

# A chromodomain protein, Chp1, is required for the establishment of heterochromatin in fission yeast

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**The chromodomain is a conserved motif that functions in the epigenetic control of gene expression. Here, we report the functional characterization of a chromodomain protein, Chp1, in the heterochromatin assembly in fission yeast. We show that Chp1 is a structural component of three heterochromatic regions—centromeres, the mating-type region, and telomeres—and that its localization in these regions is dependent on the histone methyltransferase Clr4. Although deletion of the *chp1*<sup>+</sup> gene causes centromere-specific decreases in Swi6 localization and histone H3-K9 methylation, we show that the role of Chp1 is not exclusive to the centromeres. We found that some methylation persists in native centromeric regions in the absence of Chp1, which is also true for the mating-type region and telomeres, and determined that Swi6 and Chp2 are critical to maintaining this residual methylation. We also show that Chp1 participates in the establishment of repressive chromatin in all three chromosomal regions. These results suggest that different heterochromatic regions share common structural properties, and that centromeric heterochromatin requires Chp1-mediated establishment steps differently than do other heterochromatic regions.**

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## Introduction

In eukaryotic cells, the organization of the chromatin into higher-order structures plays an important role in diverse chromosomal processes. Heterochromatin, one of the most distinct chromatin structures originally defined by cytological studies, is involved in various nuclear functions, including nuclear organization, chromosome segregation, dosage compensation, and gene regulation (Grewal and Elgin, 2002; Richards and Elgin, 2002). Heterochromatin is generally transcriptionally inert and its packaging state is epigenetically

inherited during mitosis and meiosis, independent of the primary DNA sequence (Grewal and Klar, 1996; Nakayama *et al.*, 2000). Euchromatic genes placed next to heterochromatin are subjected to a heritable inactivation leading to variegated patterns of gene expression, which is recognized as the spreading of the condensed higher-order structure (Grewal and Elgin, 2002).

In the fission yeast *Schizosaccharomyces pombe*, higher-order chromatin structure is also critical for the functional organization of heterochromatic domains, such as centromeres, the mating-type region, and telomeres (Allshire, 1996; Grewal, 2000). At centromeres, large tandem and inverted repeats (*dg* and *dh*) surrounding a unique central core are assembled into the heterochromatin structure (Takahashi *et al.*, 1992). Similarly, at the mating-type region, *mat2* and *mat3* silent donor loci and an interval called the *K* region are packaged into a heterochromatin-like structure. Marker genes inserted within these regions are transcriptionally silenced (Allshire *et al.*, 1994; Grewal and Klar, 1996). Several *trans*-acting factors are required for the complete repression of these heterochromatic regions (Allshire, 1996; Grewal, 2000). Among these factors, Swi6 and Clr4 play a crucial role in the formation of heterochromatin (Lorentz *et al.*, 1994; Ivanova *et al.*, 1998). Swi6, a homolog of heterochromatin protein 1 (HP1), contains two evolutionally conserved motifs, the chromo- and chromoshadow domains (Paro and Hogness, 1991), and functions as a dosage-critical component of heterochromatin (Nakayama *et al.*, 2000). Clr4 is a fission yeast homolog of Su(var)3–9, a protein containing both chromo- and SET domains (Ivanova *et al.*, 1998). Recently, it has been shown that Clr4 and mammalian Suv39h1 are histone H3-lysine 9 (H3-K9)-specific methyltransferases, and that Swi6 and HP1 specifically bind the methylated histone H3 through its amino-terminal chromodomain (Rea *et al.*, 2000; Bannister *et al.*, 2001; Lachner *et al.*, 2001; Nakayama *et al.*, 2001b). These findings reveal a mechanism in which the methylation of histone H3 and subsequent binding of Swi6/HP1 form a specialized higher-order chromatin structure (Nakayama *et al.*, 2001b; Grewal and Elgin, 2002).

Several lines of evidence support a role for RNA in the formation of heterochromatin (Maison *et al.*, 2002; Grewal and Moazed, 2003). The RNA interference (RNAi) pathway is generally thought to be responsible for post-transcriptional gene silencing (PTGS); in this process, a small (21–23 nucleotides) interfering RNA molecule (siRNA) triggers the degradation of the homologous mRNA (Hannon, 2002). Factors involved in RNAi include an RNase III-like enzyme (Dicer; Dcr1), an RNA-dependent RNA polymerase (RdRP; Rdp1), and an Argonaute (Ago1) protein. In *S. pombe*, deletion of any of the genes encoding the RNAi components (Ago1, Rdp1, Dcr1) causes centromere-specific defects in heterochromatin assembly, accompanied by decreases in H3-K9 methylation and Swi6 localization, and also results in the aberrant accumulation of unprocessed transcripts from centromeres (Volpe *et al.*, 2002). Specifically, the RNAi components are

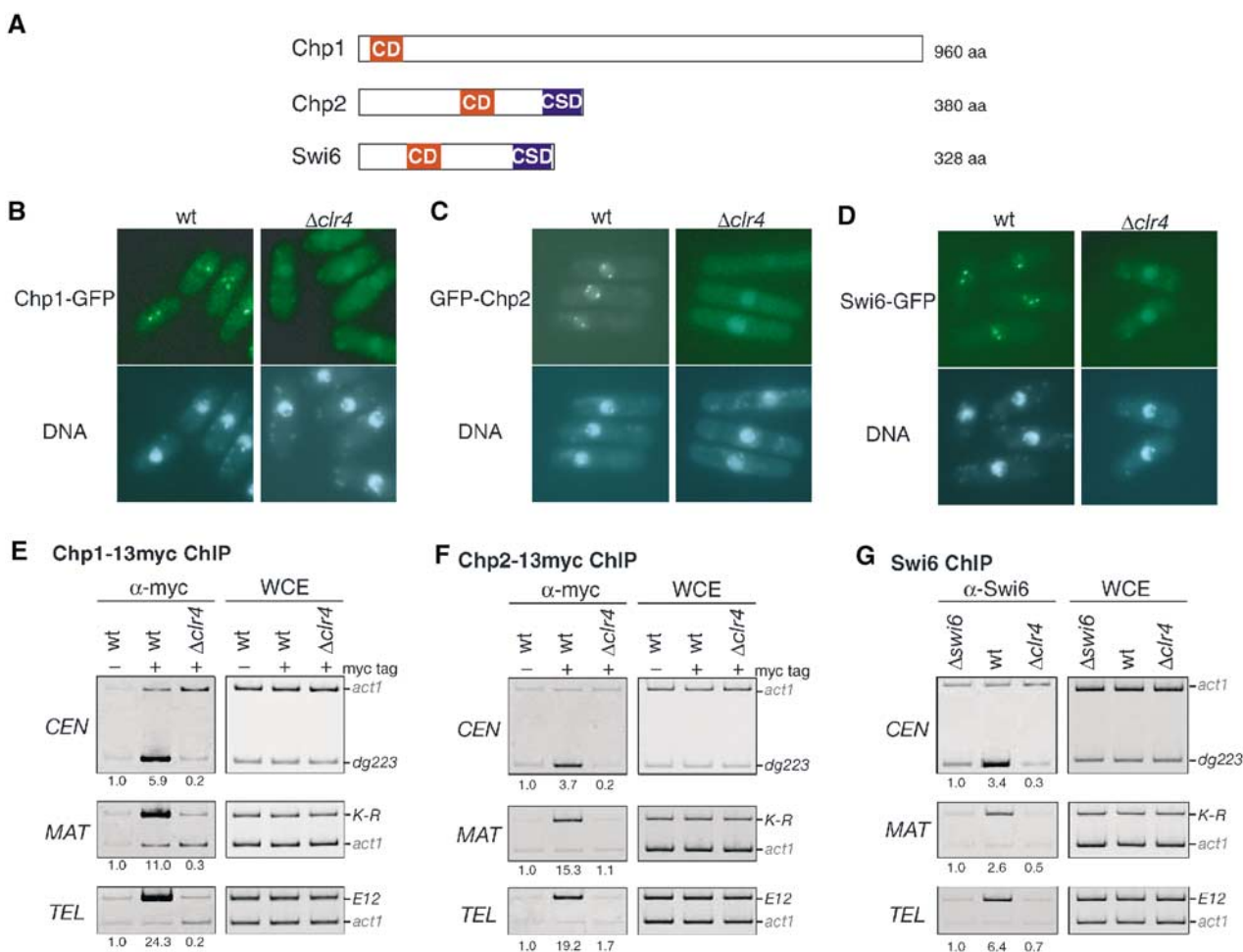
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involved in the establishment of heterochromatin assembly at the mating-type region but are dispensable for its maintenance (Hall *et al*, 2002). It has also been shown that expression of a short hairpin RNA induces the formation of repressive chromatin in a euchromatic region (Schramke and Allshire, 2003).

In *S. pombe*, at least nine chromodomain-containing proteins including Swi6 and Clr4 are present in the genome (Wood *et al*, 2002). Among these proteins, Chp1 and Chp2 are involved in transcriptional silencing at the heterochromatic regions (Figure 1A). Previous studies showed that the deletion of *chp1*<sup>+</sup> causes defects in chromosome segregation and centromeric silencing, but not in the mating-type region or telomeric silencing (Doe *et al*, 1998; Thon and Verheij-Hansen, 2000). In addition, Chp1 associates with centromeric heterochromatin and is required for H3-K9 methylation at both endogenous centromeres and an ectopic site with *cis*-acting centromeric DNA (Partridge *et al*, 2000, 2002). Consistent with the centromere-specific phenotype, a recent

report showed that Chp1 associates with Ago1, Tas3, and siRNAs to form a RITS (RNA-induced initiation of transcriptional silencing) complex (Verdel *et al*, 2004). Another protein, Chp2, shares extensive sequence similarity with Swi6 and contains both a chromo- and a chromoshadow domain (Figure 1A). In contrast to the obvious phenotypes of the  $\Delta$ *swi6* mutants, deletion of the *chp2*<sup>+</sup> gene causes weak silencing defects at centromeres, the mating-type region, and telomeres (Halverson *et al*, 2000; Thon and Verheij-Hansen, 2000). These previous reports argue that each chromodomain protein has a specialized role for different heterochromatic regions, which may be determined by differential associations. However, in terms of the domain structure characterized by H3-K9 methylation and Swi6 localization, the three heterochromatic regions, the centromeres, mating-type region, and telomeres, are fairly similar. How these chromodomain proteins cooperatively or differentially function in the formation of higher-order chromatin structure remains elusive.



**Figure 1** Chromodomain proteins Chp1 and Chp2 associate with centromeres, the mating-type region, and telomeres in a *clr4*<sup>+</sup>-dependent manner. (A) Schematic diagram showing chromodomain proteins Chp1, Chp2, and Swi6. The chromodomain (CD) and its relative, the chromoshadow domain (CSD), are shown. (B–D) GFP-tagged Chp1 (B), Chp2 (C), and Swi6 (D) formed several nuclear spots in a *clr4*<sup>+</sup>-dependent manner. Cells were stained with Hoechst33342. (E–G) Chp1, Chp2, and Swi6 associated with centromeres (CEN), the mating-type region (MAT), and telomeres (TEL) in a *clr4*<sup>+</sup>-dependent manner. (E, F) DNA isolated from an anti-myc-immunoprecipitated chromatin fraction ( $\alpha$ -myc) or whole-cell extract (WCE) was used as a template for PCR-amplifying *CEN-dg223*, *MAT-K-R*, or *TEL-E12*. The samples were prepared from either wild-type or  $\Delta$ *clr4* cells expressing Chp1-13myc or Chp2-13myc (myc tag +). As a control, wild-type cells in which Chp1 or Chp2 was not tagged (myc tag–) were used. The ratio of *act1* to *CEN-dg223*, *MAT-K-R*, or *TEL-E12* signals in the ChIP results was calculated, and the relative fold enrichments are shown beneath each lane. (G) Association of Swi6 with the indicated heterochromatic regions was assayed by ChIP as in (E, F), using anti-Swi6 antibodies. As a control, *swi6*<sup>+</sup>-deleted cells ( $\Delta$ *swi6*) were used.

In this study, we started to analyze the localization of the Chp1 and Chp2 proteins. We demonstrated that Chp1 and Chp2 are structural components of the three heterochromatic regions. Although deletion of the *chp1*<sup>+</sup> gene caused centromere-specific silencing defects, Chp1 was essential for the establishment of repressive chromatin structure in all three heterochromatic regions. The establishment function has previously been described for the components of the RNAi machinery. These results suggest that different heterochromatic regions share common structural features and imply that centromeric heterochromatin has a dynamic structure and requires frequent establishment steps.

## Results

### **Chp1 and Chp2 associate with three heterochromatic regions**

Genetics experiments revealed that Chp1 and Chp2 are involved in heterochromatic silencing (Thon and Verheine-Hansen, 2000). Although both Chp1 and Chp2 associate with centromeres (Halverson *et al*, 2000; Partridge *et al*, 2000), their precise cellular or chromosomal distribution or intermolecular dependency is not well understood. To investigate the function of these chromodomain proteins, we first analyzed their cellular distribution. In fission yeast, three heterochromatic regions, centromeres, the mating-type region, and telomeres, are visualized by immunostaining of Swi6 or GFP-fused Swi6 (Ekwall *et al*, 1995; Sadaie *et al*, 2003). Two to five discrete Swi6 foci in interphase cells represent one large cluster of all centromeres, several clusters of telomeres, and the mating-type region (Ekwall *et al*, 1995). GFP was fused to the C-terminus of Chp1 or Chp2 by introducing a *gfp*<sup>+</sup>-*kanMX6* cassette, and the fusion proteins were expressed from their native promoter. Chp1-GFP formed two to three discrete spots in interphase nuclei (Figure 1B). This is consistent with a previous report in which GFP-Chp1 was expressed from the *nmt1* promoter (Doe *et al*, 1998). In the cells expressing Chp2-GFP protein, we were unable to detect any signals, presumably because of its low abundance (data not shown). However, when GFP-Chp2 was expressed from the *nmt1* promoter on an episomal pREP41 plasmid, it appeared to form several spots in the nuclei (Figure 1C). These localization patterns were indistinguishable from the signals for Swi6 (Figure 1D and Supplementary Figure 1), suggesting that both Chp1 and Chp2 localize to the three heterochromatic regions in fission yeast. To test whether these localizations were dependent on histone H3-K9 methylation, *clr4*-deleted strains (*Δclr4*) were used for the analyses. In the *Δclr4* strain, Chp1-GFP and GFP-Chp2 did not form nuclear spots, as was previously observed for Swi6-GFP (Figures 1B–D, *Δclr4*; Ekwall *et al*, 1996). These results suggested that the Chp1 and Chp2 proteins target heterochromatic regions through interactions with each chromodomain and methylated histone H3-K9 (Supplementary Figure 2).

To further examine whether Chp1 and Chp2 associate with the centromeres (*CEN*), mating-type region (*MAT*), and telomeres (*TEL*), a chromatin immunoprecipitation (ChIP) assay was performed. For this purpose, we constructed strains expressing 13myc-tagged Chp1 or Chp2 from its native promoter. These strains did not show any of the obvious phenotypes associated with the *Δchp1* or *Δchp2* mutation, suggesting that the myc-tagged proteins functionally replaced

the wild-type proteins (data not shown). In the ChIP assay, immunoprecipitated DNA was subjected to polymerase chain reactions (PCRs) in which each primer set amplified the centromeric, mating-type region, or telomeric DNA (*dg223*, *K-R*, or *E12* in Figures 1E–G). In addition, a primer set to amplify the *act1*<sup>+</sup> gene was included as an internal control to verify the enrichment. Since each cell contains multiple copies of *dg223* (*CEN*) or *E12* (*TEL*), and a single copy of *K-R* (*MAT*), we optimized the concentration of each primer set to obtain approximately the same amount of product as that of the *act1* gene in the control PCR experiments (Figures 1E–G, WCE). Consistent with previous reports (Halverson *et al*, 2000; Partridge *et al*, 2000), an enrichment of *CEN* was observed in the anti-myc immunoprecipitates for both the Chp1- and Chp2-13myc-tagged strains, compared with the total input chromatin (WCE) or precipitates from untagged strains (Figures 1E and F). Interestingly, the Chp1- and Chp2-immunoprecipitated complexes were also enriched for *MAT* and *TEL* (Figures 1E and F). The same results were obtained for Swi6 ChIP using anti-Swi6 antibodies (Figure 1G; Nakayama *et al*, 2000, 2001a; Partridge *et al*, 2000). In agreement with the cytological analyses, the high levels of *CEN*, *MAT*, and *TEL* in the ChIP assays were abolished in *Δclr4* (Figures 1E–G; Partridge *et al*, 2000). Taken together, these results indicate that both Chp1 and Chp2 associate with the three heterochromatic domains through *clr4*<sup>+</sup>-mediated H3-K9 methylation.

### **Chromodomain proteins make distinct contributions to the formation of heterochromatin**

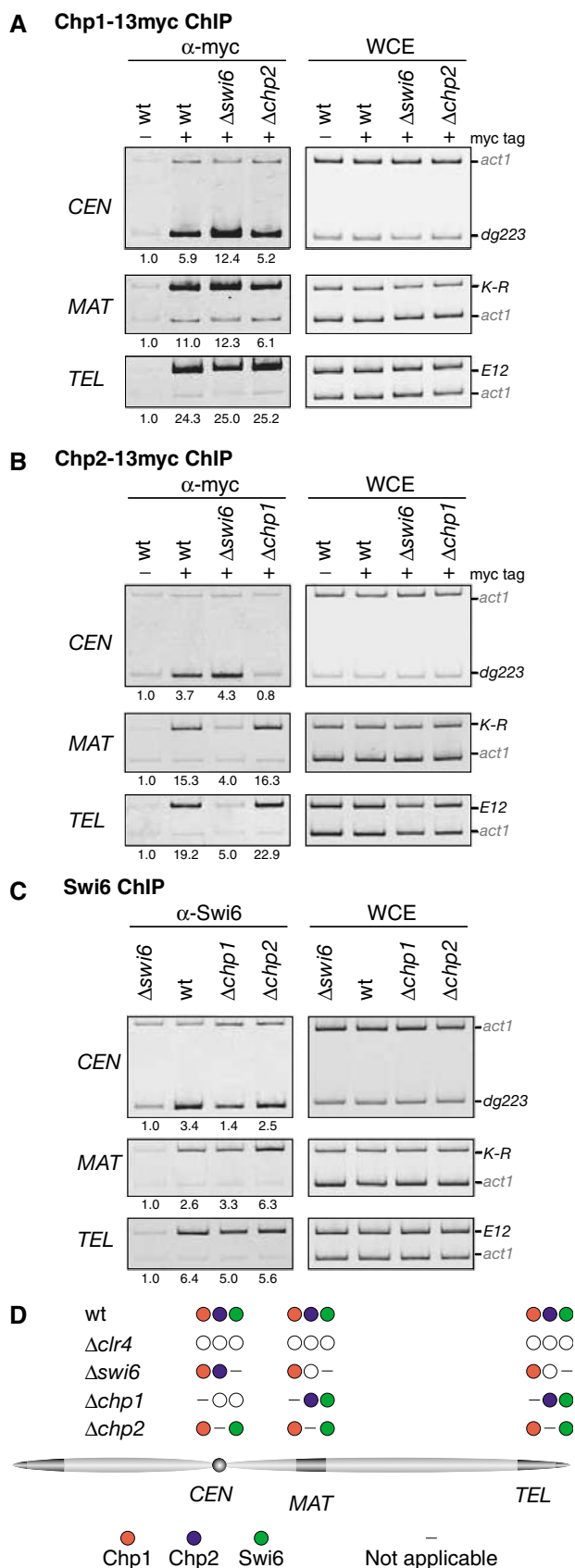
We next examined whether the heterochromatin association of Chp1, Chp2, or Swi6 was affected in each deletion mutant. It has been shown that Chp1 localization to centromeres is not affected in the *swi6* mutant, but Swi6 localization to centromeres depends on Chp1 (Partridge *et al*, 2000). The association of Chp1-13myc, Chp2-13myc, or Swi6 with heterochromatin was analyzed in the *Δchp1*, *Δchp2*, or *Δswi6* background by ChIP assay (Figure 2). We found that the association of Chp1-13myc with the three heterochromatic regions (*CEN*, *MAT*, and *TEL*) was not affected in the absence of *swi6*<sup>+</sup> or *chp2*<sup>+</sup> (Figure 2A). In contrast, the centromeric localization of Swi6 or Chp2-13myc was specifically decreased in the *Δchp1* cells (Figures 2B and C, *Δchp1*). When compared with the *Δswi6* strain, the centromeric association of Swi6 was not completely abolished in the *Δchp1* strain. Some Swi6 appeared to persist at the centromeric sequence. Interestingly, Swi6 was required for the localization of Chp2 to the mating-type region or telomeres but not to the centromeres (Figure 2B, *Δswi6*). Swi6 may act upstream of Chp2 at the mating-type region and telomeres. From these results (summarized in Figure 2D), it appears that Chp1, Chp2, and Swi6 make distinct contributions to heterochromatin formation. The changes in their heterochromatin localization were not caused by a reduction in the amount of each protein in the mutant cells, since the expression of Chp1, Chp2, or Swi6 protein was not affected in the *Δchp1*, *Δchp2*, *Δswi6*, or *Δclr4* cells (data not shown).

### **Chp1 is required for the establishment of heterochromatin**

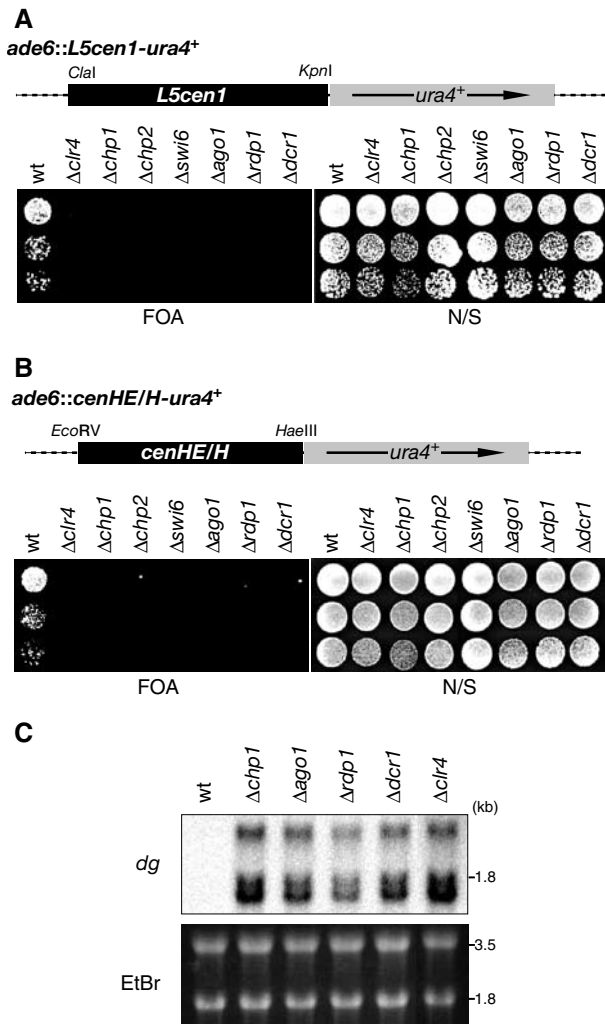
Chp1 protein plays a critical role in the localization of the Swi6 and Chp2 proteins to centromeric heterochromatin. It

has been shown that a centromere-specific silencing defect is correlated with a deficiency in the establishment of heterochromatin assembly. Factors involved in the RNAi machinery

(*rnai*) are required for the establishment of heterochromatin, and all of the  $\Delta rna1$  strains display defective gene silencing at centromeric heterochromatin, but not at the mating-type region or telomeres (Hall *et al*, 2002, 2003; Volpe *et al*, 2002). We therefore tested whether *chp1*<sup>+</sup> is required for the establishment of gene silencing. In wild-type cells, a DNA fragment from a centromere (*L5*), or the mating-type region (*cenH*) attracts heterochromatin factors when integrated at a euchromatic locus, resulting in the ectopic silencing of a neighboring reporter gene (Ayoub *et al*, 2000, 2003; Partridge *et al*, 2002). A previous report showed that Chp1 is required for the silencing and H3-K9 methylation at an ectopic site containing centromeric *L5* DNA (Partridge *et al*, 2002). If Chp1 plays a central role in the establishment of silent heterochromatin and its role is not specific for the centromeric sequences, the ectopic silencing mediated by *cis*-acting DNA from other heterochromatin regions might also be abrogated in the absence of Chp1. To test this possibility, we introduced the *L5cen1* or *cenHE/H* fragment with a *ura4*<sup>+</sup> reporter gene into a euchromatic *ade6* locus in the wild-type or  $\Delta chp1$  strain (Figures 3A and B). As the control, the same DNA constructs were introduced into the  $\Delta clr4$ ,  $\Delta swi6$ ,  $\Delta chp2$ , and  $\Delta rna1$  strains. The cells were spotted onto media containing 5-fluoroorotic acid (FOA, which is toxic to *ura4*<sup>+</sup>-expressing cells) to evaluate the *ura4*<sup>+</sup> reporter expression. As previously reported, the wild-type *ade6::L5cen1-ura4*<sup>+</sup> cells grew on FOA medium, but the other mutant cells did not, suggesting that ectopic silencing mediated by *L5cen1* was induced in the wild-type but not in the  $\Delta chp1$ ,  $\Delta clr4$ ,  $\Delta swi6$ ,  $\Delta chp2$ , or  $\Delta rna1$  strains (Figure 3A; Partridge *et al*, 2002; Volpe *et al*, 2003). The same assays were performed with strains carrying ectopic *cenHE/H* (*ade6::cenHE/H-ura4*<sup>+</sup>). We found that only the wild-type strain carrying *ade6::cenHE/H-ura4*<sup>+</sup> grew on the FOA medium (Figure 3B), indicating that ectopic silencing mediated by *cenHE/H* was not induced in the  $\Delta chp1$  and  $\Delta rna1$  strains, as is the case for ectopic *L5cen1* (Hall *et al*, 2002; Volpe *et al*, 2002). These results indicated that Chp1 protein is required for the silencing at the ectopic *L5cen1* or *cenHE/H* sequence, and also demonstrated that the silencing defect by  $\Delta chp1$  is not exclusive to the centromeric sequence. Although these results did not clarify Chp1's function in the establishment or maintenance step, subsequent experiments clearly showed that Chp1 participates in the establishment of the silent state (see below).



**Figure 2** Interdependency of chromodomain proteins to associate with heterochromatin. (A) Association of Chp1 with heterochromatic regions in the absence of *swi6*<sup>+</sup> or *chp2*<sup>+</sup>. Association of Chp1-13myc with the indicated heterochromatic regions was assayed by ChIP using anti-myc antibodies as in Figure 1. As a control, wild-type cells in which Chp1 was not tagged were used. (B) Association of Chp2 with heterochromatic regions in the absence of *swi6*<sup>+</sup> or *chp1*<sup>+</sup>. ChIP using anti-myc antibodies was performed as in (A). (C) Association of Swi6 with heterochromatic regions in the absence of *chp1*<sup>+</sup> or *chp2*<sup>+</sup>. ChIP was performed as in (A), using anti-Swi6 antibodies. As a control,  $\Delta swi6$  cells were used. (D) Summary of the results shown in (A)–(C). The localization of chromodomain proteins (filled circles) to each heterochromatic region in the indicated strains is depicted. A reduction in the heterochromatin association of the protein is indicated by an open circle.

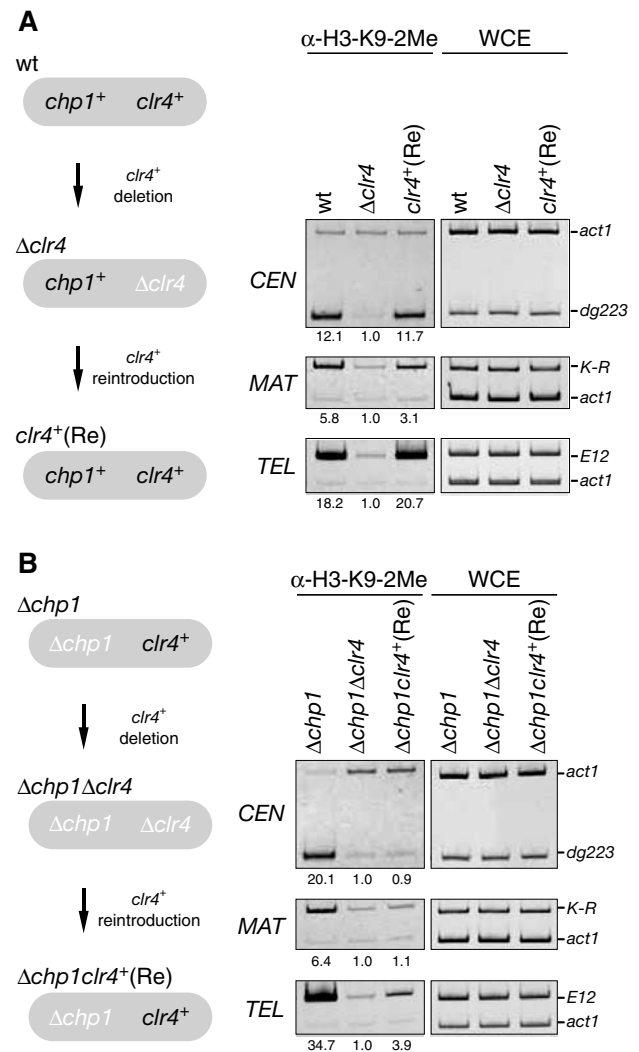


**Figure 3** *chp1<sup>+</sup>* is required for *L5cen1*- or *cenHE/H*-mediated gene silencing. (A) Deletions of *chp1<sup>+</sup>* or components of the RNAi machinery disrupt the silencing at ectopic *L5cen1*. A schematic representation of the *L5cen1-ura4<sup>+</sup>* construct inserted into the *ade6* locus is shown (top). Five-fold-diluted cultures of the indicated strains carrying *L5cen1-ura4<sup>+</sup>* were plated onto AA medium containing FOA (FOA) and nonselective (N/S) medium. (B) Deletions of *chp1<sup>+</sup>* or components of the RNAi machinery disrupted silencing at ectopic *cenHE/H. ura4<sup>+</sup>* silencing at *cenHE/H* inserted at the *ade6* locus was examined as in (A). (C) Transcripts derived from centromeric repeats were detected by Northern blotting using probes specific for centromeric *dg* repeats (top). Ethidium bromide-stained RNAs are shown as a loading control. The two major bands correspond to rRNAs (bottom).

It has been shown that deletion of components of the RNAi machinery results in the aberrant accumulation of long centromeric RNA transcripts (Volpe *et al.*, 2002). To verify the relationship between Chp1 and the RNAi factors, the centromeric transcripts in mutant strains were analyzed by Northern blotting. We found that the long centromeric transcripts accumulated in  $\Delta$ *chp1* and  $\Delta$ *clr4* (Figure 3C). The level of accumulated transcripts in the  $\Delta$ *chp1* cells was comparable to that in the  $\Delta$ *rna1* cells. Therefore, Chp1 protein was also involved in the production or processing of centromeric RNA transcripts, which might be linked to heterochromatin establishment.

We next investigated the effect of *chp1* deletion on the establishment of H3-K9 methylation at native heterochro-

matic regions. For this purpose, we performed a *clr4<sup>+</sup>* deletion/reintroduction experiment (Figures 4A and B, left panels), in which *clr4<sup>+</sup>* was deleted from either the wild-type or  $\Delta$ *chp1* strain (*clr4* deletion,  $\Delta$ *clr4*) followed by the integration of functional *clr4<sup>+</sup>* into the original locus (*clr4<sup>+</sup>* reintroduction, *clr4<sup>+</sup>* (Re)). At each step, the level of H3-K9 methylation at *CEN* (*dg223*), *MAT* (*K-R*), or *TEL* (*E12*) was monitored by ChIP analysis. In the wild-type (*chp1<sup>+</sup>*) background, *clr4<sup>+</sup>* deletion ( $\Delta$ *clr4*) caused a loss of the H3-K9 methylation at the three heterochromatic regions (Figure 4A). When *clr4<sup>+</sup>* was reintroduced, the methylation was restored to a level comparable to that of the original strain (Figure 4A). These results indicated that the reintroduced *clr4<sup>+</sup>* was functional and that the reintroduction was sufficient to establish H3-K9 methylation at the three heterochromatic regions. Similar to the wild-type strain, a deletion of *clr4<sup>+</sup>* in  $\Delta$ *chp1* cells caused the loss of H3-K9 methylation at all three heterochromatic regions (Figure 4B,  $\Delta$ *chp1 $\Delta$ *clr4*). However, in sharp contrast, the reintroduction of *clr4<sup>+</sup>* into*



**Figure 4** *chp1<sup>+</sup>* is required for the establishment of H3-K9 methylation at the three heterochromatic regions. *clr4<sup>+</sup>* was deleted from wild-type (wt) or  $\Delta$ *chp1* cells to generate  $\Delta$ *clr4* or  $\Delta$ *chp1 $\Delta$ *clr4*, respectively. Functional *clr4<sup>+</sup>* was then reintroduced (Re) and the level of H3-K9 methylation was assayed by ChIP using anti-H3-K9-2Me antibodies.*

the  $\Delta chp1\Delta clr4$  strain did not lead to the restoration of H3-K9 methylation in any of the three heterochromatic regions (Figure 4B,  $\Delta chp1clr4^+$  (Re)), except for a small amount of restored methylation in the telomeres (see Discussion). The expression of  $clr4^+$  mRNA in the  $\Delta chp1clr4^+$  (Re) strains was comparable to that in the original  $\Delta chp1$  strain (data not shown). We confirmed these results by testing another independently isolated  $\Delta chp1clr4^+$  (Re) clone to rule out the possibility that defects in the establishment of H3-K9 methylation were due to the colony isolation steps (data not shown). These results further supported the notion that Chp1 is required for the establishment of repressive chromatin at the native heterochromatic regions. The present data also demonstrate that Chp1 function is required not only for the centromeres but also for the mating-type region and telomeres.

### **Histone H3-K9 methylation in heterochromatin is maintained without Chp1 or the RNAi machinery**

Previous reports demonstrated that a mutation in *chp1* or any of the three RNAi-related genes abolishes H3-K9 methylation at a *ura4<sup>+</sup>* reporter gene inserted in centromeric outer repeat (*otr*) heterochromatin (Partridge *et al*, 2002; Volpe *et al*, 2002). Furthermore, the methylation level at centromeric *dg* repeats is reduced in each  $\Delta rna1$  strain (Volpe *et al*, 2002). Intriguingly, we found that, even in the  $\Delta chp1$  cells, histone H3 in native centromeric heterochromatin (*CEN-dg223* locus) remained methylated at lysine 9 (Figure 4B,  $\Delta chp1$ ). To clarify these differences, we compared the methylation level of H3-K9 at the *CEN-ura4<sup>+</sup>* reporter and *CEN-dg223* loci. Chromatin prepared from a strain in which a *ura4<sup>+</sup>* reporter gene was inserted into centromeric *otr* heterochromatin was immunoprecipitated with anti-dimethylated-H3-K9 antibodies, and then competitive PCR was used to determine whether the immunoprecipitates were enriched for the *CEN-ura4<sup>+</sup>* or *CEN-dg223* sequence (Figure 5). At the *CEN-ura4<sup>+</sup>* locus, H3-K9 methylation in the  $\Delta chp1$  strain was reduced to the same level as in the  $\Delta clr4$  strain (Figure 5B, *ura4<sup>+</sup>*). In contrast, at the *CEN-dg223* locus, the H3-K9 methylation level in the  $\Delta chp1$  strain was reduced, but the level was clearly higher than in  $\Delta clr4$  (Figure 5B, top panel). Several other *otr* loci, *CEN-dg660*, *-dg760*, and *-dh383*, were also subjected to the same analysis. Although the levels of the retained H3-K9 methylation were varied, we obtained similar results for all the endogenous loci (Figure 5B, bottom panel). The *chp1<sup>+</sup>* deletion did not affect H3-K9 methylation at the *MAT* and *TEL* heterochromatin (Figure 5B, top *MAT* and *TEL*). These results demonstrate that H3-K9 methylation at *CEN-dg-dh* loci is maintained, at least in part, without *chp1<sup>+</sup>*, while the H3-K9 methylation at the *ura4<sup>+</sup>* reporter locus apparently depends on *chp1<sup>+</sup>*. In  $\Delta swi6$  or  $\Delta chp2$  cells, the three heterochromatic regions were enriched in K9-methylated H3 at the same level as in wild-type cells (Figure 5B). We next examined whether H3-K9 methylation was maintained in the strains lacking RNAi genes. The results were similar to those observed in the  $\Delta chp1$  strain (Figure 5C). H3-K9 methylation was lost at the *ura4<sup>+</sup>* reporter region but was present to some extent at *CEN-dg223* in the  $\Delta rna1$  strains. There was no obvious decrease in the methylation level at *MAT* and *TEL*. Taken together, these results demonstrated that, even in the centromeric heterochromatin, some of the H3-K9 methylation is retained in the absence of Chp1 or RNAi components.

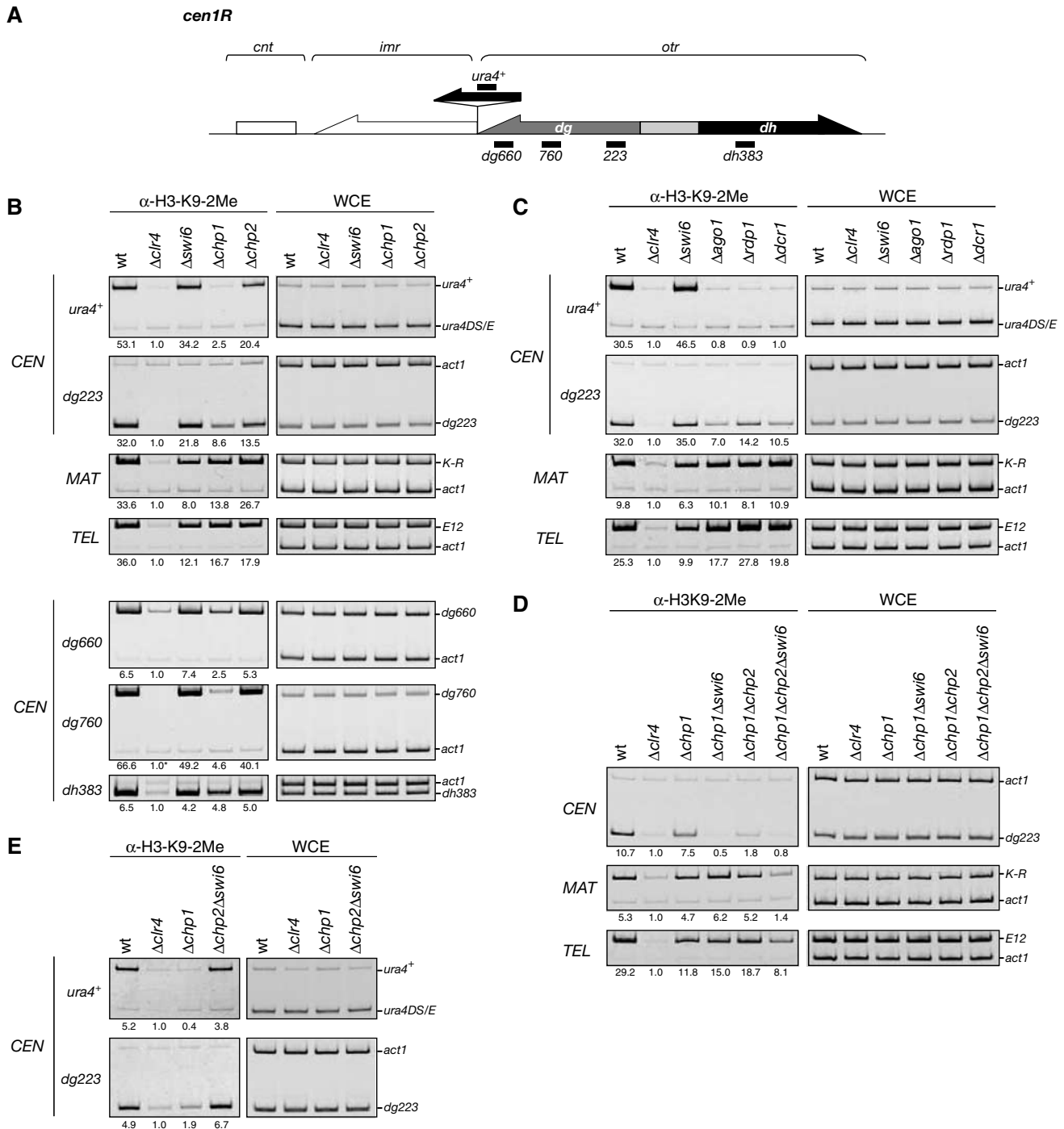
Furthermore, the differentially reduced H3-K9 methylation level at the *CEN-dg223* versus *CEN-ura4<sup>+</sup>* loci in the  $\Delta chp1$  and  $\Delta rna1$  strains suggests that both Chp1 and the RNAi machinery are involved in spreading and/or enhancing the H3-K9 methylation in centromeric heterochromatin.

### **Swi6 and Chp2 are required for the maintenance of H3-K9 methylation**

We have shown that H3-K9 methylation is maintained at all three heterochromatic regions even in the absence of Chp1 or RNAi components. We next sought to determine the factors responsible for maintaining the H3-K9 methylation. In the mating-type region, Swi6 has been shown to be required for the maintenance of H3-K9 methylation (Hall *et al*, 2002). Thus, we compared the methylation level of H3-K9 at the heterochromatic regions in the  $\Delta chp1$  and  $\Delta chp1\Delta swi6$  strains (Figure 5D). Interestingly, we found that *swi6<sup>+</sup>* deletion caused a loss of the H3-K9 methylation at *CEN (dg223)* in the  $\Delta chp1$  background (Figure 5D,  $\Delta chp1\Delta swi6$ ), suggesting that Swi6 is required for the maintenance of centromeric H3-K9 methylation in the  $\Delta chp1$  strain. Unexpectedly, the centromeric H3-K9 methylation was also severely decreased in the  $\Delta chp1\Delta chp2$  strain (Figure 5D,  $\Delta chp1\Delta chp2$ ). These results showed that both Swi6 and Chp2 are critical for maintaining the residual methylation at the centromeres. We also examined a triple-mutant strain ( $\Delta chp1\Delta chp2\Delta swi6$ ) for the maintenance of H3-K9 methylation. Interestingly, we found that, although deletion of either *swi6<sup>+</sup>* or *chp2<sup>+</sup>* did not affect the H3-K9 methylation at *MAT (K-R)* and *TEL (E12)* in the  $\Delta chp1$  background, the methylation level in these regions was severely decreased in the triple-mutant strain (Figure 5D,  $\Delta chp1\Delta chp2\Delta swi6$ ). Again, these results demonstrate that Swi6 and Chp2 are required for the maintenance of H3-K9 methylation at the three heterochromatic regions, and also indicate that Swi6 and Chp2 have overlapping functions in the maintenance of H3-K9 methylation. The synergistic effects of  $\Delta swi6$  and  $\Delta chp2$  were observed only in the  $\Delta chp1$  background (Figures 5D and E). Deletion of *chp2<sup>+</sup>* did not enhance the accumulation of centromeric transcript in the  $\Delta chp1$ ,  $\Delta swi6$ , or  $\Delta chp1\Delta swi6$  strains (Supplementary Figure 3). It should also be noted that, even in the triple-mutant strain, a reduced but detectable level of H3-K9 methylation persisted at the telomere, suggesting that another mechanism might function at the telomere (see Discussion).

### **Rik1 and Clr4 function cooperatively to promote heterochromatic H3-K9 methylation**

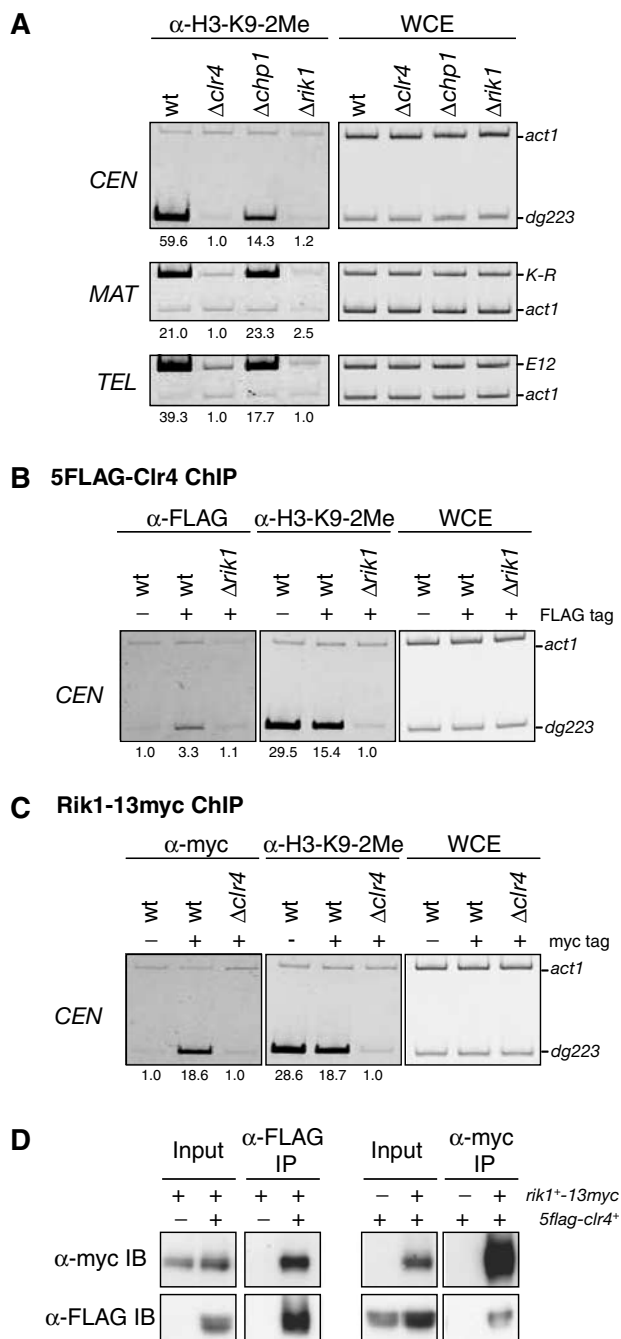
Methylation of H3-K9 is maintained without the Chp1-mediated establishment step. Although Swi6 and Chp2 cooperatively function to maintain the methyl modification, we observed residual methylation in the triple-mutant strain (Figure 5D). This suggested that other factors might help to promote methylation to the heterochromatin. Rik1, a WD-40 repeat-containing protein, is a candidate for such a factor, since it has been shown to be required for heterochromatic silencing (Allshire *et al*, 1995), and was later demonstrated to be critical for H3-K9 methylation at a *ura4<sup>+</sup>* reporter gene inserted in the centromeres or mating-type region (Nakayama *et al*, 2001b). Thus, to gain further insight into the mechanisms for maintaining H3-K9 methylation at the endogenous regions, we analyzed  $\Delta rik1$  strains. We found that H3-K9 methylation at the three heterochromatic regions (*CEN-*



**Figure 5** H3-K9 methylation is maintained at the three heterochromatic regions in the absence of Chp1 or components of the RNAi machinery. (A) Schematic diagram of the right half of centromere 1. Positions of the PCR products used to detect H3-K9 methylation in (B, C) are indicated by thick bars. (B, C) ChIP analysis was used to compare the levels of H3-K9 methylation in the indicated strains at centromeres (CEN), the mating-type region (MAT), or telomeres (TEL). (Asterisk) The ratio of *act1* to *CEN-dg760* signals present in whole-cell extract (WCE) instead of the ChIP results was used to calculate the relative fold enrichment, since the *CEN-dg760* signal in the ChIP of  $\Delta$ clr4 was undetectable. (D) Swi6 and Chp2 are required for the maintenance of H3-K9 methylation. H3-K9 methylation at centromeres (CEN), the mating-type region (MAT), and telomeres (TEL) in wild-type,  $\Delta$ clr4,  $\Delta$ chp1,  $\Delta$ chp1 $\Delta$ swi6,  $\Delta$ chp1 $\Delta$ chp2, or  $\Delta$ chp1 $\Delta$ chp2 $\Delta$ swi6 cells is shown. ChIP analysis was used to compare the level of H3-K9 methylation in the indicated strains. (E) The levels of H3-K9 methylation at centromeres in the indicated cells.

*dg223*, *MAT-K-R*, or *TEL-E12*) was reduced to a level comparable to that in  $\Delta$ clr4 (Figure 6A), suggesting that Rik1 has a critical role in H3-K9 methylation at the native heterochromatic regions. Since the phenotypes of  $\Delta$ rik1 strains are similar to those observed for the  $\Delta$ clr4 strain, we next

determined whether Rik1 contributes to the heterochromatin association of Clr4. For this experiment, strains expressing 5FLAG-tagged Clr4 from its native promoter were generated. In wild-type cells, 5FLAG-Clr4 associated with the centromeric CEN locus (Figure 6B, left panel, wt, FLAG tag+). In



**Figure 6** Clr4 and Rik1 interact with each other and act in concert to methylate H3-K9 at the three heterochromatic regions *in vivo*. (A) H3-K9 methylation at *CEN*, *MAT*, and *TEL* is abolished in  $\Delta clr4$  or  $\Delta rik1$ . (B) Clr4 associates with centromeres in a  $rik1^+$ -dependent manner. DNA isolated from the anti-FLAG- ( $\alpha$ -FLAG) or anti-K9-dimethylated H3- ( $\alpha$ -H3-K9-2Me) immunoprecipitated chromatin fraction or whole-cell extract (WCE) was used as a template for PCR. The DNA samples were prepared from either wild-type or  $\Delta rik1$  cells expressing 5FLAG-tagged Clr4. As a control, wild-type cells in which Clr4 was not tagged were used. (C) The association of Rik1 with centromeres depends on  $clr4^+$ . ChIP analysis with anti-myc antibodies was used to determine the presence of Rik1-13myc at centromeres. Wild-type,  $\Delta clr4$  cells expressing Rik1-13myc, or wild-type cells expressing untagged Rik1 were used. (D) Clr4 is co-immunoprecipitated with Rik1 *in vivo*. Anti-FLAG antibodies (left panels) or anti-myc antibodies (right panels) were used to immunoprecipitate 5FLAG-Clr4 or Rik1-13myc from a strain expressing both 5FLAG-Clr4 and Rik1-13myc (IP). 5FLAG-Clr4 or Rik1-13myc was detected by immunoblotting with anti-FLAG or anti-myc antibodies, respectively (IB).

contrast, the association of 5FLAG-Clr4 with *CEN* was disrupted in  $\Delta rik1$  cells (Figure 6B, left panel,  $\Delta rik1$ , FLAG tag +), suggesting that functional Rik1 is required for Clr4's association with centromeric heterochromatin. We further investigated Rik1's association with centromeres using strains expressing 13myc-tagged Rik1 from its endogenous promoter. Interestingly, the association between Rik1-13myc and *CEN* was detected in wild-type cells but was disrupted in  $\Delta clr4$  cells (Figure 6C). These results showed that Rik1 and Clr4 mutually affect each other's centromeric association. Finally, we examined whether Rik1 and Clr4 physically interact with each other. Whole-cell extracts prepared from a strain expressing both Rik1-13myc and 5FLAG-Clr4 were used for co-immunoprecipitation (co-IP) experiments. Strains expressing either Rik1-13myc or 5FLAG-Clr4 were used as controls. Rik1-13myc was efficiently co-precipitated with 5FLAG-Clr4, and the association was confirmed in the reciprocal IP experiments (Figure 6D). Collectively, these results suggested that Rik1 and Clr4 interact with each other and function cooperatively to promote H3-K9 methylation *in vivo*.

## Discussion

Chromodomain proteins play an important role in the formation of repressive chromatin domains. In the present study, we report the functional characterization of Chp1 protein and show that it is required for the establishment of heterochromatin and is functionally correlated with RNAi factors in *S. pombe*. Our analyses also reveal that two chromodomain proteins, Swi6 and Chp2, are involved in the maintenance of histone H3-K9 methylation.

### Chp1 and Chp2 are structural components of fission yeast heterochromatin

Consistent with the dot-like signals of the GFP fusion proteins, our ChIP analyses showed that both Chp1 and Chp2 stably associate with three known heterochromatic regions. As is the case for Swi6, this association is dependent on Clr4 histone methyltransferase. These results indicate that three chromodomain proteins, Swi6, Chp1, and Chp2, all participate in the formation of repressive chromatin at different chromosomal regions. In contrast, previous reports showed that Chp1 is crucial to centromeric silencing but not transcriptional silencing in the mating-type region or near telomeres (Thon and Verhein-Hansen, 2000). In support of this previous observation, we also found that  $\Delta chp1$  causes the delocalization of Swi6 and Chp2 specifically at centromeres. In this study, we showed that Chp1 is required for the establishment of H3-K9 methylation not only at centromeres but also at the mating-type region and telomeres (see Figure 4B). Given that Chp1 plays the same role in the different chromosomal regions, it is likely that centromeric heterochromatin needs to undergo the establishment steps more routinely than do the other regions, resulting in the centromere-specific defects of the  $\Delta chp1$  mutant. One possible explanation for the region-dependent effect might reside in the *cis*-acting DNA elements that enhance the stability of the higher-order structure. Interestingly, it has been reported that a 580-bp element *REII* located adjacent to *mat2* functions cooperatively with the *cenH* fragment to enhance silencing stability (Ayoub *et al*, 2000). A recent report also showed that ATF/CREB family proteins directly bind another *cis* element,



*REIII*, and help to nucleate heterochromatin (Jia *et al*, 2004). The presence of such additional *cis*-acting repression element(s) may cause  $\Delta$ *chp1* to have different effects on each chromosomal region. Another possibility is that the centromeric region contains transcriptionally competent elements at a high frequency, compared with the mating-type region or telomeres. Indeed, small RNAs corresponding to centromeric repeats have been cloned from *S. pombe* (Reinhart and Bartel, 2002). In addition, centromeric transcripts are produced from centromeric repeats, and these transcripts are markedly increased in RNAi mutants (this study; Volpe *et al*, 2002). Although transcriptional competency at the mating-type region or telomeres has not been extensively characterized, it is possible that the centromeric heterochromatin competes against transcriptional activities and therefore continuously requires the establishment event. Further experiments will be needed to address the importance of heterochromatin structure in the centromere functions.

### **Chp1 protein is required for the establishment of heterochromatin**

Previous reports showed that Chp1 associates with centromeres and is required for Swi6 localization and H3-K9 methylation at both centromeres and an ectopic site introduced by a centromeric *cis*-acting element (Partridge *et al*, 2000, 2002). These previous results indicated an important role of Chp1 in centromeric silencing. However, the general role of Chp1 in the assembly of silent chromatin has not been elucidated. From our results, it appears that the role of Chp1 is not exclusive to the centromeres. We showed that Chp1 is localized to three heterochromatic regions and is required for ectopic silencing mediated not only by the centromeric *cis* element *L5cen1* but also by the *cenHE/H* sequence from the mating-type region. Moreover, we demonstrated that Chp1 is required to re-establish H3-K9 methylation at all three heterochromatic regions (Figure 4B). These results suggest that Chp1 has a general role in establishing heterochromatin and also imply that the centromere-specific defect in *chp1*-deleted cells might be caused by the structural features of centromeres. Interestingly, a similar centromere-specific silencing defect is observed in RNAi mutant cells (Hall *et al*, 2002, 2003; Volpe *et al*, 2002). Elements of the RNAi machinery have been demonstrated to be required for the establishment of repressive chromatin (Hall *et al*, 2002). We also showed that aberrant centromeric transcripts accumulate in *chp1*-deleted cells, as previously observed in RNAi-deleted cells (Figure 3B). Considering these results, it appears that Chp1 is functionally correlated with the RNAi machinery. This idea is further strengthened by a recent report that Chp1, Ago1, a newly identified factor Tas3, and siRNAs form a RITS complex and are required for the H3-K9 methylation at centromeric heterochromatin (Verdel *et al*, 2004). It is thought that the RITS complex may associate with heterochromatin through sequence-specific base pairing, that is, of an siRNA–DNA or siRNA–nascent transcript, followed by the H3-K9 methylation, which may further stabilize the association (Verdel *et al*, 2004). Since aberrant centromeric transcripts accumulate in  $\Delta$ *clr4* or  $\Delta$ *chp1* cells (Figure 3C), it is possible that a stable RITS–heterochromatin association through a Chp1-methylated H3-K9 interaction is required for the efficient processing of centromeric transcripts by the RNAi machinery.

### **Methylation in heterochromatin is maintained without Chp1 or the RNAi machinery**

As described above, deletion of *chp1*<sup>+</sup> or the RNAi factor genes causes a loss of silencing and H3-K9 methylation at centromeres (Partridge *et al*, 2002; Volpe *et al*, 2002). Interestingly, we found that, by using *clr4*-deleted strains as a basal-level control, H3-K9 methylation was completely abolished at an inserted marker region, but persisted at the endogenous centromeric regions in both  $\Delta$ *chp1* and  $\Delta$ *rnai* cells. This result was confirmed using several sets of primers. These findings suggest that methyl modifications at native centromeric DNA can be maintained without the establishment step, which may partly explain why the mutations have little effect on the heterochromatin structure at the mating-type region and telomeres. For the maintenance of H3-K9 methylation, we showed that Swi6 and Chp2 play a critical role at the three heterochromatic regions. Although we did not clearly detect the centromeric localization of Swi6 or Chp2 in  $\Delta$ *chp1* cells, it appears that they are targeted to the residual H3-K9 methylation at centromeric sequences independent of Chp1 and function in the maintenance of H3-K9 methylation. In the centromeres, both Swi6 and Chp2 are required for the maintenance of the methylation in the  $\Delta$ *chp1* strain. It is likely that the reduced level or density of H3-K9 methylation in the  $\Delta$ *chp1* strain changes the centromeric associations of Swi6 and Chp2 and affects their overlapping functions. The  $\Delta$ *chp1* $\Delta$ *chp2* $\Delta$ *swi6* triple mutant displayed profound effects on all three heterochromatic regions. Notably, we found that H3-K9 methylation at the telomere (*E12*) was maintained at a clearly higher level in the triple-mutant than in the  $\Delta$ *clr4* strain. This observation indicated that telomeric heterochromatin might use another mechanism to recruit H3-K9 methylation. This possibility is supported by another finding that, in the *clr4*<sup>+</sup> deletion/reintroduction experiments, a small but detectable level of restored H3-K9 methylation was observed in the telomere sequence (Figure 4B). In addition to the heterochromatic configuration, telomeres consist of specialized DNA–protein complexes. Such telomere-specific protein(s) may have a function in recruiting H3-K9 methylation.

Another interesting observation in this study was that Rik1 plays a crucial role as a cofactor of Clr4. We showed that Rik1 associates with Clr4 and is required for its association with centromeric heterochromatin. Although it remains to be tested how Clr4 or Rik1 is recruited to heterochromatic regions, it is interesting to speculate that the Rik1–Clr4 association increases the heterochromatin binding affinity of the complex and that Swi6 and/or Chp2 promote this heterochromatin binding. Finally, we should point out that the methylation levels at an endogenous element and an inserted marker region were different in the  $\Delta$ *chp* and  $\Delta$ *rnai* strains. These differences in H3-K9 methylation state might be attributable to histone exchange. It is likely that nucleosomal histone H3 in the *ura4*<sup>+</sup> marker region is subjected to active replacement throughout the cell cycle, as is the case for *Drosophila* H3.3, which is thought to be a plausible way to remove methyl modifications (Ahmad and Henikoff, 2002). This result also supports the above idea that heterochromatin always competes against transcriptional activities, and suggests that Chp1 and RNAi factors are required for the spreading and/or enhancement of H3-K9 methylation from the *cis*-acting region to the surrounding region. Further studies are

necessary to elucidate the molecular mechanism of how Chp1 and RNAi factors spread the H3-K9 methylation.

## Materials and methods

### Strains and plasmids

Deletion and tagging to produce GFP, 13myc, or 5FLAG fusion proteins of endogenous *chp1*<sup>+</sup>, *chp2*<sup>+</sup>, *clr4*<sup>+</sup>, and *rik1*<sup>+</sup> were performed by a PCR-based gene targeting protocol (Bahler *et al*, 1998; Krawchuk and Wahls, 1999). To express GFP-Chp2 fusion protein from a multicopy plasmid, an EGFP coding sequence was amplified by PCR and cloned into the pREP41 plasmid (Basi *et al*, 1993) to create pYB228. cDNA of the *chp2*<sup>+</sup> coding sequence was amplified by PCR and inserted downstream of the EGFP gene of pYB228 to construct pYB232. For integration of an *LScen1-ura4*<sup>+</sup> or *cenHE/H-ura4*<sup>+</sup> fragment into the *ade6* locus, PCR-amplified *ura4*<sup>+</sup> (−187 to +1.24 kb) was cloned into the *PstI-SalI* site of the pBluescript SK(−) (pYB265). A PCR-amplified *Clal-KpnI* fragment from *cen1* (*LScen1*) (Partridge *et al*, 2002) or *EcoRV-HaeIII* fragment (*cenHE/H*) (Ayoub *et al*, 2000) was cloned into the *SmaI* site of pYB265 to create pYB277 or pYB285, respectively. The *BamHI-SpeI* fragment from *ade6*<sup>+</sup> was cloned into pBluescript SK(−) (pYB286) and *BamHI-SalI* fragments from pYB277 or pYB285 were inserted into the *NdeI* locus in *ade6*<sup>+</sup> of pYB286 to generate pYB289 or pYB288, respectively. pYB289 and pYB288 were digested with *XhoI* and used for the transformation of wild-type and mutant cells. For the *clr4*<sup>+</sup> reintroduction experiments, the *clr4*<sup>+</sup> genomic sequence was PCR-amplified and cloned into the pCRII-TOPO plasmid (*clr4*-pTP). A hygromycin resistance gene (*hyg*<sup>r</sup>) was inserted into *clr4*-pTP using the *EcoRV* and *NotI* sites (*clr4-hyg*-pTP). The *clr4-hyg*-pTP plasmid was linearized by digesting it with the *HpaI* site in the *clr4*<sup>+</sup> genomic region and introduced into the  $\Delta$ *clr4* strains. All other strains were constructed by standard genetic crosses. The strains used in this study are listed in Supplementary Table 1.

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### Chromatin immunoprecipitation

ChIP was performed as described previously (Nakayama *et al*, 2000). To immunoprecipitate 13myc- or 5FLAG-tagged proteins, anti-c-myc (9E10, Roche) or anti-FLAG (M2, Sigma) monoclonal antibodies were used. To immunoprecipitate Swi6 or histone H3 dimethylated at K9, anti-Swi6 or anti-H3-K9-2Me monoclonal antibodies were used. PCR products were separated on a 5% nondenaturing polyacrylamide gel, stained with ethidium bromide, and photographed by Printgraph equipped with Image Saver HR (ATTO). The primer sets used are listed in Supplementary Table 2.

### RNA isolation and Northern hybridization

Total RNA was extracted from cells as described by Schmitt *et al* (1990). Purified total RNA samples were separated on a 1.25% agarose gel containing 6.7% formaldehyde, blotted to nylon membranes, crosslinked, and hybridized with PCR-amplified centromeric *dg* sequence (Volpe *et al*, 2002).

### Co-immunoprecipitation

Cells expressing both FLAG-tagged Clr4 and myc-tagged Rik1 were collected, and extracts were prepared as described (Iida and Araki, 2004). FLAG-Clr4 or Rik1-myc was immunoprecipitated with and detected by an anti-FLAG antibody or anti-c-myc antibody, respectively.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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