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The Nucleation and Maintenance of Heterochromatin by a Histone Deacetylase in Fission Yeast

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Summary

Posttranslational modifications of histones play an essential role in heterochromatin assembly. Whereas the role of Clr4/Suv39h-mediated methylation of histone H3 at lysine 9 (H3K9) in heterochromatin assembly is well studied, the exact function of histone deacetylases (HDACs) in this process is unclear. We show that Clr3, a fission yeast homolog of mammalian class II HDACs, acts in a distinct pathway parallel to RNAi-directed heterochromatin nucleation to recruit Clr4 and mediate H3K9 methylation at the silent mating-type region and centromeres. At the *mat* locus, Clr3 is recruited at a specific site through a mechanism involving ATF/CREB family proteins. Once recruited, Clr3 spreads across the 20 kb silenced domain that requires its own HDAC activity and heterochromatin proteins including Swi6/HP1. We also demonstrate that Clr3 contributes to heterochromatin maintenance by stabilizing H3K9 trimethylation and by preventing histone modifications associated with active transcription, and that it limits RNA polymerase II accessibility to naturally silenced repeats at heterochromatin domains.

Introduction

The organization of genetic information into distinct chromatin domains plays a critical role in all aspects of DNA biology in eukaryotes. Chromosomes are organized into two major types of chromatin domains, gene-rich and transcriptionally competent euchromatin and gene-poor and transcriptionally repressed heterochromatin. Except for certain interstitial sites on the chromosome arms, heterochromatin is generally associated with repetitive DNA sequences present at centromeres and telomeres and has crucial roles in maintaining genome integrity, regulation of gene expression, and nuclear organization (Hall and Grewal, 2003; Jia et al., 2004b; Cam et al., 2005). Genetic and biochemical studies in several different organisms have shed considerable light on the establishment and maintenance of heterochromatin domains. Importantly, histones and their posttranslational modifications have emerged as key mediators of heterochromatin assembly, involving hypoacetylation of histone tails and, in particular, H3K9 methylation (Ekwall, 2004; Grewal and Rice, 2004; Kurdistani and Grunstein, 2003).

In the fission yeast *Schizosaccharomyces pombe*, large blocks of heterochromatin associated with centromeres, telomeres, and the silent mating-type regions are essential for functional organization of these chromosomal domains. Heterochromatin, assembled at the tandem and inverted arrays of centromeric *dg* and *dh* repeats that surround the unique central core, mediates the recruitment of cohesin that is important for centromeric cohesion and proper segregation of chromosomes (Cam et al., 2005; Ekwall, 2004). Similarly, formation of heterochromatin across a 20 kb domain at the mating-type region containing the *mat2* and *mat3* donor loci and the interval between them, known as *K*-region (*mat2/3* region), is critical for transcriptional silencing and suppression of recombination (Grewal and Klar, 1997). Significantly, heterochromatin promotes communication between distal chromosomal regions by facilitating long-range chromatin interactions that influence nonrandom selection of *mat2* and *mat3* donor loci during mating-type switching (Jia et al., 2004b).

Several factors involved in heterochromatin formation have been identified in fission yeast. These factors include histone-modifying enzymes and structural heterochromatin proteins. Clr3 and Clr6 are homologous to mammalian class II and class I histone deacetylases (HDACs), respectively, while spSir2 belongs to a special class of deacetylases requiring the nicotinamide adenine dinucleotide (NAD^+) cofactor for its catalytic activity (Ekwall and Ruusala, 1994; Thon et al., 1994; Grewal et al., 1998; Shankaranarayana et al., 2003; Freeman-Cook et al., 2004). Clr4, an H3K9-specific methyltransferase and member of the highly conserved Suv39h protein family, has been implicated in silencing and heterochromatin formation in several different organisms (Ivanova et al., 1998; Rea et al., 2000; Nakayama et al., 2001). Deacetylases and methyltransferases cooperate to establish a specific histone modification pattern that is essential for recruitment of heterochromatin proteins. In particular, H3K9me serves as a binding site for chromodomain proteins such as Swi6, Chp1, and Chp2, the chromosomal association of which is important for heterochromatin formation (Bannister et al., 2001; Nakayama et al., 2001; Partridge et al., 2002; Sadaie et al., 2004; Cam et al., 2005).

Recent studies have suggested the involvement of the RNAi machinery in targeting heterochromatin to specific chromosomal loci in *S. pombe* (Hall et al., 2002; Volpe et al., 2002) and other organisms (reviewed in Matzke and Birchler [2005]). Double-stranded RNAs produced either via bidirectional transcription of repetitive elements or with the help of RNA-dependent RNA polymerases (Rdp1) are processed into small interfering RNAs (siRNAs) by the RNaseIII-like ribonuclease Dicer (Dcr1). siRNAs, known to associate with the RNA-induced initiation of transcriptional silencing (RITS) complex consisting of Chp1, Ago1, and Tas3, are thought to provide locus specificity for guiding heterochromatin

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and RNAi machineries to cognate repeated sequences (Noma et al., 2004; Verdel et al., 2004). At the mating-type region, the *cenH* DNA element, which shares 96% sequence identity with centromeric repeats (Grewal and Klar, 1997), is an RNAi-dependent heterochromatin nucleation center (Hall et al., 2002; Noma et al., 2004). In addition to the RNAi pathway, the ATF/CREB family proteins Atf1 and Pcr1, which specifically localize to a region containing two heptamer binding sites near the *mat3* locus, are capable of initiating heterochromatin assembly (Jia et al., 2004a; Kim et al., 2004). Atf1/Pcr1 and the RNAi machinery act in parallel pathways to nucleate H3K9me, which then spreads across the entire silent mating-type interval surrounded by boundary elements (*IR-L* and *IR-R*) (Noma et al., 2001; Jia et al., 2004a). Despite noticeable progress in understanding the mechanism of heterochromatin assembly, several key issues related to the targeting of chromatin-modifying activities and their precise function remain unresolved.

In this study, we investigate the role of the Clr3 HDAC in heterochromatin assembly. Whereas it is known that Clr3 is important for heterochromatin-mediated silencing (Ekwall and Ruusala, 1994; Thon et al., 1994; Grewal and Klar, 1997), it is not understood how Clr3 is recruited to its target loci. Moreover, while it has been shown that Clr3 and Clr4 synergistically promote H3K9me (Nakayama et al., 2001), the exact function of Clr3 and its HDAC activity in heterochromatin assembly has not been analyzed.

Results

Localization of Clr3 to the *mat* Locus Is Dependent on Swi6 and Chp2

To understand the role of Clr3 in heterochromatic silencing at the *mat* locus, we first examined its localization at a *ura4*$^+$ reporter gene (*Kint2::ura4*$^+$) inserted within the *mat2/3* interval (Grewal and Klar, 1997). To this end, we constructed a strain expressing the Clr3 protein fused to a FLAG epitope tag at its carboxy terminus (Clr3-$[FLAG]_3$). Clr3-$(FLAG)_3$ is fully functional (data not shown) and expressed under the control of native regulatory elements. Chromatin immunoprecipitation (ChIP) analysis showed that Clr3-$(FLAG)_3$ was preferentially enriched at *Kint2::ura4*$^+$ as compared to the control mini-*ura4* (*ura4DS/E*) at an endogenous euchromatic location, suggesting that Clr3 specifically localizes to the silenced chromosomal region (Figure 1).

It has been shown that the chromodomain proteins Swi6, Chp1, and Chp2 localize to heterochromatic loci including the *mat* locus, centromeres, and telomeres. In contrast to Swi6 and Chp2 proteins that are required for stable maintenance of heterochromatin and silencing at the *mat* locus, Chp1, a component of the RITS RNAi complex (Verdel et al., 2004), is dispensable for maintenance of the preassembled heterochromatic state at this locus and is required only for the initial targeting of heterochromatin (Noma et al., 2004; Sadaie et al., 2004). Based on these findings, we investigated whether lack of Swi6, Chp1, or Chp2 affects Clr3 localization at the *mat* locus. Remarkably, loss of Swi6 and Chp2 but not Chp1 completely abolished the localization of Clr3 at *Kint2::ura4*$^+$ (Figure 1). These analyses suggest that stable binding of Clr3 at the *mat* locus is independent of the RNAi pathway but depends upon other factors involved in heterochromatin formation such as Swi6 and Chp2.

Clr3 Is Distributed across the Entire Silent Mating-Type Interval

We have previously shown that H3K9me and Swi6 are present across the entire *mat2/3* interval (Noma et al., 2001). The Swi6-dependent localization of Clr3 prompted us to analyze its distribution at high resolution across the *mat2/3* interval. ChIP analysis with primers distributed across the entire silent mating-type region was performed as described previously (Noma et al., 2001). Our results revealed that Clr3 is indeed enriched throughout the 20 kb heterochromatic domain surrounded by the *IR-R* and *IR-L* boundary elements but is absent at the surrounding euchromatic regions (Figure 2A).

Heterochromatin Assembly at the *mat* Locus Involves the Recruitment and Spreading of Clr3

Heterochromatin formation at the *mat2/3* region has been shown to involve nucleation of heterochromatin at specific DNA elements essential for the recruitment of histone-modifying activities and subsequent Swi6-dependent spreading of heterochromatin and its associated factors such as the RITS complex (Hall et al., 2002; Noma et al., 2004). To determine whether Clr3 is initially targeted to a specific nucleation site, we performed ChIPs at the *mat2/3* region in a *swi6* mutant strain. In the absence of Swi6, Clr3 localization was confined to a small region near the *mat3* locus (Figure 2A), which is distinct from the *cenH* element responsible for RNAi-mediated targeting of heterochromatin to this region.

We next investigated whether loss of Clr3 HDAC activity affects its localization. A strain expressing the mutant Clr3 (*clr3-735*) tagged with the FLAG epitope was constructed. The Clr3-735 protein contains a mutation near its active site and expresses at a level comparable to that of wild-type protein, but mutant cells are defective in histone deacetylation and silencing at the *mat2/3* locus (see Figure S1 with the Supplemental Data available with this article online) (Grewal et al., 1998; Noma and Grewal, 2002). ChIP analysis revealed that the Clr3 mutant protein was mainly restricted to the nucleation site adjacent to the *mat3* locus and the spreading of Clr3 across the *mat2/3* region was severely affected (Figure 2A). We also determined the effect of Sir2 on Clr3 localization. Loss of Sir2 compromises heterochromatin spreading at the *mat* locus (Shankaranarayana et al., 2003). Surprisingly, except for a small but reproducible enrichment of Clr3 at the nucleation site, Clr3 was virtually absent from the entire *mat2/3* region in a *sir2Δ* strain (Figure 2A). These data, together with results showing defects in Swi6 localization at the *mat* locus in *clr3-735* cells (see below; Figure S2), suggest that Clr3 is initially recruited to the silent *mat* interval at a nucleation site located near the *mat3* locus. Subsequently, Clr3 spreads across the entire *mat2/3* interval that is dependent upon its own HDAC activity, Swi6 and Sir2 proteins, and possibly other factors, such as Chp2, involved in heterochromatin assembly.

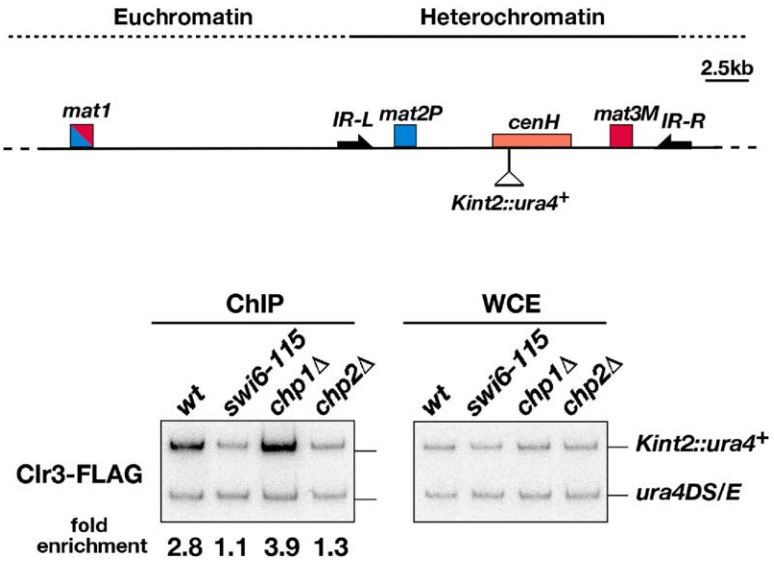

Figure 1. Localization of Clr3 at the Silent Mating-Type Locus Requires Swi6 and Chp2

The physical map of the mating-type locus denoting the *Kint2::ura4*$^+$ insertion site (top). A 20 kb heterochromatin domain flanked by inverted repeats, *IR-L* and *IR-R*, encompasses *mat2P* and *mat3M* as well as *cenH*, which is highly homologous to centromeric repeats. Localization of Clr3 at *Kint2::ura4*$^+$ in wild-type and indicated mutant background cells. ChIP analyses using anti-FLAG antibody were carried out against Clr3-$(FLAG)_3$. DNA isolated from immunoprecipitated fractions (ChIP) or whole-cell extracts (WCE) was analyzed by competitive PCR, where one primer pair simultaneously amplifies *Kint2::ura4*$^+$ and *ura4DS/E* fragments. The ratio of signal intensities between *Kint2::ura4*$^+$ and *ura4DS/E* in immunoprecipitated fractions was normalized to that in whole-cell extracts and shown underneath each lane as "fold enrichment."

Clr3 Interacts with Swi6

Swi6 serves as a platform to recruit a variety of protein complexes such as cohesin and the Swi2-containing recombination complex (Jia et al., 2004b; Nonaka et al., 2002). Since Clr3 spreading requires Swi6, we tested whether Clr3 interacts with Swi6. Extracts from strains expressing either Clr3-Myc alone or both HA-Swi6 and Clr3-Myc fusion proteins were used to perform immunoprecipitation with an anti-HA antibody. Western blot analysis of the precipitated fractions with anti-Myc antibody revealed Clr3 interaction with HA-Swi6 (Figure 2B). Similarly, when we used antibodies against Swi6 to perform immunoprecipitation, Clr3-Myc was detected in the precipitated fraction, whereas no signal was present in control fractions precipitated with an anti-GST antibody (Figure 2B). The fact that only a small fraction of the total Clr3 protein coimmunoprecipitated with Swi6 might be a consequence of the highly dynamic nature of Swi6 at heterochromatic loci, in which Swi6 interacts transiently with Clr3 (Cheutin et al., 2004). Moreover, while Swi6 localizes specifically to heterochromatic domains (Cam et al., 2005), Clr3 is widely distributed at euchromatic loci, in addition to its localization at heterochromatin (Wiren et al., 2005), and thus might also account for such modest Clr3-Swi6 interaction. Nevertheless, interaction between Clr3 and Swi6, along with Chp2-mediated binding of Clr3 to chromatin (Figure 1), might be necessary for the spreading of heterochromatin.

Atf1/Pcr1 Factors Are Required for Targeting Clr3 to its Nucleation Site at the *mat* Locus

We next sought to analyze *cis*- and *trans*-acting factors involved in targeting Clr3 to its nucleation site near the *mat3* locus. The Clr3 nucleation center encompasses the *REIII* silencer element and two Atf1/Pcr1 binding sequences, one of which resides within the *REIII* element (Thon et al., 1999; Jia et al., 2004a) (Figure 3). To determine precisely the site of Clr3 binding within this region, we performed high-resolution ChIP mapping in a *swi6* mutant background. Our analysis revealed a peak of Clr3 enrichment that coincided exactly with the *REIII* element. The heptamer sequence (5′-ATGACGT-3′) within *REIII* corresponds to a site where Atf1/Pcr1 was found to be most highly enriched, and deletion of *REIII* or Atf1/Pcr1 affects heterochromatin assembly at the *mat* locus (Jia et al., 2004a). We therefore examined a possible involvement of Atf1/Pcr1 in Clr3 recruitment at the nucleation site. In double mutant cells lacking Pcr1 and Swi6, the localization of Clr3 was almost completely abolished from *REIII* (Figure 3). To further confirm the involvement of Atf1/Pcr1 in recruiting Clr3 to the *mat* locus, we constructed a mutant strain carrying a deletion of the heptamer sequence within the *REIII* element. Remarkably, deletion of the heptamer Atf1/Pcr1 binding site resulted in severe loss of Clr3 from the *REIII* element to levels comparable to those lacking Atf1/Pcr1 (Figure 3). Taken together, these observations suggest that ATF/CREB family proteins are essential for recruiting Clr3 to the *mat* locus via binding of Atf1/Pcr1 to a heptamer sequence (that we have named *CAS*, *C*lr3 *a*ttracting *s*equence).

Clr3 Acts in Parallel to the RNAi Pathway to Nucleate Heterochromatin

Parallel pathways involving Atf1/Pcr1 proteins and the RNAi machinery control heterochromatin nucleation at the *mat2/3* region (Jia et al., 2004a). To further analyze the role of Clr3 in the Atf1/Pcr1-dependent pathway of heterochromatin nucleation, we combined the *clr3Δ* with mutations in components of the RNAi pathway and analyzed the effects of double mutant combination on H3K9 methylation. Whereas H3K9me levels at *Kint2::ura4*$^+$ were not affected in *clr3Δ*, *dcr1Δ*, or *atf1Δ* single mutants compared to wild-type, H3K9me was completely abolished in a *clr3Δ dcr1Δ* double mutant strain (Figure 4A). These results suggest that Clr3 operates in a pathway parallel to RNAi to nucleate heterochromatin at the *mat* locus. Importantly, loss of both Clr3 and Atf1 did not dramatically reduce the levels of H3K9me compared to those of *clr3Δ dcr1Δ* (Figure 4A) or *atf1Δdcr1Δ*

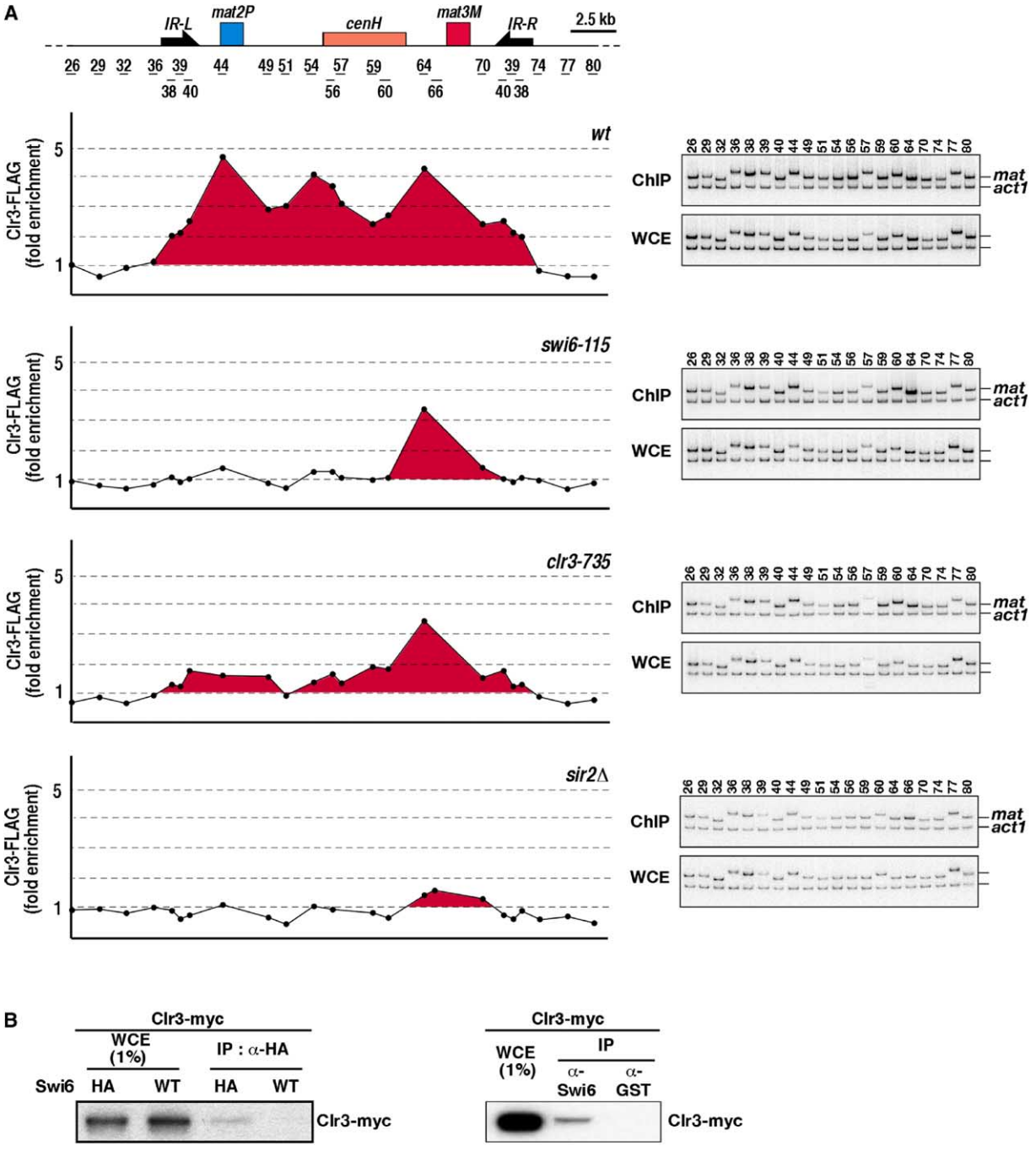

Figure 2. Distribution of Clr3 across the *mat2/3* Region and Interaction between Clr3 and Swi6

(A) The map of the mating-type region indicating the PCR fragments amplified in ChIP experiments is shown (top). ChIP using anti-FLAG antibody was performed with Clr3-$(FLAG)_3$ expressing strains in wild-type, *swi6-115*, *clr3-735*, or *sir2*Δ background. DNA recovered from ChIP and WCE samples was analyzed by multiplex PCR, whereby the *mat* region and *act1* (internal amplification control) were simultaneously amplified from the same template DNA. The fold enrichment of Clr3-$(FLAG)_3$ was calculated by normalizing ratios of the *mat* region and *act1* signals in ChIP fractions to those in WCE. Filled color indicates areas with fold enrichment above 1.0.

(B) Clr3 interacts with Swi6 in vivo. Extracts prepared from the indicated strains were incubated with anti-HA antibody (left) or with either anti-Swi6 or anti-GST antibody (right). Immunoprecipitated fractions were analyzed by Western blotting with anti-Myc antibody (9E10 or A-14, Santa Cruz) to detect Clr3-Myc. Lanes labeled WCE contain the equivalent of 1% of the input protein.

cells (Jia et al., 2004a), indicating that Clr3 and Atf1 are operating in the same pathway.

We have demonstrated previously that the *cenH* element is essential for RNAi-dependent heterochromatin nucleation at the *mat2/3* region (Hall et al., 2002). Cells carrying a replacement of *cenH* with a *ura4*$^+$ reporter gene (*KΔ::ura4*$^+$) can exist in two metastable, expressed (*ura4-on*) and silenced (*ura4-off*), epigenetic

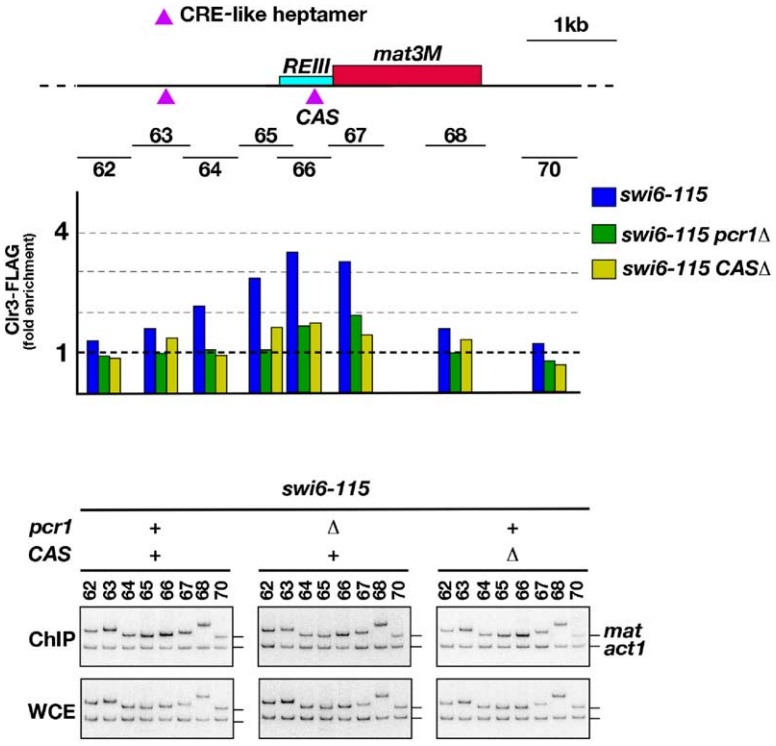

Figure 3. Atf1/Pcr1 Recruits Clr3 to *REIII*

Close-up view of a region between *cenH* and *mat3M* (top). *mat3M*, *REIII*, and two Atf1/Pcr1 binding sites are indicated by a red box, a blue box, and purple triangles, respectively. PCR fragments amplified in ChIP experiments are indicated by numbered horizontal bars. Localization of Clr3 at *REIII* was analyzed by ChIP using the indicated strains (bottom). Relative enrichment values for each PCR fragment in different strain backgrounds were plotted.

states (Nakayama et al., 2000). Cells carrying the silenced *ura4-off* epigenetic state maintained by the Atf1/Pcr1 pathway (Jia et al., 2004a) are enriched for Swi6 and H3K9me at the *mat2/3* region (Hall et al., 2002; Nakayama et al., 2000). We crossed a *clr3Δ* strain with a *KΔ::ura4*$^+$ (*ura4-off*) strain to determine whether Clr3 is critical for maintaining high levels of H3K9me in the absence of RNAi-mediated heterochromatin nucleation. Indeed, loss of Clr3 resulted in the inability of *KΔ::ura4*$^+$ cells to maintain a stable *ura4-off* state (data not shown) and caused a dramatic reduction in the H3K9me level at *KΔ::ura4*$^+$ (Figure 4B).

Loss of the RNAi machinery does not completely abolish H3K9me at centromeric repeats, suggesting an additional mechanism(s) maintaining basal methylation levels (Sadaie et al., 2004). Similar to the *mat* locus, a pathway involving Clr3 might be responsible for targeting H3K9me at centromeres, in addition to the RNAi pathway. Remarkably, whereas *clr3Δ* or *dcr1Δ* single mutant strains still maintained H3K9 methylation, H3K9me at centromeric repeats was almost completely abolished in *clr3Δdcr1Δ* double mutant cells (Figure 4C).

RNAi- and Clr3-Based Mechanisms Independently Recruit Clr4 to the *mat* Locus

To further analyze the involvement of the RNAi- and Clr3-dependent pathways in the nucleation of H3K9me, we tested the effects of mutations in Clr3 and RNAi factors on Clr4 recruitment. ChIP analyses showed that, whereas Clr4 levels at the silent mating-type region of cells carrying either *dcr1Δ* or *clr3Δ* alleles were comparable to those in wild-type cells, Clr4 localization was completely abolished in *clr3Δdcr1Δ* cells (Figure 4D). Furthermore, in *KΔ::ura4*$^+$ cells containing a deletion of the *cenH* element required for RNAi-mediated heterochromatin nucleation, deletion of *clr3* alone resulted in delocalization of Clr4 from the *mat2/3* locus (Figure 4E). We conclude that parallel mechanisms involving Clr3 and the RNAi machinery are involved in targeting Clr4 and H3K9me to the *mat2/3* region.

Clr3 Affects the Dynamics of H3K9 Methylation and Swi6 Localization

In higher eukaryotes, distinct H3K9 methylation (mono [H3K9me1], di [H3K9me2], or tri [H3K9me3]) states correlate with different degrees of gene silencing (Grewal and Rice, 2004). For instance, H3K9me3 is enriched at constitutive heterochromatin, while H3K9me1 and H3K9me2 are mainly associated with silenced loci at euchromatic domains (Peters et al., 2003; Rice et al., 2003). Despite a loss of heterochromatic silencing, we noticed a consistent increase in H3K9me2 levels in *clr3Δ* cells (Figures 4A and 4C), which prompted us to investigate the effects of *clr3Δ* on the dynamics of H3K9 methylation at the *mat* locus. ChIP analyses with antibodies that selectively recognize different H3K9 methylation states detected trimethylation in addition to dimethylation at the *Kint2::ura4*$^+$ reporter in wild-type cells (Figure 5A). However, in *clr3Δ* cells, H3K9me3 was significantly reduced, while there was a substantial increase in H3K9me1. Furthermore, H3K9me2 levels were slightly elevated (Figure 5A). Interestingly, identical modification patterns were also observed in a *swi6* mutant, consistent with Swi6 involvement in Clr3 spreading (Figure 5A). In contrast to mammalian cells where distinct enzymes seem to catalyze different methylated states of H3K9 (Peters et al., 2003; Rice et al., 2003), we found that Clr4 is responsible for all three states of

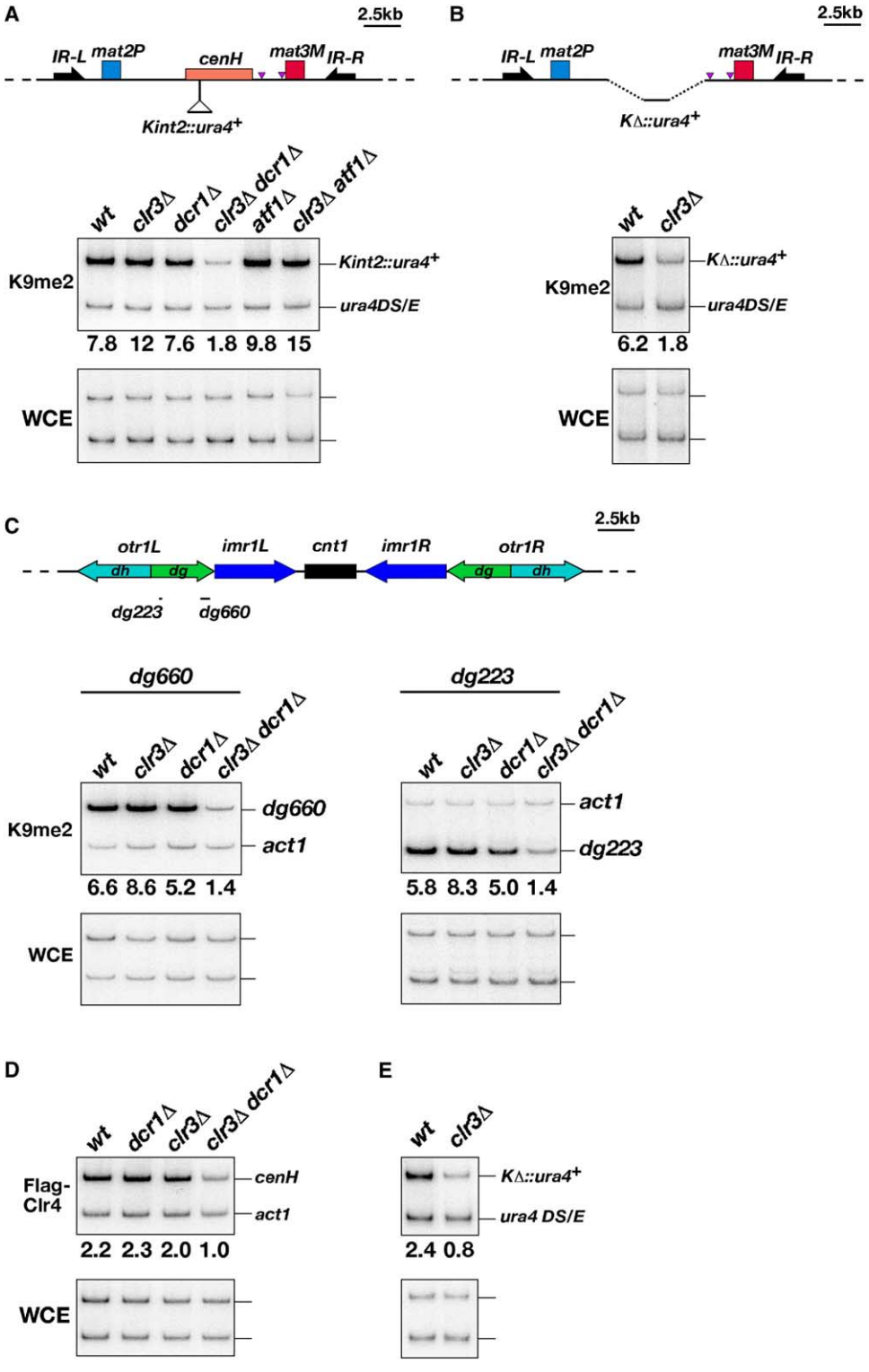

Figure 4. Clr3 Acts in a Redundant Manner to the RNAi Pathway to Nucleate Heterochromatin at the *mat* Locus

(A) Effects of *clr3*Δ and *dcr1*Δ or *atf1*Δ on H3K9me at the *mat* locus. A schematic diagram of *Kint2::ura4*$^+$ with Atf1/Pcr1 consensus binding sites indicated by purple triangles is shown. H3K9me levels at *Kint2::ura4*$^+$ in the indicated strain backgrounds were examined by ChIP using anti-H3K9me2 antibody.

(B) Clr3 is essential for H3K9me at the *mat* locus in cells lacking the *cenH* repeat. Schematic diagram of *K*Δ*::ura4*$^+$ carrying a replacement of the *cenH*-containing region with *ura4*$^+$. Purple triangles denote Atf1-Pcr1 binding sites. H3K9me2 levels at *K*Δ*::ura4*$^+$ in wild-type and *clr3*Δ cells were examined by ChIP.

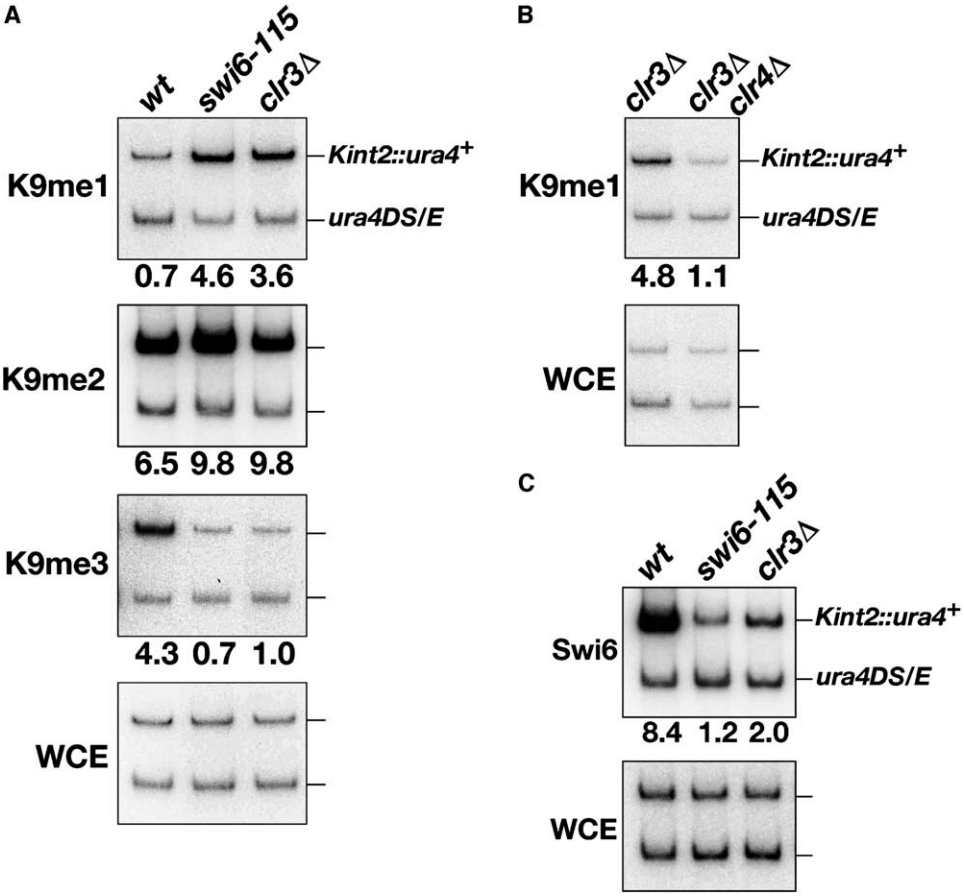

Figure 5. Clr3 Affects the Degrees of H3K9 Methylation and Swi6 Localization at the *mat2/3* Locus

(A) Degrees of H3K9 methylation are altered in *swi6-115* and *clr3*Δ mutants. H3K9me1, H3K9me2, and H3K9me3 levels at *Kint2::ura4*$^+$ in wild-type and mutant strains were examined by ChIP using antibodies specific for H3K9me1, H3K9me2, and H3K9me3, respectively.

(B) Clr4 is required for monomethylation of H3K9. H3K9me1 levels at *Kint2::ura4*$^+$ in *clr3*Δ and *clr3*Δ*clr4*Δ mutants were examined by ChIP using an antibody specific for H3K9me1.

(C) Clr3 is important for Swi6 localization at the *mat* locus. Swi6 levels at *Kint2::ura4*$^+$ in wild-type and mutant strains were examined by ChIP.

H3K9 methylation in *S. pombe*. H3K9me1, which was observed in *clr3Δ* cells, was completely abolished in a *clr3Δclr4Δ* double mutant strain (Figure 5B).

We next explored whether loss of Clr3 affects Swi6 binding at the *mat* locus. Deletion of *clr3* resulted in severely reduced Swi6 levels at *Kint2::ura4*$^+$ even though Swi6 expression was not affected (Figure 5C; Figure S3). Unlike wild-type cells in which Swi6 was preferentially enriched throughout the *mat2/3* interval, the levels of Swi6 were drastically reduced across this entire heterochromatic domain in *clr3Δ* cells (Figure S2). We also studied the effects of mutations in *clr3* on Swi6 localization at outer (*otr*) centromeric repeats where the RNAi machinery plays a predominant role in targeting H3K9me and heterochromatin formation. Loss of Clr3 activity also resulted in reduction in Swi6 levels at *otr* repeat loci, although the effect was less severe than that observed at the *mat* locus (data not shown) (Nakayama et al., 2001). Based on these results, we conclude that Clr3 is required for efficient binding of Swi6 to heterochromatic loci.

Swi6 Delocalization in *clr3*Δ Cells Correlates with Aberrant Histone Modifications

The loss of H3K9me3 observed in *clr3Δ* cells could, in principle, be responsible for the decrease in Swi6 localization at the *mat* locus. Alternatively, modifications of residues adjacent to the H3K9me mark might interfere

(C) Clr3 acts in a separate pathway parallel to the RNAi to nucleate H3K9me at centromeric repeats. A schematic diagram of cen1 is shown with the positions of amplified PCR fragments (*dg660* and *dg223*) indicated by horizontal bars. H3K9me2 levels at centromeric repeats in the indicated strains were examined by ChIP.

(D) Effects of *clr3*Δ and *dcr1*Δ on Clr4 localization at the *mat* locus. Cells expressing $(FLAG)_3$-Clr4 were used to perform ChIP with anti-FLAG antibody in indicated strain backgrounds.

(E) Clr3 is essential for Clr4 localization at the *mat* locus in cells lacking the *cenH* repeat. Clr4 levels at *KΔ::ura4*$^+$ in $(FLAG)_3$-Clr4 expressing wild-type and *clr3*Δ cells were examined by ChIP.

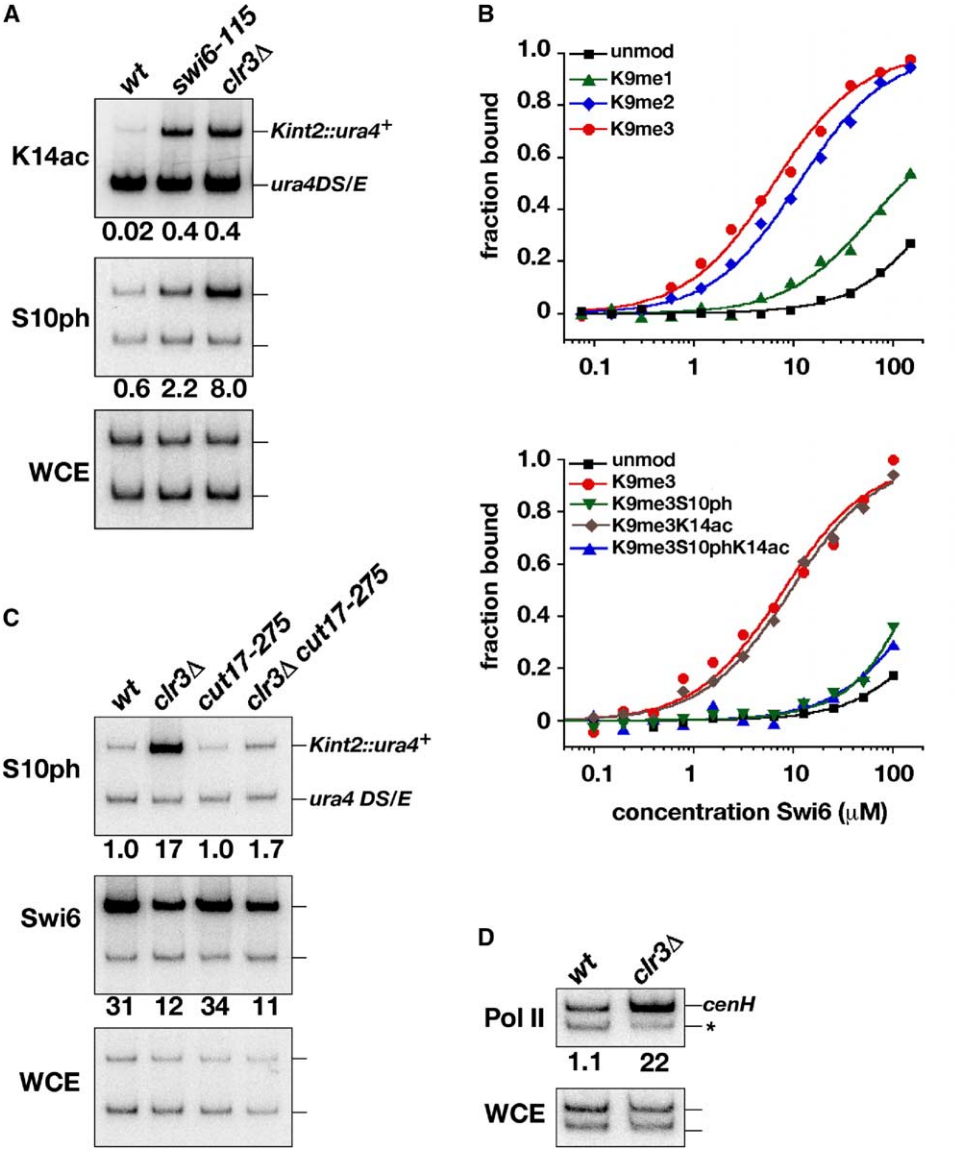

Figure 6. Lack of Clr3 Causes Aberrant Histone Modification Pattern and Increased Pol II Occupancy at the *mat* Locus

(A) Levels of H3K14ac and H3S10ph at *Kint2::ura4*$^+$ are elevated in *clr3*Δ and *swi6-115* mutants. H3K14ac and H3S10ph levels at *Kint2::ura4*$^+$ in the indicated strains were examined by ChIP.

(B) In vitro binding analysis of Swi6 to modified H3 peptides. The interaction of Swi6 with the indicated peptides was analyzed by fluorescence polarization measurements.

(C) Increased H3S10ph in *clr3*Δ mutant is related to the Cut17-Ark1 pathway. Levels of H3S10ph and Swi6 at *Kint2::ura4*$^+$ in the indicated strain backgrounds were examined by ChIP. Because of the temperature sensitivity of *cut17-275* mutants, cells were grown at 25°C before processing.

(D) Pol II occupancy at the naturally silenced *cenH* element is strongly reduced by Clr3-mediated silencing. Pol II occupancy was measured by ChIP using 8WG16 antibody that recognizes the CTD repeat domain of the largest subunit of Pol II.

with Swi6 binding (Fischle et al., 2003). Therefore, we analyzed the modification status of H3K9me neighboring residues in the *clr3Δ* mutant strain by ChIP analysis. Consistent with previous studies (Bjerling et al., 2002; Noma and Grewal, 2002; Wiren et al., 2005), deletion of *clr3* resulted in an increase in acetylation at H3K14 (H3K14ac) (Figure 6A), a mark of active chromatin that is absent at heterochromatic loci. Furthermore, the levels of H3S10 phosphorylation (H3S10ph), another modification mark associated with active chromatin as well as mitotic chromosomes (Nowak and Corces, 2004), were also increased at the *mat* locus (Figure 6A). Similar changes were observed in the *swi6* mutant; however, the effect on H3S10ph was weaker than in the *clr3* mutant (Figure 6A). Intriguingly, in these mutant cells, H3S10ph levels at *Kint2::ura4*$^+$ were higher than those at the *ura4DS/E* locus located at the endogenous euchromatic site (Figure 6A), consistent with a special-

ized mechanism targeting H3S10ph to heterochromatic loci (Petersen et al., 2001). Thus, lack of Clr3 results in aberrant histone modifications at the *mat2/3* region, which might in turn be responsible for Swi6 delocalization.

To directly explore whether changes in the histone modification pattern in *clr3* mutant might prevent Swi6 binding to chromatin, we performed fluorescence polarization binding assays to determine the interaction of Swi6 with a series of modified H3 peptides. These analyses showed that Swi6 has only limited preference for H3K9me1 over the unmodified H3 tail. Much stronger binding strength was observed for H3K9me2 and H3K9me3, which showed the strongest interaction (Figure 6B). Binding experiments with peptides carrying multiple modifications further indicated that the presence of S10ph adjacent to the strongest Swi6 binding mark, H3K9me3, severely interferes with Swi6 interaction with H3K9me (Figure 6B). In contrast, H3K14ac had no effect on Swi6 binding to the H3K9me3 mark. These results suggest that, besides loss of H3K9me3, reduction in Swi6 levels at the *mat* locus in the *clr3* mutant might be due to elevated levels of H3S10ph interfering with binding of Swi6 to methylated H3K9. We therefore tested whether the increase in H3S10ph in *clr3* mutant cells was responsible for the reduction in Swi6 at the *mat* locus in vivo. Our analyses revealed that a temperature-sensitive mutation in the survivin homolog Cut17/Bir1 (*cut17-275*), which is known to bind centromeric repeats and is required for proper localization of fission yeast aurora kinase Ark1 (Morishita et al., 2001), almost completely abolished H3S10ph at the *mat* locus in *clr3Δ* cells (Figure 6C). However, this loss of H3S10ph failed to restore Swi6 localization (Figure 6C), suggesting that, besides H3S10ph, additional mechanisms are responsible for Swi6 delocalization in *clr3Δ* cells.

Clr3 Restricts RNA Polymerase II Accessibility to Heterochromatic Repeat Elements

To understand the function of Clr3 in transcriptional silencing and to explore whether aberrant histone modification patterns in a *clr3* mutant background correlate with an increased access of the basal transcription machinery, we investigated the effect of *clr3Δ* on binding of RNA polymerase II (Pol II) to the naturally silenced *cenH* element located within the *mat2/3* domain. ChIP analysis was performed using antibodies that recognize the carboxy-terminal repeat domain (CTD) of the largest subunit of Pol II. As shown in Figure 6D, we observed a dramatic (20-fold) increase in Pol II occupancy at the *cenH* element, suggesting that Clr3 HDAC is required to prevent recruitment of the basal transcription machinery to sequences at heterochromatic loci. Together, our analyses point to a role for Clr3 in preventing improper histone modifications and enhanced occupation of the basal transcription apparatus that could directly or indirectly destabilize heterochromatic structures.

Discussion

Recent studies have considerably advanced our understanding of heterochromatin assembly. While these studies have primarily focused on factors that trigger heterochromatin formation, such as repetitive DNA sequences and RNAi, with the subsequent methylation of H3K9 by Clr4/Suv39h and the recruitment of Swi6/HP1, the role of HDACs in this process is not entirely clear. Here, we used the fission yeast silent mating-type locus as a model system to dissect the roles of Clr3 HDAC in heterochromatin formation. We demonstrate that Clr3 acts in a pathway parallel to the RNAi mechanism to recruit Clr4 and nucleate heterochromatin assembly. Clr3 is recruited to the *mat* locus at a specific site by a mechanism involving ATF/CREB family DNA binding proteins, whereas spreading of Clr3 across a 20 kb silenced domain is dependent on its HDAC activity and the heterochromatin machinery including the Swi6 protein. Clr3 also plays a critical role in heterochromatin maintenance by stabilizing H3K9 trimethylation and preventing aberrant histone modifications. We propose that Clr3 has dual roles at the silent mating-type locus: to nucleate heterochromatin and to maintain appropriate histone modification patterns, thereby limiting access to the basal transcription apparatus including RNA Pol II.

Recruitment and Spreading of Clr3 across the Silent Mating-Type Locus

Based on our analyses, the process of Clr3 localization at the *mat* locus can be divided into at least two steps: the initial recruitment of Clr3 to the site near the *mat3* locus and the subsequent spreading of Clr3 across the *mat2/3* region (Figure 7). The initial recruitment of Clr3 to the *mat* locus involves the newly identified *CAS* sequence as well as Atf1/Pcr1, which is known to localize to the *CAS* sequence and is required for heterochromatin formation (Jia et al., 2004a). While we do not know presently whether Atf1/Pcr1 is sufficient to nucleate heterochromatin or whether additional factors are required, we note that loss of Sir2, which shares targets with Clr3 (Wiren et al., 2005), results in severe defects in localization of Clr3 at the *mat* locus.

Although a substantial amount of Clr3 is localized to the nucleation site in a *swi6* mutant, it fails to spread across the *mat2/3* region. Likewise, Clr3 is confined to the nucleation site in a *clr3-735* mutant that lacks Clr3 HDAC activity. Since we found that Swi6 is largely delocalized from the *mat* interval in the *clr3-735* mutant (Figure S2), these results collectively suggest a positive feedback loop in which deacetylation of histones by Clr3 directly or indirectly helps stabilizing Swi6 association with chromatin, which in turn facilitates the spreading of Clr3 through its interaction with Swi6 (Figure 2B). This mechanism is reminiscent of the positive feedback model that has been suggested for Clr4 and Swi6 spreading (Hall et al., 2002) (see Figure 7). Since Clr3 is essential for stable Swi6 localization and recruitment of Clr4 (this study), we suggest that Clr3 is an essential component of the same regulatory loop. This interpretation is supported by findings that *Drosophila* Su(VAR) 3-9 genetically and physically interacts with HP1 (Schotta et al., 2002) and HDAC1 (Czermin et al., 2001) and that class II HDACs associate with HP1α and SUV39H1 in mammalian cells (Zhang et al., 2002).

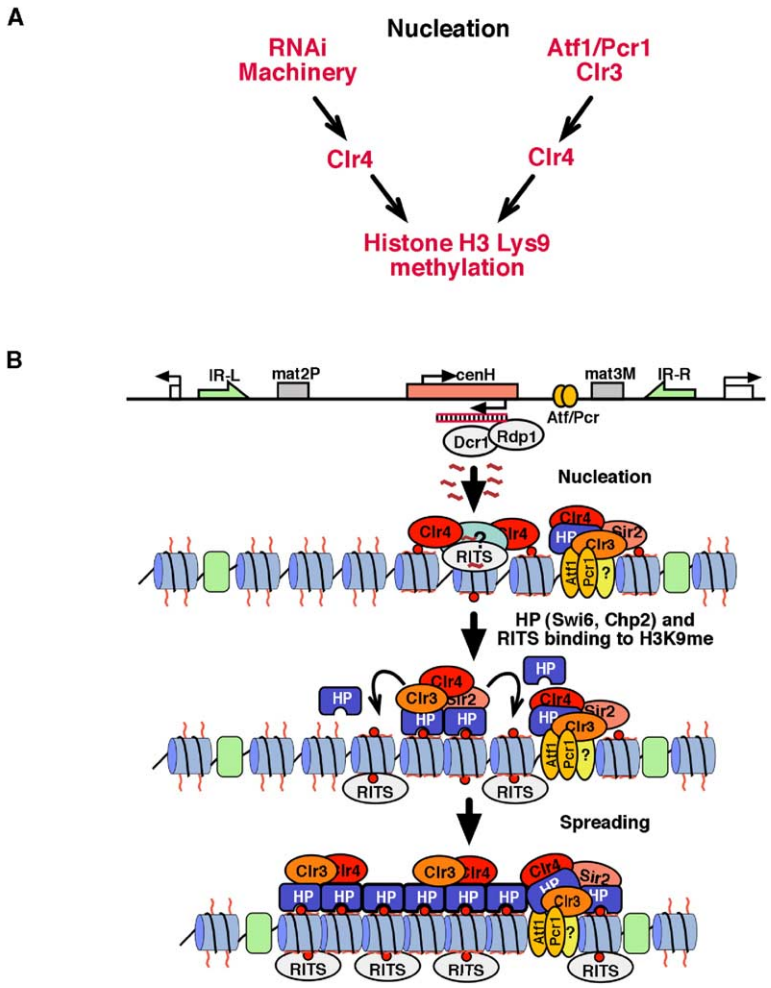

Figure 7. Model for Heterochromatin Assembly at the Silent Mating-Type Region

(A) Redundant pathways initiate Clr4-mediated H3K9 methylation, the convergence of which, facilitated by Swi6 and HDACs, contributes to establishment and maintenance of heterochromatin at the silent mating-type region. Clr4 is recruited independently, either by the RNAi machinery or by Atf1/Pcr1, which cooperates with Clr3 to nucleate H3K9 methylation.

(B) At the *mat2/3* region, two major *cis* elements, *cenH* and *CAS*, act as nucleation centers recruiting Clr4 via the RNAi and Atf1/Pcr1/Clr3 pathways, respectively. In the RNAi-based mechanism, transcripts (large red lines) generated by *cenH* are processed into siRNAs (small red lines). siRNAs specify the targeting of H3K9me (red circles) either via RITS or an uncharacterized protein complex (blue oval). In the Clr3-based mechanism, Atf1/Pcr1 bound to *CAS*, along with unidentified factors (yellow oval) and Sir2, recruit Clr3, which in turn cooperates with heterochromatin proteins (HPs) such as Swi6 and Chp2 to promote H3K9me by Clr4. Swi6 and Chp2 recognize H3K9me, bind to chromatin, and recruit Clr3 and perhaps other HDACs. Together, these factors promote H3K9me by Clr4, thereby creating additional binding sites. This spreading process continues until the flanking *IR-L* and *IR-R* boundaries (green arrows/boxes) are encountered. Once heterochromatin is formed, it allows stable localization of RITS and Clr3, ensuring effective maintenance of heterochromatic structures.

Role of Clr3 in Regulating Histone Modifications and Heterochromatin Maintenance

Apart from its role in the initial targeting of heterochromatin, Clr3 is critical for protecting the *mat2/3* region from access by the basal transcription apparatus including Pol II and probably other cellular factors (Figure 6). The increased occupancy of transcription-associated factors in *clr3Δ* cells, presumably caused by unregulated accessibility of "open" chromatin, could in general destabilize heterochromatin structures (Ahmad and Henikoff, 2001). Besides, our analyses uncovered a role for Clr3 in maintaining H3K9me3, which is a conserved mark at constitutive heterochromatin essential for efficient silencing (Peters et al., 2003; Wang et al., 2003). Loss of silencing in *clr3Δ* cells correlates with a dramatic reduction in H3K9me3 levels and a concomitant increase in H3K9me1 levels, while H3K9me2 remains largely unaffected. These results indicate that trimethylation of H3K9 is a crucial step during the assembly of constitutive heterochromatin. Since peptides bearing K9me1 are poor substrates for Swi6 binding in vitro, the depletion of high-affinity binding sites for Swi6 in *clr3Δ* cells might be causally related to heterochromatin assembly defects at the *mat2/3* region.

Our experiments also revealed a dramatic increase in H3S10ph and H3K14ac at the *mat2/3* locus in *clr3Δ* mutant cells (Figure 6A). Indeed, it has been suggested that phosphoacetylation of histone H3 might be a general mechanism for relieving HP1-mediated repression in higher eukaryotes (Mateescu et al., 2004). Whereas we found that H3S10ph occludes Swi6 from interacting with H3K9me3 in vitro (Figure 6B), our analysis of cells carrying a mutation in Survivin/Cut17, which we found is required for H3S10ph at the *mat2/3* locus presumably via its interaction with Aurora kinase Ark1 (Morishita et al., 2001; Petersen et al., 2001), indicate that, besides H3S10ph, additional mechanisms must be responsible for Swi6 delocalization. We favor the view that H3K14ac, which does not directly interfere with Swi6 binding to methylated H3K9 in vitro (this study), induces an overall open chromatin structure that provides greater access to chromatin-modifying factors and causes reduction in H3K9me3 levels, which in turn might prevent Swi6 localization at the *mat2/3* region. Indeed, substitution of H3K14 with alanine, which mimics H3K14ac, results in delocalization of Swi6 (Mellone et al., 2003). It is possible that deacetylation of histones by Clr3 is essential for higher-order folding of the *mat2/3* region (Jia et al., 2004b), which, in addition to promoting long-range chromatin interactions essential for mat-

ing-type switching, might also stabilize Swi6 binding to chromatin. Consistent with this interpretation, mutations in Clr3 have been shown to affect mating-type switching (Grewal et al., 1998; Thon et al., 1994), and defects in higher-order chromatin assembly were also observed in mouse cells treated with the HDAC inhibitor Trichostatin A (Maison et al., 2002).

Heterochromatin Assembly at the Mating-Type Locus and Its Relationship to Other Systems

Our present work suggests the existence of at least two distinct mechanisms for the initial targeting of heterochromatin at the *mat* locus and at centromeres. At the *mat* locus, heterochromatin can be nucleated either by the RNAi machinery acting at the *cenH* element or by the sequence-specific DNA binding proteins Atf1/Pcr1, which cooperate with Clr3 to nucleate H3K9 methylation at the *CAS* site (Figure 7). While some silencing factors act specifically in RNAi- or DNA-based nucleation mechanism, the core heterochromatin machinery, including Swi6, Clr4, and HDACs, participates in heterochromatin assembly nucleated by both pathways. The involvement of multiple HDACs is intriguing but might reflect the need to deacetylate multiple residues during heterochromatin assembly. Sir2, which is known to deacetylate H3K9 (Shankaranarayana et al., 2003) and thus probably precedes Clr4 methylation of H3K9, might have a more direct effect on H3K9 methylation. In the RNAi-based mechanism, siRNAs generated by the processing of transcripts derived from *cenH* likely provide the sequence specificity for the targeting of heterochromatin nucleation complex, whereas, in the DNA-based mechanism, Atf1/Pcr1 together with Clr3 could independently recruit Clr4, which mediates methylation of H3K9. H3K9me establishes binding sites not only for Swi6 and Chp2 but also for the RITS complex through the Chp1 chromodomain (Noma et al., 2004). Chromatin bound Swi6 and Chp2 presumably serve as a platform for the recruitment of HDACs and Clr4 to methylate H3K9 on adjacent nucleosomes, thereby allowing heterochromatin proteins and their associated histone-modifying enzymes to spread across the entire heterochromatic domain (Hall et al., 2002). Consistent with this model, spreading of Clr3 and Clr4-mediated H3K9 methylation from nucleation sites to surrounding sequences requires Swi6, and Chp2 is required for Clr3 localization at the *mat* locus (Hall et al., 2002) (this study). Moreover, it has been demonstrated that the histone-modifying enzymes Clr3, Clr4, and Sir2 are required for efficient spreading of heterochromatin across the *mat2/3* region, in addition to their participation in the initial nucleation of heterochromatin complexes (Freeman-Cook et al., 2004; Noma et al., 2004; Shankaranarayana et al., 2003) (this study).

The mechanisms described above share many parallels with assembly of heterochromatin domains in other systems. For example, in budding yeast, silent chromatin assembly is initiated by recruitment of Sir proteins to specific silencer elements by DNA binding factors. The Sir protein complex then spreads to nearby sequences in a manner that requires physical coupling of the histone-modifying enzyme Sir2 with the structural proteins Sir3 and Sir4 (Rusche et al., 2003). Furthermore, studies from higher eukaryotes have implicated both DNA- and RNAi-based mechanisms in targeting heterochromatin. Whereas RNAi is important for targeting heterochromatin to repetitive sequences (Matzke and Birchler, 2005), DNA binding factors such as Kruppel-associated box (KRAB)-zinc finger repressors and E2F-Rb mediate assembly of heterochromatic structures at the promoters of specific genes during development (Nielsen et al., 2001; Schultz et al., 2002). Coincidentally, assembly of silenced chromatin by KRAB and E2F-Rb also requires the recruitment of HDACs and methyltransferases activities as well as HP1 to establish repression. However, the precise sequence of recruiting histone-modifying enzymes with respect to the targeting of HP1 is not clear. Based on our present and earlier studies (Hall et al., 2002), it is plausible that DNA binding factors could initially recruit HDACs. Once recruited, HDACs and their associated corepressors cooperate with Swi6/HP1 to recruit methyltransferase to the target locus. Similar modes of H3K9me nucleation involving DNA- and RNAi-based mechanisms might operate in other organisms. Their convergence might be necessary to facilitate efficient heterochromatin formation at specific loci. However, such redundancy in higher eukaryotes would likely complicate genetic analysis that attempts to uncover single factor contribution, such as the role of RNAi, in heterochromatin formation.

Experimental Procedures

Yeast Strains

The strains expressing epitope-tagged Clr3 were constructed using the PCR tagging method. To express Swi6 or Clr4 fused to HA or FLAG epitope at their amino terminus, we replaced endogenous copies of these genes with DNA fragments carrying open reading frames of these genes fused to epitope tags. *HA-swi6*$^+$ and *FLAG-clr4*$^+$ are expressed under the control of native promoters. Standard conditions were used for growth, sporulation, and tetrad analysis. To construct the *CASΔ* strain, we first inserted an *ura4*$^+$ marker gene at the EcoRV site near *mat3M* and then replaced the *ura4*$^+$ with a DNA fragment lacking the *CAS* sequence by homologous recombination.

ChIP

ChIPs were performed as previously described (Nakayama et al., 2000). Antibodies used were the following: Swi6 (Nakayama et al., 2000); mono-, di-, or tri-methylated H3K9 (Peters et al., 2003; Upstate); H3S10ph (Allis lab); H3K14ac (Upstate); anti-CTD of largest subunit of Pol II (Covance); or anti-FLAG M2 (Sigma). Results shown are representative of multiple independent experiments.

Immunoprecipitation and Western Analysis

Exponentially growing cells were harvested, resuspended in an equal volume of 2× HC buffer (200 mM HEPES [pH 7.4], 300 mM KCl, 2 mM EDTA, 40% glycerol, 2 mM PMSF) supplemented with protease inhibitor cocktail (Roche), and disrupted with acid-washed glass beads (Sigma) at 4°C. Cell lysates recovered by adding 1× HC buffer were centrifuged at 15,000 rpm for 20 min, precleared using IgG-Sepharose beads (Amersham), and used to perform immunoprecipitation overnight at 4°C. Immunoprotein complexes were recovered by incubation with protein G Sepharose beads for 1 hr and washed three times with 1× HC buffer before subjection to Western analysis.

Binding Assays

Swi6 fused to an amino-terminal His-tag was expressed in *E. coli* and purified by Ni-affinity and gel filtration chromatography. Fluorescence polarization binding assays were performed under condi-

tions of 20 mM imidazole (pH 7.0), 25 mM NaCl, and 2 mM DTT and in the presence of 100 nM fluorescein-labeled peptide following a previously described protocol (Jacobs et al., 2001).

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.molecule.org/cgi/content/full/20/2/173/DC1/.

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