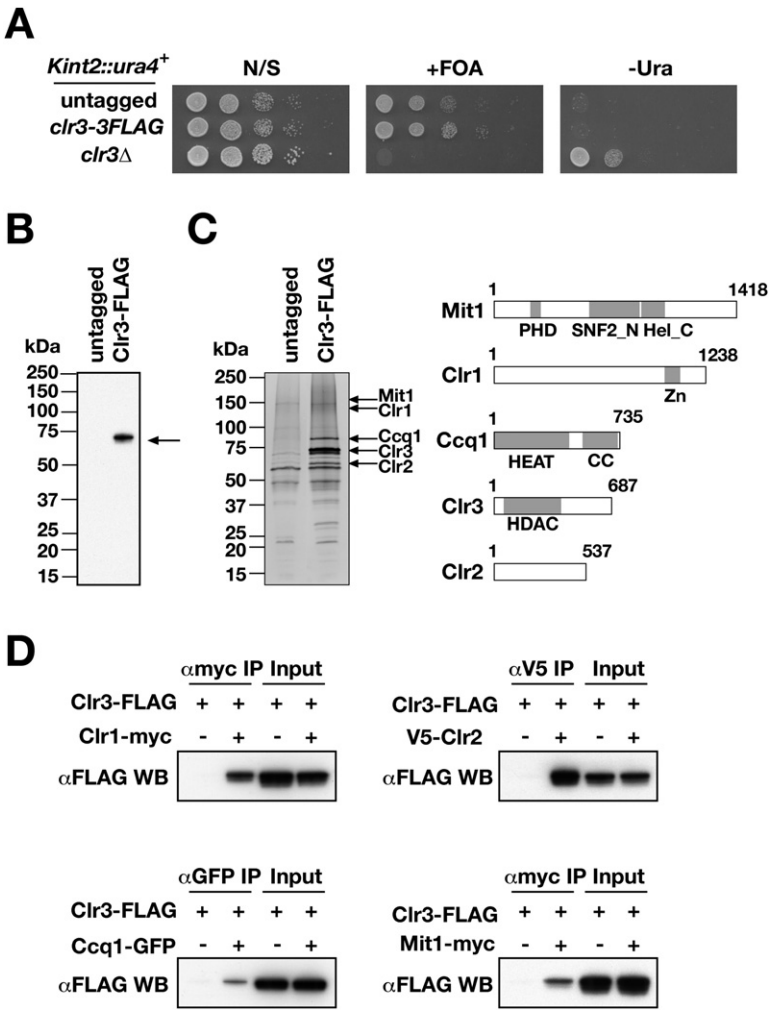

Figure 1. Purification of Clr3 and Identification of Its Interacting Partners

(A) Clr3-FLAG is functional. Ten-fold serial dilution of untagged, Clr3-FLAG, and *clr3*Δ strains carrying *Kint2*::*ura4*$^+$ marker at the silent *mat* locus were spotted onto nonselective (N/S), uracil-lacking (-Ura), and FOA-containing (+FOA) plates.

(B) The expression of Clr3-FLAG. Extracts from untagged and Clr3-FLAG strains were analyzed by western blotting. The arrow indicates protein band corresponding to Clr3-FLAG.

(C) Clr3 purification and schematic representation of Clr3 binding proteins. Extracts from untagged and Clr3-FLAG strains were subjected to FLAG purification procedure, and Clr3 binding proteins were visualized by silver staining. The bands were excised from gel and subjected to mass spectrometry. CC, coiled-coil domain; HDAC, histone deacetylase; HEAT, HEAT repeat; Hel_C, helicase C-terminal domain; PHD, PHD finger; SNF2_N, SNF2 N-terminal domain; Zn, C2H2-type Zinc finger.

(D) Extracts prepared from strains expressing tagged proteins were incubated with indicated antibody, and immunoprecipitated (IP) fractions were analyzed by western blotting using anti-FLAG antibody. Lanes labeled "input" show the equivalent of 10% extracts used in IP lanes.

2005). To identify the global targets of SHREC and determine its mode of recruitment, we utilized ChIP-chip analyses to map at high resolution all SHREC components across the *S. pombe* genome. We found prominent binding peaks of Mit1, Clr1, Clr2, and Clr3 associated with major heterochromatin domains including centromeres, subtelomeres, rDNA, and the *mat* locus, while those of Ccq1 were restricted to telomeres (Figure 2). Detailed examination of the major heterochromatic regions revealed the ubiquitous presence of SHREC components throughout the entire heterochromatic domains (Figure 3A). In particular, the binding profiles of SHREC components at centromeres, subtelomeres, and the *mat* locus are highly similar to H3K9me2 and Swi6 distributions reported previously (Cam et al., 2005), except Clr2, which is also enriched at the central core (*cnt*) domain of centromeres, regions devoid of H3K9me2 and Swi6 (Figure 3A). This result suggests that in addition to their roles at heterochromatin, SHREC components, notably Clr2, might have a role in chromatin organization at the *cnt* domain, the site of kinetochore assembly.

SHREC also localizes to a number of euchromatic sites (Figures 2 and 3B). These sites include protein-coding genes, noncoding RNAs, and intergenic regions. Additionally, SHREC associates with Tf2 retrotransposons and some solo LTRs sequences that are believed to be packaged into repressive chromatin (Hansen et al., 2005). We noticed that some SHREC components, in particular Clr3, localize to several sites seemingly unaccompanied by other components, which might reflect the dynamic nature of SHREC, or that these components can act independently in certain chromosomal contexts. However, the consistent colocalization patterns at most genomic locations among the four SHREC components, namely Clr1, Clr2, Clr3, and Mit1, but not Ccq1 due to its restricted binding profile at the chromosomal end regions, further support the notion that these proteins may constitute the core quartet of SHREC and that Ccq1, similar to Atf1/Pcr1 required for targeting Clr3 to a nucleation site at the *mat* locus (Yamada et al., 2005), mediates the recruitment of SHREC to the telomeric loci.

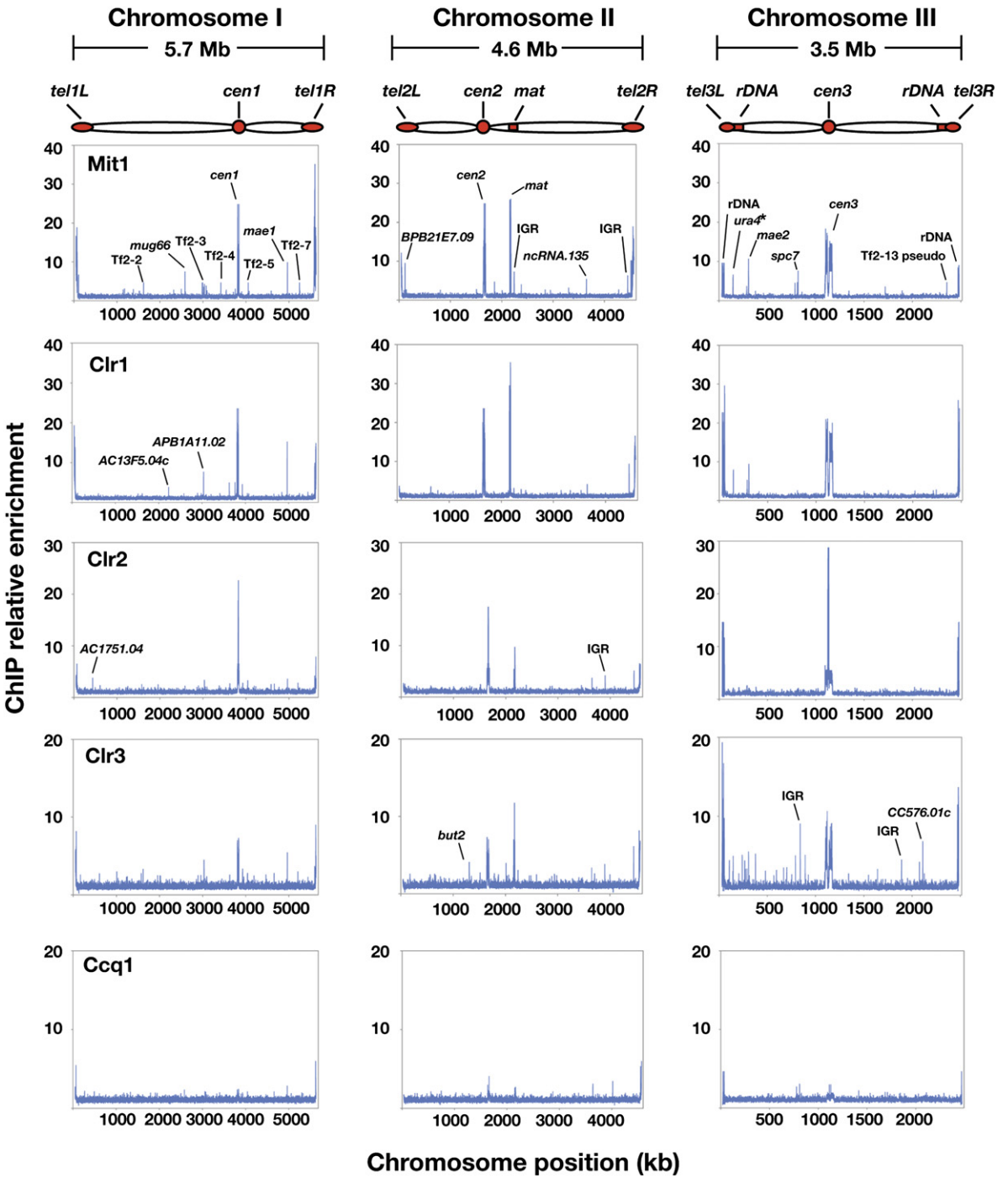

Figure 2. Genome-Wide Distribution Maps of Clr3 and Its Interacting Proteins

ChIP-chip analyses were performed to determine chromosomal distribution profiles of SHREC components. Relative enrichments of Mit1, Clr1, Clr2, Clr3, and Ccq1 are plotted in alignment with the map of each chromosome. Relative enrichment values shown are averages of results from two independent experiments. *ura4**, enrichment at *ura4*$^{+}$ locus, reflects crosshybridization to *otr1R::ura4*$^{+}$ present in strains used for ChIP analyses. *cen*, centromere; *mat*, the mating-type locus; *tel*, telomere.

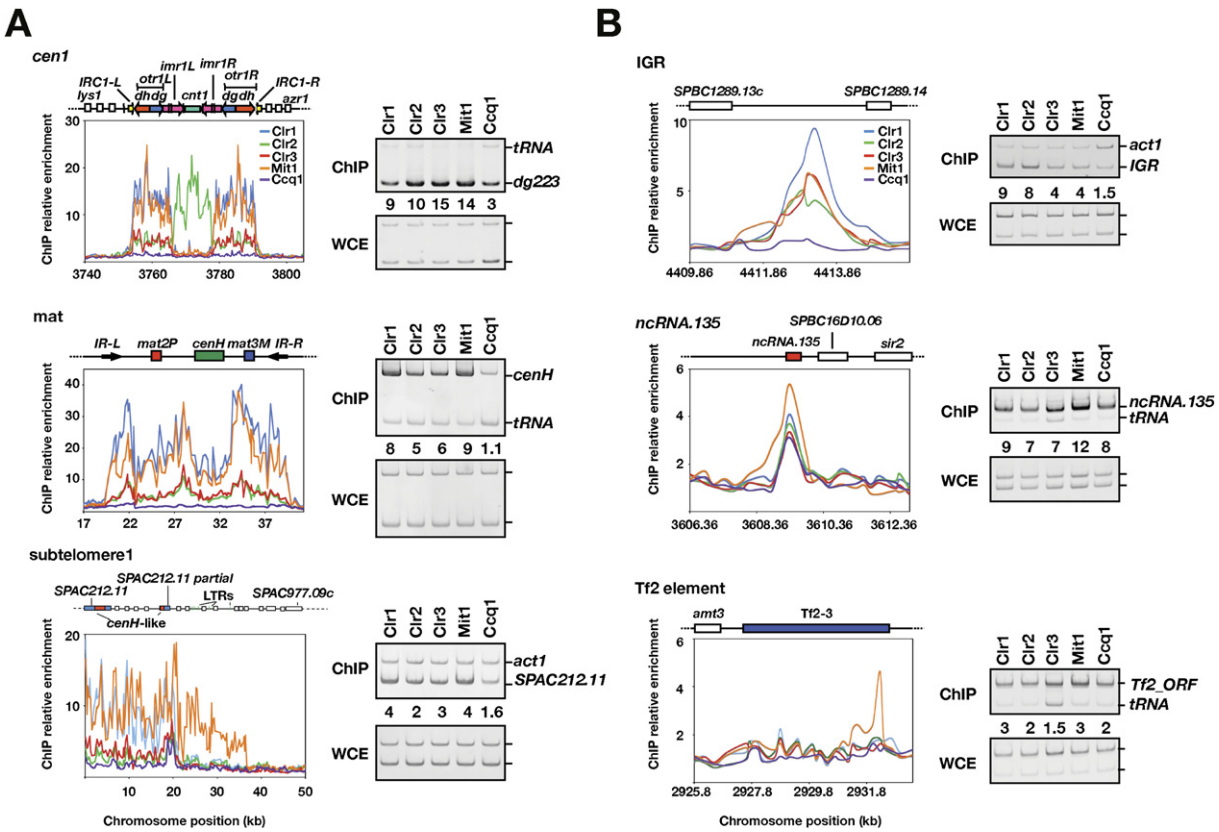

Figure 3. SHREC Localizes to Both Heterochromatic and Euchromatic Loci

(A and B) Distributions of SHREC components at major heterochromatic domains (A) and at euchromatic loci (B). Conventional ChIP analyses of Mit1, Clr1, Clr2, Clr3, and Ccq1 at different loci were performed to confirm results from ChIP-chip experiments. DNA isolated from chromatin-immunoprecipitated (ChIP) or whole-cell extract (WCE) fractions were analyzed by multiplex PCR with primers designed to amplify SHREC target sequences and the *act1* or *tRNA* controls. Intensity ratios of PCR products in ChIP and WCE lanes were used to calculate relative enrichment values shown below each lane (see Noma et al., 2001 for details). Sequences analyzed included the following: *dg* repeat (*dg223*), *cenH* element (*cenH*), a telomere-associated gene that contains a *dh*-like element fused to its open-reading frame (*SPAC212.11*), an intergenic region (*IGR*), noncoding RNA (*ncRNA*), and Tf2 retrotransposon (Tf2).

Ccq1 and Taz1 Cooperate to Recruit SHREC to the Telomere Ends

Fission yeast employs at least two pathways to maintain gene silencing at telomeres: Taz1, a telomere binding protein, and RNAi machinery that acts through *dh* repeat-like sequences embedded within subtelomeric regions (Cooper et al., 1997; Kanoh et al., 2005). Immunofluorescence analysis previously showed colocalization of Ccq1 with Taz1 to telomeres (Flory et al., 2004), and our ChIP analysis showed binding of Ccq1 to the telomere ends and, to lesser extent, subtelomeres (Figures 3 and S3). Since Ccq1 interacts with SHREC, we hypothesized that SHREC also localizes to the telomere ends. ChIP analyses revealed that all four core components of SHREC were enriched at telomeres (Figure 4A). Importantly, SHREC was highly enriched at the telomere-associated sequences (TAS), which are occupied by Taz1 (Kanoh et al., 2005). Moreover, SHREC localization pattern overlapped significantly with that of Taz1, suggesting that Taz1, in cooperation with Ccq1, could be responsible for recruiting SHREC to the telomere ends. Consistent with this idea, the levels of Clr3 at telomeres were reduced to the same extent in mutant strains disrupted for either Ccq1 or Taz1, while the levels of Ccq1 at telomeres, relative to those in wild-type cells, were unchanged in *clr3*Δ cells but decreased in *taz1*Δ cells (Figure 4B). However, Clr3 enrichment levels, though further reduced relative to those in single *ccq1* and *taz1* mutant cells, still remained at telomeres in *ccq1*Δ *taz1*Δ cells (Figure 4B). This result points to an additional pathway, such as RNAi, which has been implicated in telomeric silencing (Kanoh et al., 2005), operated in conjunction with the Taz1 pathway to recruit SHREC to telomeres. We found that Clr3 localization at telomere ends was completely abolished in cells defective in both Taz1 and RNAi pathways. However, defect in RNAi pathway had no impact on Ccq1 localization (Figure 4B). These results support the hypothesis of SHREC recruitment to the telomere ends by dual Taz1/RNAi pathways. While Taz1 seems to cooperate with Ccq1 to directly recruit SHREC, the RNAi pathway most likely acts indirectly

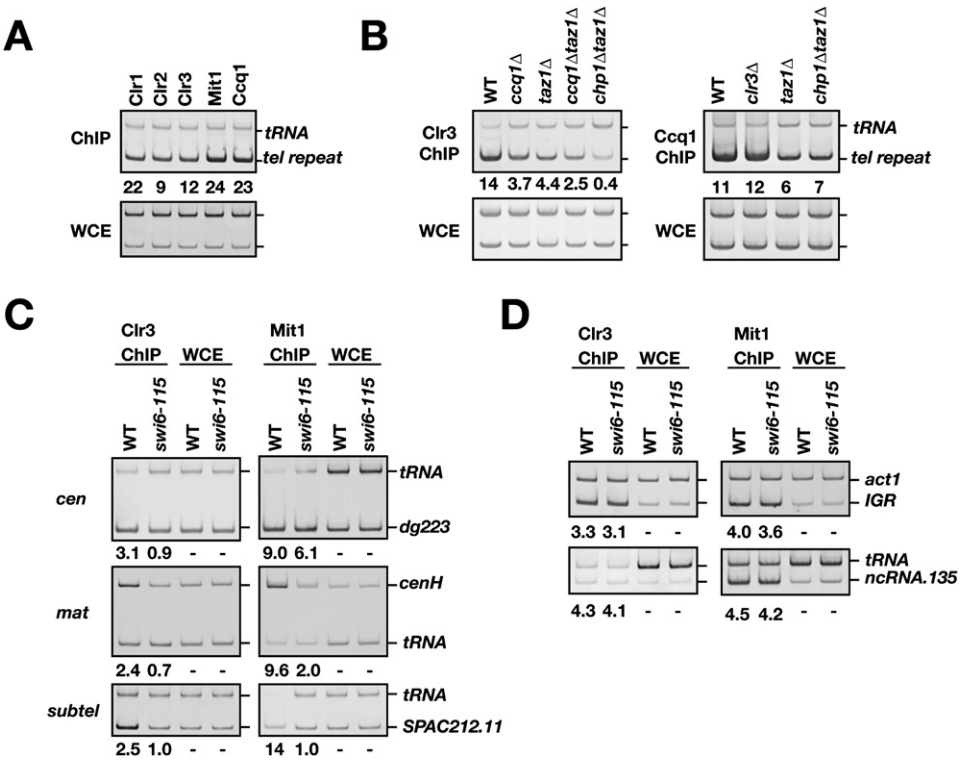

Figure 4. Swi6-Dependent and -Independent Binding of SHREC to Chromatin

(A) ChIP analysis of SHREC at a telomeric end of chromosome 1.

(B) Ccq1 and Taz1 contribute to Clr3 recruitment to telomere ends. Effects of indicated mutations on Clr3 (left panel) and Ccq1 (right panel) localizations at telomeric repeats were tested by ChIP.

(C) Stable binding of Clr3 and Mit1 to heterochromatic loci requires Swi6/HP1.

(D) Clr3 and Mit1 localize to euchromatic regions independently of Swi6/HP1. Intensity ratio of PCR products in ChIP and WCE lanes were used to calculate relative enrichment values shown below each lane. A mitochondrial *tRNA* or *act1* gene was used as a control in ChIP assays.

by targeting H3K9me and Swi6, which in turn serve as a platform for SHREC localization (see Discussion).

Swi6-Dependent and -Independent Localization of SHREC to Chromatin

Clr3 can be recruited to the *mat* locus via the Atf1/Pcr1 pathway independent of Swi6 but needs to interact with Swi6 to spread throughout the silent *mat* interval (Yamada et al., 2005). We determined whether similar mechanisms operate to localize SHREC to other heterochromatic and euchromatic loci. Levels of Clr3 and Mit1 were dramatically reduced at subtelomeres in *swi6* mutant strains (Figure 4C), indicating that localization of SHREC across subtelomeric regions also requires Swi6. Moreover, although our analysis showed reduction in Clr3 and Mit1 localization at centromeric repeats, Mit1 binding could still be detected (Figure 4C). These data are consistent with the idea that SHREC components are recruited to centromeric repeats independent of Swi6, but their stable binding and spreading requires Swi6. In contrast to heterochromatic loci, SHREC recruitment to euchromatic sites was unaffected in the absence of Swi6, as shown by Clr3 and Mit1 localization at a locus encoding a noncoding RNA and an intergenic region (Figure 4D). These results indicate the existence of distinct mechanisms for localizing SHREC to heterochromatin and euchromatin. Whereas SHREC localization at heterochromatic loci involves a combination of sequence-specific targeting machinery and the sequence-independent spreading via the H3K9me-Swi6 platform, euchromatic loci seem to rely solely on the Swi6-independent mechanism to recruit SHREC to specific sites.

SHREC Is an Effector Complex for Silencing Transcription at Heterochromatic Loci

The ubiquitous presence of SHREC at all heterochromatin domains indicated possible involvement of SHREC in silencing heterochromatic transcription. Indeed, reporter genes inserted at major heterochromatin domains including pericentromeric repeats, the silent *mat* locus, telomeres, and rDNA loci were derepressed in strains lacking any individual core component of SHREC (Figure 5A). Deletion of *ccq1*, however, affected silencing of markers inserted at telomeres, consistent with its localization pattern (Figure 2), but any detectable silencing defects at other heterochromatic loci were not observed (Figure 5A).

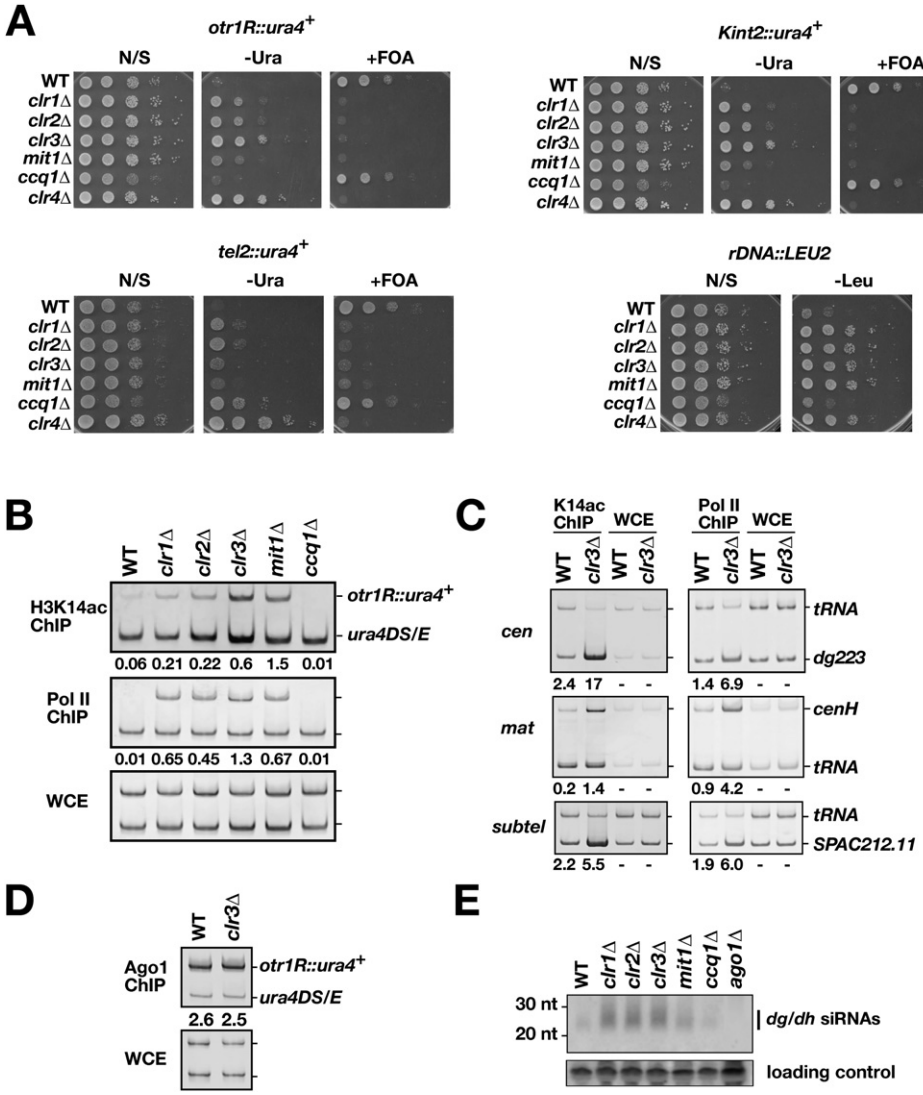

Figure 5. SHREC Is Essential for Heterochromatic Transcriptional Silencing

(A) Silencing defects of reporters inserted at pericentromeric repeats (*otr1R::ura4*$^+$), silent *mat* locus (*Kint2::ura4*$^+$), telomeric region (*tel2::ura4*$^+$), or tandem rDNA repeats (*rDNA::LEU2*) in SHREC mutants were examined by serial dilution analyses on nonselective (N/S), uracil-lacking (-Ura), and FOA-containing (+FOA) medium.

(B) H3K14ac and Pol II occupancy at *otr1R::ura4*$^+$ are enhanced in SHREC mutants. ChIPs were carried out using anti-H3K14ac and anti-Pol II antibodies.

(C) Heterochromatic repeats show elevated levels of H3K14ac and Pol II in *clr3*Δ background.

(D) Deletion of *clr3* does not affect RITS localization at *otr1R*::*ura4*$^+$. Cells expressing tagged Ago1 (myc-Ago1) were used to perform ChIPs. Comparable levels of Ago1 at *otr1R::ura4*$^+$ were detected in wild-type and *clr3*Δ cells.

(E) Deletions of SHREC components result in elevated levels of siRNAs corresponding to *dg* and *dh* repeats. siRNAs from each strain were analyzed by northern blot analysis with a probe specific for *dg*/*dh* sequences.

The weak telomeric silencing defect observed in *ccq1*Δ cells is most likely due to redundant pathways contributing to recruitment of SHREC to telomeres (Figure 4).

In *S. pombe*, Pol II localizes to heterochromatin to direct transcription of repeat elements, the transcripts of which serve as substrates for production of siRNAs (Cam et al., 2005; Djupedal et al., 2005; Kato et al., 2005). However, many heterochromatin functions require the assembly of condensed chromatin, which limits Pol II occupancy to repeat elements. Mutations in Clr3 or defects in its localization (e.g., in *swi6* mutant cells) correlate with increases in histone acetylation and Pol II levels at heterochromatic loci (Yamada et al., 2005). Because Clr3 is a core component of SHREC, we hypothesized that Clr3 affects heterochromatic silencing primarily through SHREC, and defects in other core components of SHREC would similarly

destabilize heterochromatin. Indeed, we found increases in H3K14ac levels and greater Pol II occupancy at the reporter embedded within pericentromeric heterochromatin (*otr1R::ura4*$^{+}$) in mutant strains lacking SHREC components (Figure 5B). As expected, *ccq1Δ* did not affect H3K14ac or Pol II occupancy at *otr1R::ura4*$^{+}$ (Figure 5B). The resultant "open" chromatin structures, due to impaired SHREC function, also allowed enhanced Pol II occupancy at naturally silenced heterochromatic repeats, as indicated by increased levels of Pol II and H3K14ac at these sequences (Figure 5C). Based on these analyses, we conclude that SHREC serves as an effector complex for transcriptional silencing of heterochromatic repeats by limiting the occupancy of Pol II machinery.

SHREC Is Not Required for RNAi-Mediated Posttranscriptional Silencing In *Cis*

In addition to the transcriptional regulatory activity of SHREC, RNAi machinery processes repeat transcripts into siRNAs (Cam et al., 2005; Motamedi et al., 2004; Noma et al., 2004; Sugiyama et al., 2005; Verdel et al., 2004). We investigated whether SHREC affects RNAi-mediated silencing of heterochromatic repeats. The role of SHREC in transcriptional silencing could be decoupled from the *cis*-PTGS function of the RNAi machinery since impaired SHREC had no effect on the localization of RITS Ago1 subunit at heterochromatin (Figure 5D). Enhanced transcriptional-machinery occupancy at heterochromatic repeats in SHREC defective cells, however, should result in elevated repeat transcripts and, thus, corresponding increase in siRNA production. Indeed, we observed increased levels of siRNAs corresponding to centromeric repeats in SHREC mutant strains (Figure 5E).

Clr3 and Mit1 Activities Are Required for Transcriptional Regulatory Function of SHREC

We next explored whether SHREC mediates transcriptional silencing through enzymatic activities associated with Clr3 and Mit1, HDACs, and SNF2 ATPase homologs, respectively. For this purpose, we employed yeast strains that carry a substitution mutation either in the HDAC domain of Clr3 (Clr3^{D232N}) or the ATP binding domain of Mit1 (Mit1^{K587A}). These mutations have no effect on the protein levels of Clr3 (Yamada et al., 2005) and Mit1 (Figure S4). However, both *clr3*^{D232N} and *mit1*^{K587A} mutant alleles alleviated silencing of a marker gene inserted at pericentromeric repeats (Figure 6A), and at naturally silenced repeat elements embedded within heterochromatin domains at the *mat* locus (*cenH*), centromeres (*dg223*) and subtelomeres (*SPAC212.11*) (Figure 6B). Loss of silencing occurred at the transcriptional level since we detected elevated levels of both Pol II and H3K14ac at repeat elements in the mutant strains (Figure 6C). To test whether Clr3 has HDAC activity and whether the D232N substitution abolishes this activity, we affinity-purified both Clr3 and Clr3^{D232N} and measured their HDAC activities. Whereas Clr3 activity, which is sensitive to trichostatin A (TSA), was readily detected, Clr3^{D232N} mutant is defective in HDAC activity (Figures 6D and S5). Similarly, while ATPase activity could be detected in wild-type Mit1 protein, this activity is abrogated in the Mit^{K587A} mutant (Figure S6). We also noticed that Mit1-K587A mutation did not affect Clr3 activity in vitro (Figure 6D). These results suggest that HDAC and ATP-dependent nucleosome-remodeling factors might act in concert via SHREC to maintain sequences coated with heterochromatin in a repressed state.

SHREC Regulates Chromatin Organization at a Heterochromatin Domain

Based on results showing that Clr3 and Mit1 activities are critical for heterochromatic TGS, we hypothesized that SHREC might be involved in facilitating proper positioning of nucleosomes required to assemble repressive chromatin. We explored this possibility by comparing micrococcal nuclease (MNase) digestion patterns at the silent *mat* region in wild-type, *clr3*^{D232N}, and *mit1*^{K587A} strains. Our analysis revealed dramatic changes in MNase digestion patterns across a broad region consistent with altered nucleosome positioning in strains defective in either Clr3 or Mit1 activity (Figure 6E). Notably, in mutant strains, there were pronounced differences in MNase digestion patterns near *CAS*, an Atf1/Pcr1-dependent recruiting site for Clr3 to the *mat* locus (Yamada et al., 2005). SHREC also affects nucleosome positioning at a region downstream of the *CAS* toward *mat2P*, consistent with the ubiquitous presence of SHREC and its critical role in maintaining TGS across this region. Mutant cells also displayed noticeable differences in MNase digestion pattern at *mat3M*, the silencing of which requires SHREC components (Ekwall and Ruusala, 1994; Thon et al., 1994; Thon and Klar, 1992). Despite similar overall changes in nucleosome positioning patterns in both mutants, we noticed that chromatin structure was relatively less perturbed in *mit1*^{K587A} cells than in *clr3*^{D232N} cells (Figure 6E). This result is in agreement with a slightly weaker silencing defect observed in *mit1*^{K587A} cells than in *clr3*^{D232N} (Figure 6A), indicating involvement of additional redundant remodeling factors in heterochromatic silencing. Taken together, these data suggest that a critical aspect of condensed heterochromatin formation involves proper positioning of nucleosomes by activities such as SHREC.

DISCUSSION

Heterochromatin possesses a remarkable ability to repress transcription and recombination across large chromosomal domains. The binding of HP1 to H3K9me and their subsequent spreading across the entire heterochromatin domain are critical for heterochromatin to exert long-range repressive effects. The oligomerization of HP1 may mediate chromatin condensation (Grewal and Elgin, 2002). However, recent evidence, especially from fission yeast, has illuminated a more complex heterochromatin landscape, with Swi6/HP1 not only being dynamically bound to heterochromatin (Cheutin et al., 2004;

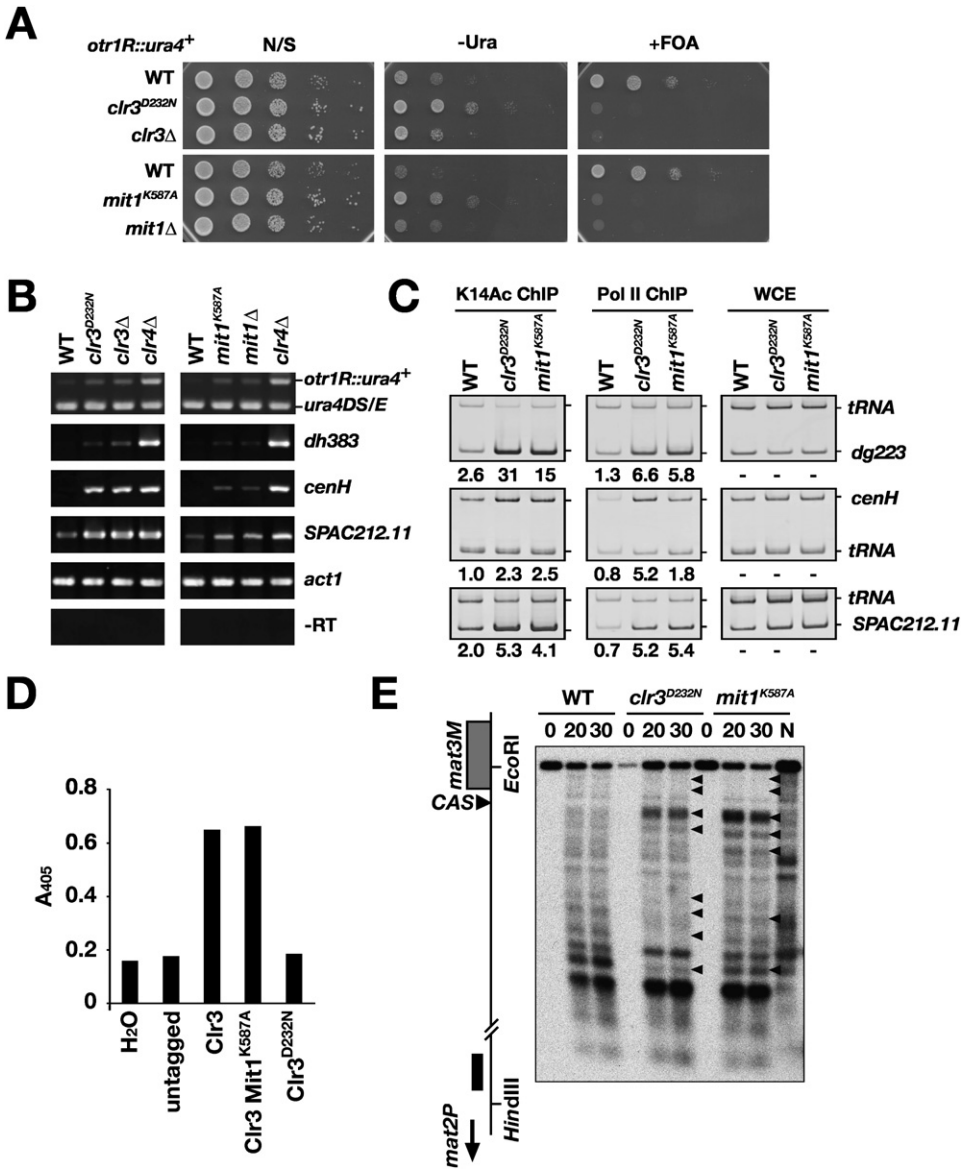

Figure 6. Clr3 and Mit1 Activities Are Essential for SHREC-Mediated Transcriptional Silencing

(A) Mutation in Clr3-HDAC domain ($Clr3^{D232N}$) or Mit1 ATP binding domain ($Mit1^{K587A}$) results in loss of *otr1R*::*ura4*$^+$ silencing.

(B) Mutant strains show accumulation of transcripts derived from heterochromatic repeats as measured by RT-PCR analysis. Primers used were *dh* repeats (*dh383*), *cenH* element (*cenH*), and telomere-associated *SPAC212.11* locus (*SPAC212.11*). *act1* was used as a control. *otr1R*::*ura4*$^+$ expression was also analyzed and compared with an endogenous *ura4* carrying a small deletion (*ura4DS/E*). -RT, no reverse transcription.

(C) $Clr3^{D232N}$ and $Mit1^{K587A}$ mutants show elevated levels of H3K14ac and Pol II occupancy at heterochromatic repeats, as determined by ChIP assays.

(D) Clr3 has HDAC activity that is abolished by $Clr3^{D232N}$ mutation. Purified Clr3 from cells expressing either Clr3-FLAG or $Clr3^{D232N}$-FLAG was used to perform HDAC assay. Effect of *mit1*^{K587A} mutation on Clr3-HDAC activity was investigated by purifying Clr3-FLAG from cells expressing $Mit1^{K587A}$ protein.

(E) Clr3 and Mit1 activities are critical for proper positioning of nucleosomes at the silent *mat* locus. MNase-treated chromatin fractions from indicated strains were digested with EcoRI and HindIII and then analyzed by Southern blotting with a *mat*-specific probe (black bar). Lane N indicates naked DNA treated with MNase. The diagram shows the positions of restriction enzyme sites, *CAS* (Clr3 attracting sequence) and the *mat3M* locus. Arrows indicate MNase sensitive or insensitive sites in mutant strains.

Festenstein et al., 2003) but also acting as recruiting platform for factors that can facilitate or restrict transcription (Yamada et al., 2005; Zofall and Grewal, 2006). Heterochromatin also allows RNAi machinery to become stable component of extended domains to actively target repeat-sequence-derived transcripts for degradation (Cam

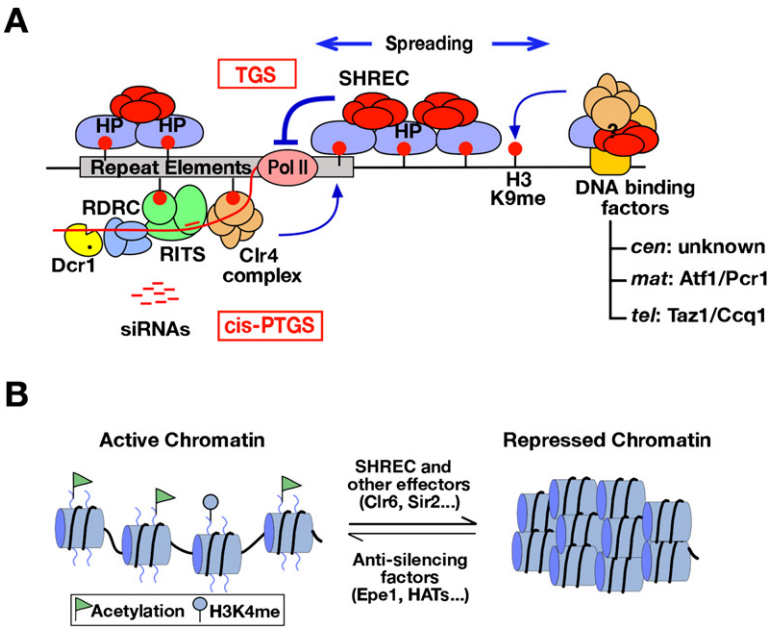

Figure 7. Models Showing Mechanisms of SHREC Recruitment to Heterochromatic Loci and the Role of SHREC in Assembly of Higher-Order Chromatin Structures

(A) Distinct DNA binding factors recruit SHREC to different heterochromatic regions. RNAi machinery (such as RITS, RDRC, and Dicer), which target transcripts produced by *dg* and *dh* elements present at all major heterochromatic loci to generate siRNAs, and chromatin bound SHREC along with other factors independently target Clr4 complex to methylate H3K9. H3K9me not only facilitates stable tethering of RNAi machinery to chromatin via RITS but also allows heterochromatin proteins (HPs) such as Swi6 and Chp2 to be recruited to chromatin. H3K9me bound HPs create a self-assembling platform that allows spreading of SHREC across the entire domain to affect heterochromatic TGS.

(B) SHREC via activities associated with Clr3 and Mit1 facilitates organization of nucleosomes into condensed higher-order structures that are essential for various heterochromatin functions. Other chromatin-modifying activities such as HDACs and nucleosome-remodeling factors also likely participate in assembly of higher-order chromatin structures. Chromatin state is determined by dynamic balance between activities that lead to the assembly of nucleosomes into condensed structures and activities associated with anti-silencing factors such as Epe1 and HATs that favor the disassembly of condensed structures to a more open chromatin.

et al., 2005). Although these studies have considerably advanced our understanding of heterochromatin, it is still not clear how condensed heterochromatic structures refractory to transcription and other factors are established. Here we describe the identification of SHREC as a TGS effector complex that has an important role in shaping the heterochromatin landscape. SHREC localizes throughout heterochromatin domains and to various genetic elements dispersed across euchromatic regions. Moreover, SHREC restricts the occupancy of transcriptional machinery that depends upon its two distinct activities associated with Clr3 and Mit1. These analyses suggest that SHREC functions as a versatile effector complex that could be targeted to different loci via specialized recruitment mechanisms to assemble repressive higher-order chromatin structures.

SHREC Recruitment to Heterochromatic and Euchromatic Loci

Our analyses suggest the involvement of distinct mechanisms for recruiting SHREC to different genomic locations. At major heterochromatic loci where transcription and recombination are repressed across large regions, the stable binding of SHREC requires Swi6/HP1, which is known to coat these domains (Cam et al., 2005; Kanoh et al., 2005; Noma et al., 2001; Partridge et al., 2000). However, SHREC recruitment to heterochromatic loci is not solely dependent on Swi6/HP1. SHREC can also be targeted to heterochromatic regions via sequence-specific DNA binding proteins such as Atf1/Pcr1, independent of Swi6/HP1 (Yamada et al., 2005). We find that both Swi6-dependent and -independent mechanisms mediate SHREC recruitment to all major heterochromatic loci (Figure 7A).

Telomeres are specialized structures that protect chromosome ends. It has been shown previously that Taz1, a telomere binding protein, contributes to heterochromatin assembly (Cooper et al., 1997; Kanoh et al., 2005). However, how Taz1 mediates heterochromatin formation and silencing was not known. Our results suggest Taz1-dependent recruitment of SHREC to the telomere ends as one likely mechanism of telomeric silencing. Taz1 appears to function cooperatively with another telomere binding protein Ccq1 to recruit SHREC to telomeric loci. Although it is still unknown whether Taz1 interacts directly with SHREC, we found that Ccq1 associates with Clr3 and likely is directly involved in recruiting SHREC to telomeres. Once recruited, SHREC components may cooperate with DNA binding factors to facilitate the recruitment of Clr4, as suggested previously (Yamada et al., 2005), resulting in H3K9me and Swi6 recruitment, thus establishing a platform for the spreading of SHREC across an entire domain. Additionally, RNAi factors acting through *dh*-like elements (*SPAC212.11*) recruit H3K9me and Swi6, which in turn could also mediate the recruitment and spreading of SHREC. Consistent with this argument, we find that Taz1/Ccq1 acts in a parallel pathway to RNAi to recruit SHREC to subtelomeric regions, and SHREC spreading throughout heterochromatin domains requires Swi6. Taz1/Ccq1- and RNAi-based recruitments of SHREC at telomeres resemble mechanisms operating at the silent

mat region, at which Atf1/Pcr1 and RNAi machinery independently nucleate heterochromatin (Jia et al., 2004a; Noma et al., 2004). While Atf1/Pcr1 targets Clr3 to a specific site, H3K9me-Swi6 platform established by both DNA- and RNAi-based mechanisms is essential for spreading of Clr3 across entire heterochromatin domains (Yamada et al., 2005). Similar DNA-based mechanism(s) operated in conjunction with the RNAi machinery likely mediates SHREC recruitment to rDNA arrays and pericentromeric regions.

SHREC also localizes to several sites dispersed across euchromatic regions of the *S. pombe* genome, which include protein-coding genes, noncoding RNAs, and Tf2 retrotransposons. However, the mechanism(s) of SHREC recruitment to these loci is not known. One component of SHREC, Clr1, contains Zn fingers that could potentially bind DNA to target SHREC to different loci. Moreover, PHD domains have been shown to specifically bind H3K4 di- and trimethylation (Mellor, 2006). Thus, it is possible that Mit1 mediates SHREC recruitment to chromatin via its PHD domain.

Our analyses also revealed an unexpected enrichment of Clr2 at the *cnt* domain of centromeres. The recruiting mechanism and the role of Clr2 at *cnt* domain are not known. Clr2 could contribute to histone modifications such as H4 hypoacetylation (Hayashi et al., 2004; Walfridsson et al., 2005), which is known to impact kinetochore assembly, by acting as a loading platform for recruitment of other SHREC components whose localization at *cnt* may be cell-cycle regulated.

Possible Mechanism for SHREC-Mediated Transcriptional Silencing

Studies from several different systems have suggested that, in general, heterochromatic sequences are less accessible to factors involved in different aspects of DNA transactions (Eissenberg, 2001; Sun et al., 2001). Moreover, Elgin and colleagues have demonstrated that the formation of condensed heterochromatic structures correlates with the establishment of regularly spaced nucleosomal arrays indicative of higher-order chromatin organization (Sun et al., 2001). While long-range nucleosome ordering associated with heterochromatin packaging is known to require HP1 (Cryderman et al., 1998), the molecular mechanism responsible for these structural changes, however, remains undefined. In this regard, it is interesting that SHREC contains both HDAC (Clr3) and SNF2 family ATPase (Mit1). Our analyses suggest that SHREC distributed across heterochromatin domain via H3K9me-Swi6/HP1 platform mediates the assembly of condensed chromatin by facilitating organization of nucleosomes into higher-order structures (Figure 7B). This process depends upon activities associated with both Clr3 and Mit1 since mutations in these factors that disrupt their functions also abolish heterochromatic silencing and alter nucleosome positioning, resulting in "open" chromatin structures.

The HDAC activity of Clr3, in principle, could be required for establishing "histone code" for recruitment of Swi6/HP1. Our previous analysis, however, has shown that acetylation of H3K14, which is the main target of Clr3 (Wiren et al., 2005), does not directly interfere with Swi6 binding to H3K9me2 or H3K9me3 peptides (Yamada et al., 2005). Nonetheless, since hyperacetylated histones can disrupt higher-order chromatin compaction (Tse et al., 1998), it is possible that removal of acetyl group from histones by Clr3 is critical for Mit1 function and intramolecular nucleosome interactions required for higher-order chromatin organization. Indeed, it has been shown recently that histone acetylation inhibits both ACF-mediated nucleosome sliding and the folding of nucleosome arrays in vitro (Shogren-Knaak et al., 2006). The assembly of higher-order structures, therefore, may require concerted action of HDACs and nucleosome-remodeling enzymes operating in tandem. SHREC may be the cellular apparatus that could fulfill such a task critical for transcriptional silencing. In this model, heterochromatin defects caused by H3K14 to alanine substitution (Mellone et al., 2003), which mimics H3K14ac, could be manifestation of defects in nucleosome packaging.

Given that SHREC components and RNAi machinery both contribute to stabilizing heterochromatic structures, small RNAs produced in *cis* could have a structural role in further compaction of higher-order structures. As such, similar defects in maintenance of higher-order structures in pericentromeric heterochromatin were observed in mouse cells subjected to RNase treatment or HDAC inhibitors (Maison et al., 2002), and siRNAs contribute to higher-order chromatin organization in *S. pombe* and *Drosophila* (Grimaud et al., 2006; Hall et al., 2003; Sugiyama et al., 2005).

In addition to transcriptional silencing, SHREC-mediated chromatin organization is expected to have wide-ranging implications for other chromosomal processes. SHREC via its activities may orchestrate assembly of specialized structures essential for centromere and telomere functions (Kanoh and Ishikawa, 2003; Pidoux and Allshire, 2004). SHREC components are also required for the developmentally controlled choice of donor mating-type cassettes during the switching process and for recombination suppression considered necessary to prevent ectopic recombination between repeat elements (Hall and Grewal, 2003; Jia et al., 2004b; Thon et al., 1994).

Relationship of SHREC-Mediated Heterochromatic Repression to Other Systems

Multienzyme SHREC complex containing HDAC and chromatin remodeling factors is reminiscent of the Mi2/NuRD (nucleosome-remodeling histone deacetylase) silencing complex, which, among other factors, contains HDAC1/2 and SNF2 ATPase Mi2 (Bowen et al., 2004; Feng and Zhang, 2003). Similar to SHREC, targeting of Mi2/NuRD to its target loci is also mediated by multiple recruitment mechanisms. Besides sequence-specific DNA binding factors, both complexes utilize sequence-independent platforms for binding to chromatin (Schultz

et al., 2002; Yamada et al., 2005; this study). While H3K9me-Swi6 provides a loading platform for SHREC, methylated DNA bound by MBD (methyl-CpG binding domain) proteins is believed to target NuRD (Bowen et al., 2004). NuRD has been shown to maintain epigenetic silencing of specific genes (Kehle et al., 1998). However, the precise contribution of HDAC and remodeling activities of NuRD in gene silencing in vivo, and their role in assembly of constitutive heterochromatin are not well understood. To this end, our analysis suggests that activities associated with both Clr3 and Mit1 are required for SHREC-mediated heterochromatic silencing.

Several SNF2-related proteins, possessing similarity to Mit1, have been shown to play an important role in silencing, especially silencing induced by DNA methylation in higher eukaryotes. For instance, ATRX, which possesses a PHD-like Zn finger and SNF2-like ATPase domains, localizes to repetitive sequences such as pericentromeric heterochromatin and rDNA arrays and is required for maintenance of DNA methylation at repeated sequences (Gibbons et al., 2000). In *Arabidopsis*, mutations in SNF2 (DDM1 and DRD1) and HDAC (HDA6) proteins result in the upregulation of transposable elements and a certain subset of endogenous genes (Aufsatz et al., 2002; Chan et al., 2006; Huettel et al., 2006; Kanno et al., 2004, 2005; Lippman et al., 2003). While DRD1 is essential for RNA-directed de novo DNA methylation (Huettel et al., 2006; Kanno et al., 2004), DDM1 cooperates with HDA6 to stably maintain heterochromatin at domains containing transposable elements (Aufsatz et al., 2002; Lippman et al., 2003). DDM1 and HDA6 could also form a complex similar to SHREC that not only regulates heterochromatic silencing but is also targeted to euchromatic targets including retrotransposons and LTRs located near genes. In mammals, loss of Lsh/HELLS, closely related to DDM1, results in hyperacetylation of histones and substantial DNA hypomethylation at repetitive sequences such as the long interspersed element (LINE) and short interspersed element (SINE) (Dennis et al., 2001). These findings suggest that transposon suppression by SNF2 and HDAC may be conserved from fission yeast to mammals. Further analysis of SHREC-mediated higher-order chromatin assembly in fission yeast may have direct implications for mechanisms of heterochromatic silencing in higher eukaryotes.

EXPERIMENTAL PROCEDURES

Strains

Strains expressing epitope-tagged proteins (Clr1-[13x]myc, Clr3-[3x]FLAG, Clr3^{D232N}-[3x]FLAG, Ccq1-GFP and Mit1-[13x]myc) and deletion strains (*clr1*Δ, *clr2*Δ, *clr3*Δ, *ccq1*Δ, and *mit1*Δ) were constructed by a PCR-based method. To construct a strain expressing Clr2 tagged at its N terminus, a DNA fragment containing 5′UTR of *clr2*$^+$, V5 epitope tag, and *clr2* ORF was used to replace the endogenous *clr2*. The resultant strain expresses V5-Clr2 under its native promoter. To generate *mit1*^{K587A} mutant, wild-type strain was transformed with a DNA fragment consisting of mutated *mit1* fragment fused to myc tag and *Kan*^{R} cassette. The mutation was confirmed by DNA sequencing.

Immunoaffinity Purification

Whole-cell extracts (WCEs) from untagged control or Clr3-FLAG were purified by FLAG immunoaffinity purification protocol as described (Jia et al. 2005). The eluted fractions were visualized by silver staining and subjected to mass spectrometry.

Immunoprecipitation

WCEs from strains expressing tagged proteins were incubated with anti-myc (A-14, SantaCruz), anti-V5 (A190-120A, Bethyl), or anti-GFP (ab290, Abcam) antibody. Bound proteins were recovered by adding protein G sepharose beads. After washing, eluted proteins were subjected to western blotting using anti-FLAG M2 antibody (Sigma).

ChIP and ChIP-Chip Analysis

ChIP and ChIP-chip were carried out as previously described (Cam et al., 2005) using anti-myc (A-14, Santa Cruz), anti-V5 (ab9116, Abcam), anti-GFP (ab290, Abcam), anti-Pol II (8WG16, Covance) or anti-histone H3 K14ac (07-353, Upstate) antibody. Microarray data can be accessed at National Cancer Institute (http://pombe.nci.nih.gov/) and at NCBI GEO under the accession number GSE6568.

RNA Analyses

Total RNA samples were subjected to RT-PCR using Onestep RT-PCR kit (Qiagen). For siRNA detection, small RNAs (< 200 nt) were purified with mirVana miRNA isolation kit (Ambion). Twenty micrograms of small RNA were resolved on a 15% denaturing acrylamide gel and subjected to northern blotting analysis with ^{32}P-labeled single-stranded RNA probes (~50 nt) corresponding to *dg* and *dh* repeats.

HDAC and Nucleosome-Mapping Assays

Clr3-FLAG, Clr3^{D232N}-FLAG or Clr3-FLAG mit1^{K587A} were affinity purified using anti-FLAG M2 antibody (Sigma), and the purified fractions were subjected to HDAC activity assay using a colorimetric HDAC activity assay kit (BioVision). Nucleosome mapping was carried out by MNase digestion assay as described previously (Jia et al., 2004b).

Supplemental Data

Supplemental Data include six figures and can be found with this article online at http://www.cell.com/cgi/content/full/128/3/491/DC1/.

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