Phosphorylation of Swi6/HP1 regulates transcriptional gene silencing at heterochromatin

Atsushi Shimada,[1,2] Kohei Dohke,[1,2,6] Mahito Sadaie,[3,7] Kaori Shinmyozu,[4] Jun-Ichi Nakayama,[3] Takeshi Urano,[5] and Yota Murakami[1,8]

[1]Laboratory of Signal Transduction, Department of Cell Biology, Institute for Virus Research, Kyoto University, Kyoto, Kyoto 606-8507, Japan; [2]Laboratory of Cell Regulation and Molecular Network, Division of Systemic Life Science, Graduate School of Biostudies, Kyoto University, Kyoto, Kyoto 606-8501, Japan; [3]Laboratory for Chromatin Dynamics, RIKEN Center for Developmental Biology, Kobe, Hyogo 650-0047, Japan; [4]Proteomics Support Unit, RIKEN Center for Developmental Biology, Kobe, Hyogo 650-0047, Japan; [5]Department of Biochemistry, Shimane University School of Medicine, Izumo, Shimane 693-8501, Japan

Heterochromatin protein 1 (HP1) recruits various effectors to heterochromatin for multiple functions, but its regulation is unclear. In fission yeast, a HP1 homolog Swi6 recruits SHREC, Epe1, and cohesin, which are involved in transcriptional gene silencing (TGS), transcriptional activation, and sister chromatid cohesion, respectively. We found that casein kinase II (CK2) phosphorylated Swi6. Loss of CK2-dependent Swi6 phosphorylation alleviated heterochromatic TGS without affecting heterochromatin structure. This was due to the inhibited recruitment of SHREC to heterochromatin, accompanied by an increase in Epe1. Interestingly, loss of phosphorylation did not affect cohesion. These results indicate that CK2-dependent Swi6 phosphorylation specifically controls TGS in heterochromatin.

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Heterochromatin is a silent chromatin structure of centromeres and telomeres whose formation is specified by the methylation of histone H3 Lys 9 (H3K9me) that binds heterochromatin protein 1 (HP1). Heterochromatin dynamically participates in many nuclear functions that are regulated by coordinated recruitment of effectors to heterochromatin by HP1/Swi6 (Grewal and Jia 2007). It is recently shown that HP1 has various modifications and that phosphorylation of HP1γ by protein kinase A participates in euchromatic domain gene activation through interaction with Ku70 (Lomberk et al. 2006). Based on these observations, the existence of an HP1-mediated "silencing subcode" that underlies the instructions of the histone code is postulated (Lomberk et al. 2006). But it is totally unknown whether such HP1 modifications indeed regulate heterochromatin function through differential recruitment of effectors.

Fission yeast is a good model organism for heterochromatin analysis because of its well-conserved but simple heterochromatin. In fission yeast, the HP1 homolog Swi6 recruits silencing complex SHREC (Yamada et al. 2005; Sugiyama et al. 2007) and transcriptional activator Epe1 (Zofall and Grewal 2006; Isaac et al. 2007) for control of heterochromatic transcriptional gene silencing (TGS). In addition, Swi6 recruits cohesin that is involved in sister chromatid cohesion (Bernard et al. 2001; Nonaka et al. 2002). However, the regulatory mechanism for the recruitment is not understood.

Results and Discussion

In attempts to identify novel heterochromatin-related genes based on the localization of their gene products (see Supplemental Material) in fission yeast, we found that the disruption of *ckb1* alleviated the silencing of marker genes inserted in centromeric heterochromatin (Fig. 1A,B). The extent of the silencing defects in *ckb1Δ* cells was comparable with that caused by disruption of other heterochromatic genes, including *clr4*, a histone H3K9-specific histone methyltransferase; *clr3*, a histone deacetylase and a subunit of SHREC; and *swi6*, a HP1 homolog (Fig. 1B).

Consistent with the silencing defects observed in Fig 1B, *ade6* or *ura4* mRNA was increased in *ckb1Δ* cells (Supplemental Fig. S1). The mRNA increase largely occurred at the transcriptional level, since deletion of *ckb1* caused a significant increase in RNA polymerase II (RNAPII) on the marker gene, as judged by the chromatin immunoprecipitation (ChIP) assay (Supplemental Fig. S2A). At centromeric *dh* repeats, RNAPII transcribes bidirectional noncoding RNA (ncRNA) (Djupedal et al. 2005; Kato et al. 2005); the ncRNA is processed to siRNA that is important for RNAi-dependent heterochromatin formation (Volpe et al. 2002). The reverse transcript was transcribed in, while the forward transcription (*dh*for) was repressed by, heterochromatin (Volpe et al. 2002), as indicated by an increase of *dh*for transcription in *clr4Δ* and *swi6Δ* cells (Supplemental Fig. S1). In *ckb1Δ* and *clr3Δ* cells, *dh*for transcript levels were also significantly increased to levels similar to those observed in other mutants (Supplemental Fig. S1).

We next used a ChIP assay to analyze the centromeric heterochromatin structure in *ckb1Δ* cells by measuring the levels of H3K9me and Swi6. Both H3K9me and Swi6 were enriched on centromeric repeats (*dg223*) in *ckb1Δ* cells similar to wild-type or *clr3Δ* cells (Fig. 1C). This indicates that the heterochromatin structure at the native heterochromatin locus is preserved in both mutants, despite their alleviated silencing of *dh*for transcription (Supplemental Fig. S1). In contrast, levels of H3K9me and Swi6 at *imr::ura4* were significantly

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Present addresses: [6]Graduate School of Natural Sciences, Nagoya City University, Mizuho-ku, Nagoya 467-8601, Japan; [7]Cambridge Research Institute, Cancer Research UK, Robinson Way, Cambridge CB2 0RE, United Kingdom.
[8]Corresponding author.
E-MAIL yota@virus.kyoto-u.ac.jp; FAX 81-75-751-3975.
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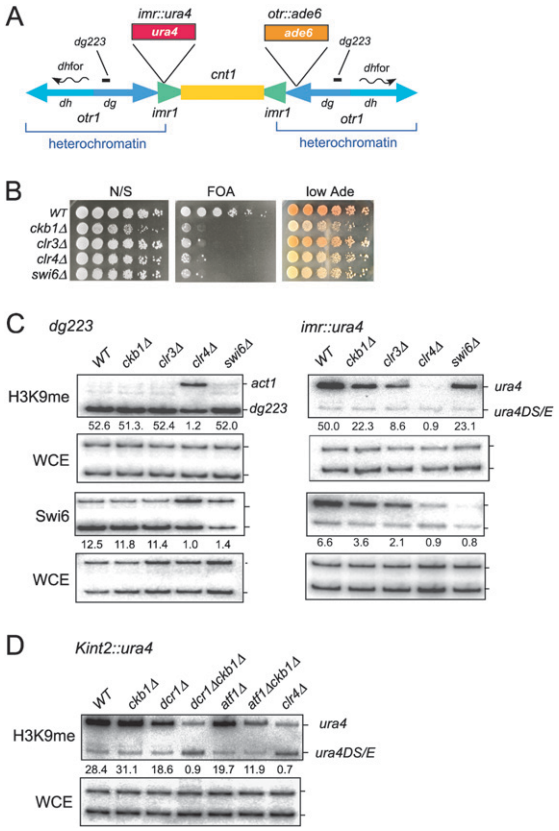

Figure 1. The Ckb1 mutant showed defects in heterochromatic TGS but not in heterochromatin structure. (*A*) Schematic representation of *imr::ura4* and *otr::ade6* in chromosome 1 used for silencing assay. A heterochromatic ncRNA (*dh*for) and the position of amplified PCR fragment (*dg223*) are also indicated. (*B*) Silencing at centromeric heterochromatin of *ckb1Δ* strain and other heterochromatic mutants. The marker genes inserted in heterochromatin are repressed by the H3K9-me/Swi6-dependent heterochromatin, resulting in red colony formation on low-adenine plates (repression of *ade6*) and resistance to FOA (repression of *ura4*) in wild-type cells. Disruption of the silencing caused white or pink colony formation and FOA sensitivity as observed in mutant cells. (*C*) Heterochromatin structure at centromeric repeats (*dg223*) and inserted marker gene (*imr::ura4*) in the indicated strains were analyzed by measuring levels of H3K9me and Swi6 using ChIP assay with antibodies against dimethyl H3K9 and Swi6. The relative enrichment at the target loci (*dg223* or *imr::ura4*) signal against the euchromatic region (*act1* and *ura4DS/E* minigene) is normalized to WCE (whole-cell extract) signal and shown *below* each lane. Note that larger amounts of samples were loaded on the gel for ChIP assay for H3K9me in *clr4Δ* cells and for Swi6 in *Δswi6* cells at *dg223* for easier quantification. (*D*) Ckb1 functions in the Atf1 pathway at the mating locus heterochromatin formation. Enrichment of H3K9me on the *ura4* gene inserted in the mating locus (*Kint2::ura4*) was analyzed by ChIP assay in the indicated strains. Relative enrichment at *Kint2::ura4* against euchromatic *ura4DS/E* is shown *below* each lane as *A*.

decreased in *clr3Δ* and *ckb1Δ* cells (Fig. 1C). Because heterochromatin formation on the marker gene depends on Clr3-dependent spreading of the H3K9me and Swi6 from flanking heterochromatin (Yamada et al. 2005), this result suggests that Ckb1 is involved in the spreading of heterochromatin.

The similarity of the phenotypes between *ckb1Δ* and *clr3Δ* cells (Fig. 1B,C) suggests that Ckb1 and Clr3 function similarly in heterochromatin formation/function. Two parallel pathways operate in heterochromatin formation at the mating locus: a transcription factor, Atf1/Pcr1-dependent pathway, and an RNAi-directed pathway (Jia et al. 2004; Yamada et al. 2005). H3K9me on the *ura4* inserted at the mating locus heterochromatin (*Kint2::ura4*) was hardly affected by deletion of either *atf1* or *dcr1*, an essential component of the RNAi-directed pathway (Fig. 1D). Clr3 acts in the Atf1/Pcr1-pathway, since H3K9me on *Kint2::ura4* was diminished in *dcr1Δclr3Δ* but not in *atf1Δclr3Δ* cells (Yamada et al. 2005). Similarly, H3K9me on *Kint2::ura4* was diminished in *dcr1Δckb1Δ* but still retained in *atf1Δckb1Δ* cells (Fig. 1D). Therefore, Clr3 and Ckb1 function similarly in Atf1/Pcr1-dependent heterochromatin formation at the mating locus.

Since Ckb1 is a regulatory subunit of casein kinase II (CK2) (Roussou and Draetta 1994), the above results suggest that CK2 phosphorylated heterochromatic proteins in order to regulate TGS. Considering that CK2 phosphorylates *Drosophila* HP1 in vitro (Zhao and Eissenberg 1999) and that Swi6 is a phospho-protein in vivo (Bailis et al. 2003), We analyzed the phosphorylation of Swi6 in *ckb1Δ* cells and in *orb5-19* cells, which have temperature-sensitive CK2 activity (Snell and Nurse 1994). The mobility of Swi6 in the Western blot increased in *ckb1Δ* cells, resulting in multiple bands (Fig. 2A). The mobility of Swi6 in *orb5-19* cells was increased even at permissive temperatures and was enhanced at 36°C (Fig. 2A). The mobility changes in both mutants were quite similar to those observed with calf intestine alkaline phosphatase (CIP)-treated extract (Fig. 2B). The CIP-induced mobility shift was suppressed by the addition of a phosphatase inhibitor (Fig. 2B), indicating that this shift was caused by Swi6 dephosphorylation.

We next examined whether Swi6 was phosphorylated by CK2 in vitro. CK2 was precipitated with an anti-Flag antibody from cells expressing Flag-tagged Cka1 (also known as Orb5) (Snell and Nurse 1994), a catalytic subunit of CK2 (Roussou and Draetta 1994; Snell and Nurse 1994). CK2 prepared from wild-type cells phosphorylated casein efficiently, while that from *ckb1Δ* cells phosphorylated casein less efficiently (Fig. 2C) as reported previously (Roussou and Draetta 1994), though both CK2 preparations contained a similar amount of Cka1 (Supplemental Fig. S3). Similarly, CK2 efficiently phosphorylated bacteria-prepared Swi6 in a Ckb1-dependent manner, resulting in slower migrating bands in SDS-PAGE (Fig. 2C). In addition, we found that the under-phosphorylated form of Swi6 was coprecipitated with Cka1-Flag (Fig. 2D), further supporting that Swi6 is a direct target of CK2 in vivo.

To analyze the role of CK2-directed phosphorylation in vivo, we introduced a series of amino acid substitutions at 10 potential CK2 phosphorylation sites (Pinna 2002) of genomic *swi6* (Fig. 3A) using PCR-based mutagenesis (see the Supplemental Material). In these mutants, we substituted the series of the putative phosphorylation sites with alanine, thereby preventing phosphorylation. Mutations at the five CK2 sites located in the N-terminal half (S18-117A in Fig. 3A) resulted in an increase in mobility, whereas mutant Swi6 harboring S18A and S24A showed a slight mobility change, suggesting different contribution of each phosphorylation site to the mobility change. The lesser mobility change in *ckb1Δ* cells than that of Swi6-S18-117A would reflect the partial phosphorylation of Swi6 by residual activity of CK2 in *ckb1Δ* cells (Fig. 2A,C). Conversely, mutations at the five CK2 sites located in the C-terminal region of Swi6 (Fig.

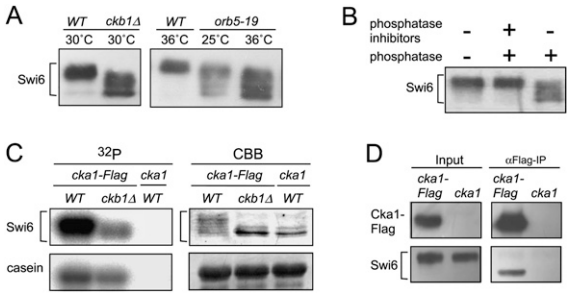

Figure 2. CK2 phosphorylates Swi6 in vitro and in vivo. (*A*) Cell extracts were prepared from wild-type, *ckb1Δ*, and *orb5-19* cells grown at the temperatures indicated. *orb5-19* is a mutant of the catalytic subunit of CK2 (Cka1) that displays temperature-sensitive CK2 activity (Snell and Nurse 1994). Swi6 proteins in the extracts were analyzed by Western blotting. (*B*) Swi6 immunoprecipitated from wild-type cell extracts were incubated with or without CIP. Phosphatase inhibitors were added where indicated. Reaction mixtures were separated with SDS-PAGE, and Swi6 was detected by Western blotting. (*C*) Bacterially produced Swi6 or dephosphorylated α-casein was incubated with Flag-tagged Cka1-containing CK2, immunoprecipitated from the strains indicated. Immunoprecipitates from cells without tagged Cka1 were used as a control. The reaction products were analyzed by SDS-PAGE. Swi6 and α-casein were detected by autoradiography and Coomassie Brilliant Blue (CBB) staining. (*D*) Cka1 was immunoprecipitated from cells expressing Cka1 with or without Flag-tag. The precipitated Flag-Cka1 and Swi6 were detected by Western blotting.

3A) (*swi6-S192-274A*) did not influence the mobility of Swi6 (Fig. 3B). The glutamic acid substitutions (*S18-24E*, *S18-52E*, or *S18-117E*), which were used to mimic the negative charge of phosphorylated serine, did not affect the mobility of Swi6 in the gel (Supplemental Fig. S4A,B). This implies that the mutated sites are targets for phosphorylation and the increase of negative charge by phosphorylation is responsible for the slow mobility of Swi6 in the gel.

Mass spectrometric analysis of Swi6 prepared from wild-type and *ckb1Δ* cells showed that five potential CK2 phosphorylation sites (S18, S24, S46, S52, and S117) in the N-terminal half are really phosphorylated in vivo, and the phosphorylation of four of them (S18, S24, S46, and S117) decreased in *ckb1Δ* cells (Supplemental Fig. S5). Specially, clear phosphorylation at S18 and S24 was detected in wild-type cells, but not in *ckb1Δ* cells. In contrast, only one site (S212) out of five potential CK2 phosophorylation sites in the C-terminal half was phosphorylated (Supplemental Fig. S5). Although the mass spectrometric analysis was not quantitative and might not detect all phosphorylation sites, the results are consistent with those obtained with phosphorylation-site mutants described above (Fig. 3A,B; Supplemental Fig. S4A,B).

swi6-S192-274A mutations did not affect silencing at the centromere (*imr::ura4*), while *swi6-S18-24A* and *S18-117A* mutants displayed decreases in silencing (Fig. 3C). Since both mutants indicate a similar level of silencing defects, phosphorylation of S18 and S24 seems to be important for TGS. In contrast, the glutamic substitution mutants (*swi6-S18-24E*, *swi6-S18-52E*, *swi6-S18-117E*) moderately alleviated silencing (Supplemental Fig. S4C), implying that phosphorylated serine itself is involved in Swi6-dependent TGS. Notably, as the number of glutamic substitutions increased, silencing defects appeared to increase (Supplemental Fig. S4C). This suggests that most of all CK2 phosphorylation sites in the N-terminal half of Swi6 contribute to silencing. All together, these results show that phosphorylation by CK2 at the N-terminal region of Swi6 is required for heterochromatic silencing at centromeres. Therefore, we used *swi6-S18-117A* as the nonphosphorylated mutant of *swi6* throughout the remainder of this study.

Another HP1 homolog, Chp2, also plays an important role in heterochromatin (Halverson et al. 2000; Thon and Verhein-Hansen 2000; Sadaie et al. 2008). Gel mobility analyses suggested that, like Swi6, Chp2 was phosphorylated in CK2-dependent manner in vivo (Supplemental Fig. S6A,B), indicating that phosphorylation of Chp2 is under the control of CK2. However, Chp2 was not phosphorylated by CK2 in vitro (Supplemental Fig. S6C), despite the existence of many putative phosphorylation sites (Supplemental Fig. S7A), for unknown reasons. Mutations at putative CK2 phosphorylation sites in the N-terminal half (*chp2-S15-172A*) or the middle (*chp2-S249-300A*) of Chp2 did not affect the mobility of the protein in the gel or the silencing of centromeric heterochromatin (Supplemental Fig. S7B,C). Mutant Chp2, harboring amino acid substitutions from serine to alanine or to glutamic acid at phosphorylation sites in the chromo-shadow domain (S323 and S354, Supplemental Fig. S7A), showed marked instability in vivo, which caused significant silencing defects (Supplemental Fig. S7B,C). Therefore, we could not definitively determine whether Chp2 is a direct target of CK2. Determination of the in vivo phosphorylation sites of Chp2 by mass spectrometric analysis was not done, since expression of Chp2 is much lower than Swi6 (Sadaie et al. 2004), and we could not get enough Chp2 proteins for the analysis.

swi6-S18-117A mutant cells showed similar phenotypes to *ckb1Δ* cells with regard to TGS and heterochromatin structure. Both cell types displayed increased transcription from marker genes inserted in the heterochromatin (Fig. 4A) accompanied by an increased occupancy of RNAPII

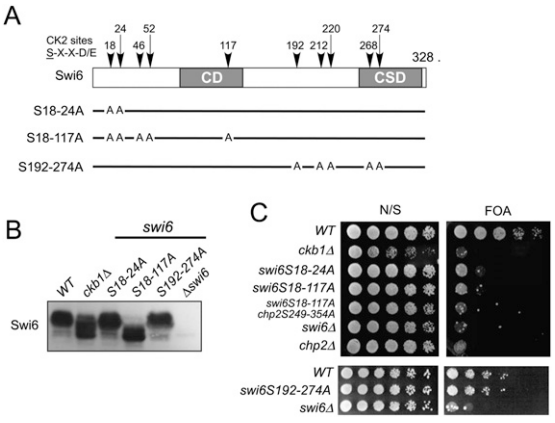

Figure 3. Mutants of the putative CK2 phosphorylation sites of Swi6 showed defects in phosphorylation and heterochromatic silencing. (*A*) Schematic diagram of the structure of Swi6 and the location of mutations introduced at the putative CK2 phosphorylation sites (S-X-X-D/E) in each mutant. The amino acid substitutions from serine (S) to alanine (A) were introduced into the genomic *swi6* gene by PCR-mediated mutagenesis and homologous recombination (see the Supplemental Material). (*B*) Western blotting analysis of Swi6 in the extract prepared from the strains indicated. (*C*) Heterochromatic silencing of the indicated strains were analyzed as described in Figure 1B.

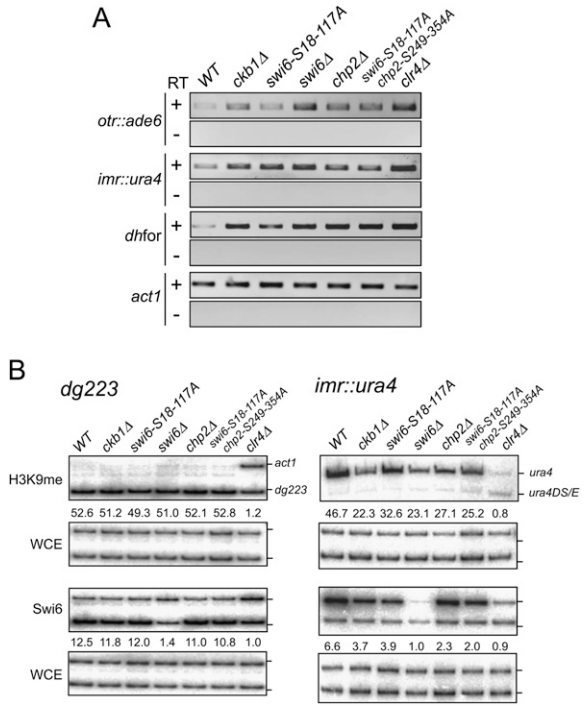

Figure 4. A CK2 phosphorylation sites mutant of Swi6 showed a similar phenotype to *ckb1Δ* cells. (*A*) The level of transcripts generated from marker genes (*otr::ade6* and *imr::ura4*) and centromeric noncoding RNA (*dh*for) in the strains indicated were analyzed by RT–PCR using total RNA prepared from the indicated strains in the presence (+) or absence (−) of RT. *act1* RNA was analyzed as a control. (*B*) The heterochromatin structure at the centromeric repeats (*dg223*) and the inserted marker gene (*imr::ura4*) in the indicated strains were analyzed by measuring levels of H3K9me and Swi6 using the ChIP assay. The level of RNAPII on *imr::ura4* was also analyzed. The relative enrichment at target loci signal (*dg223* or *imr::ura4*) against the euchromatic region (*act1* and *ura4DS/E*) is normalized to WCE (whole-cell extract) signal and shown *below* each lane. Note that larger amounts of samples were loaded on the gel for ChIP assay of *clr4Δ* cells at *dg223* for easier quantification.

(Supplemental Fig. S2B), increased centromeric ncRNA (Fig. 4A, *dh*for), and unaffected centromeric heterochromatin (Fig. 4B, *dg223*), but disturbed spreading of heterochromatin on the inserted *ura4* gene (Fig. 4B, *imr::ura4*). These results indicate that the phenotypes observed in *ckb1Δ* cells are mainly caused by decreased phosphorylation of Swi6 by CK2. Furthermore, immunofluorescence analysis indicated that both wild-type Swi6 and Swi6-S18-117A protein formed similar multiple foci in nuclei, which represents the heterochromatic loci. This confirms the preservation of heterochromatin structure in *swi6-S18-117A* cells (Supplemental Fig. S8).

As shown in Figure 1, the phenotypes of the *ckb1Δ* mutant resemble those of *clr3Δ*. Clr3 is a subunit of SHREC, an effector complex for heterochromatic TGS and localizes at heterochromatic domains in a Swi6- and Chp2-dependent manner (Yamada et al. 2005; Sugiyama et al. 2007; Sadaie et al. 2008). Therefore, our results suggest that SHREC requires CK2-dependent phosphorylation of Swi6 to localize at the heterochromatic domain. To examine this hypothesis, localization of Clr3 and Mit1, another subunit of SHREC (Sugiyama et al. 2007), was analyzed in *ckb1Δ* and *swi6-S18-117A* cells (Fig. 5A). In wild-type cells, both Clr3 and Mit1 were enriched on centromeric repeats; this localization was decreased in *swi6Δ* or *chp2Δ* cells, though substantial Mit1 was retained in *swi6Δ* cells, as reported previously (Sadaie et al. 2008). Similarly, Clr3 and Mit1 localization was decreased in *ckb1Δ* and *swi6-S18-117A* mutants, although this decrease was less in *swi6-S18-117A* than in *ckb1Δ* cells. Introduction of *chp2-S249-354A* to *swi6-S18-117A* mutants reduced Clr3 and Mit1 enrichment to a level similar to that observed in *ckb1Δ* cells. These results support the idea that phosphorylation of Swi6 by CK2 is required for efficient recruitment of SHREC. We could not detect coprecipitation of Clr3 with Swi6 or Chp2 in vivo (Sadaie et al. 2008), though Swi6 had been shown to coprecipitate with Clr3 (Yamada et al. 2005). We speculate that the interaction of Clr3 with Swi6 is indirect and/or dynamic and difficult to detect in vivo. Interestingly, Clr3 could be pulled down from cell extract by GST-Chp2, but not by Swi6 (Sadaie et al. 2008). Thus, CK2-dependent phosphorylation of Chp2 may also contribute to the recruitment.

Swi6 also recruits Epe1, which antagonizes TGS and spreading of heterochromatin when it is overexpressed (Ayoub et al. 2003; Zofall and Grewal 2006; Isaac et al. 2007). We examined the effect of various mutations on

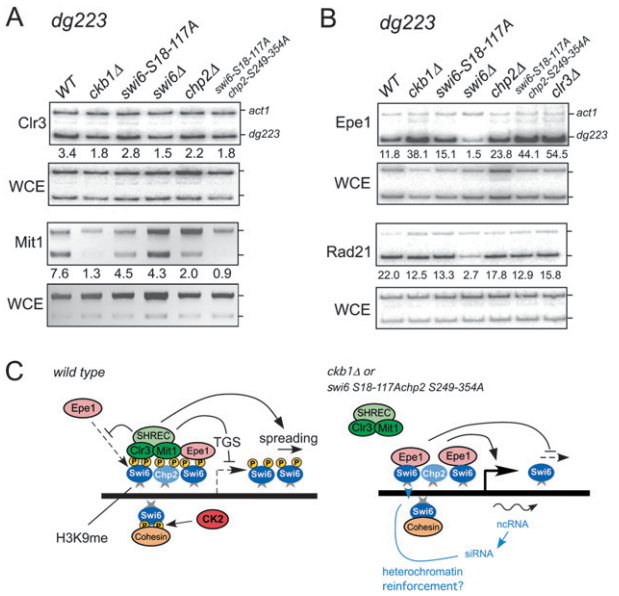

Figure 5. CK2-dependent Swi6 phosphorylation regulates binding of the effector to heterochromatin. (*A*) Localization of Clr3 and Mit1, components of the silencing complex SHREC, on centromeric repeats was examined in the indicated strains by ChIP assay. The Flag-tagged *clr3* gene or Myc-tagged Mit1 was introduced into each strain, and a ChIP assay was performed with an anti-Flag or anti-Myc antibodies. Relative enrichment at target locus signal (*dg223*) against the euchromatic region (*act1*) is normalized to WCE (whole-cell extract) signal and is indicated *below* each lane. (*B*) Localization of Epe1 and Rad21 on centromeric repeats (*dg223*) was analyzed by ChIP assay. Either Flag-tagged Epe1 or myc-tagged Rad21 was used for ChIP assay. Relative enrichment at target locus signal (*dg223*) against the euchromatic region (*act1*) is normalized to WCE (whole-cell extract) signal and is indicated *below* each lane. (*C*) Model for the effect of CK2-dependent phosphorylation of Swi6 (and Chp2) on the association of effectors (SHREC, Epe1, and Rad21) with heterochromatin. The effects of the hyperphosphorylated state in wild-type cells (*top* panel), the effects of the hypophosphorylated state in *ckb1Δ* cells, or the effects of phosphorylation site mutants of *swi6* and *chp2* (*bottom* panel) are indicated. For details, see text.

the localization of Epe1 (Fig. 5B, top panel). Localization of Epe1 at centromeric repeats was significantly decreased in *swi6Δ* cells, but not in the other mutants. This indicates that Epe1 requires Swi6 for its heterochromatin localization, but does not require its CK2-dependent phosphorylation. Intriguingly, enrichment of Epe1 tends to inversely correlate with that of Clr3 (Fig. 5A,B). Localization of Epe1 increased in *clr3Δ*, *ckb1Δ*, and *swi6-S18-117A chp2-S249-354A* strains (Fig. 5B); in the latter two mutants, localization of Clr3 was severely reduced to the same level as that in *swi6Δ* cells (Fig. 5A). This indicates that loss of Clr3 at the heterochromatin increases Epe1 recruitment. Association of Epe1 with heterochromatin might be inhibited by deacetylation of histones and/or chromatin remodeling by SHREC, which contains Clr3 and a chromatin remodeling factor (Sugiyama et al. 2007).

The cohesin complex densely localizes at centromeric heterochromatin in a Swi6-dependent manner to establish centromeric sister chromatid cohesion (Bernard et al. 2001; Nonaka et al. 2002). The loss of centromeric cohesion by the disruption of heterochromatin caused hypersensitivity to TBZ, an inhibitor of spindle assembly, and an increase in cells showing lagging chromosome during mitosis (Supplemental Fig. S9; Supplemental Table S1, see *clr4Δ* and *swi6Δ*). *ckb1Δ*, *swi6-S18-117A*, *chp2Δ*, and *swi6-S18-117Achp2-S249-354A* strains exhibited no increase in cells showing lagging chromosome (Supplemental Table S1). Furthermore, these mutants showed a similar sensitivity to TBZ as wild-type cells, except *ckb1Δ* cells, which were slightly sensitive to TBZ (Supplemental Fig. S9). Consistently, these mutations retained a significant amount of Rad21, a subunit of the cohesion complex, at centromeric repeats, as measured by ChIP assay, while loss of *swi6* diminished Rad21 (Fig. 5B). *ckb1Δ* cells also retained Rad21 to the same extent as other mutants, suggesting that TBZ sensitivity of *ckb1Δ* cells is not due to the decrease of cohesion but probably due to the other defects caused by *ckb1* deletion (Roussou and Draetta 1994). Thus, CK2-dependent phosphorylation of Swi6 does not affect Swi6-dependent establishment of sister chromatid cohesion at centromere.

The effects of Swi6 phosphorylation by CK2 are summarized in Figure 5C. Phosphorylation of Swi6 by CK2 (and probably CK2-dependent phosphorylation of Chp2) promotes association of the Clr3-containing silencing complex, SHREC; the associated SHREC, in turn, limits association of the antisilencing factor Epe1. As a result, TGS at heterochromatin is enhanced through the alteration of chromatin structure induced by SHREC. As SHREC enhances the spreading of heterochromatin (Yamada et al. 2005), while Epe1 antagonizes it (Zofall and Grewal 2006; Trewick et al. 2008), the spreading of heterochromatin is also enhanced by CK2-directed phosphorylation of Swi6. Conversely, dephosphorylation of Swi6 (and probably of Chp2) results in the release of SHREC from heterochromatin, which causes the increase of Epe1 on heterochromatin. This suppresses TGS and the spreading of heterochromatin through altered chromatin structure, and finally causes the increased transcription of ncRNA and marker genes. The increase in ncRNA transcription may contribute to the maintenance of H3K9me and Swi6 in "active" heterochromatin, since ncRNA triggers H3K9 methylation via an RNAi-dependent heterochromatin formation pathway (Volpe et al. 2002). Importantly, the change in the phosphorylation state of Swi6 does not affect localization of cohesin at heterochromatic repeats. TGS at centromeric heterochromatin is also alleviated without affecting Swi6 localization in mutants of Hsk1-Dfp1 kinase, which regulates the initiation of DNA replication (Bailis et al. 2003). However, *hsk1-dfp1* mutants display chromosomal segregation defects caused by mislocalization of Rad21 at centromeric heterochromatin. In addition, although Hsk1-Dfp1 interacts with and phosphorylates Swi6 in vitro, Swi6 mobility is not altered in *hsk1-dfp1* mutants (Bailis et al. 2003). Thus, it is not clear whether Swi6 is a target of the kinase in vivo for the observed TGS defects.

Our results suggest that heterochromatic structure can be regulated between "silent" and "active" states, by changing the CK2-dependent phosphorylation of Swi6, without affecting other functions. CK2 plays a role in a number of important cellular processes (Ahmed et al. 2002; Pinna 2002; Litchfield 2003), including DNA damage repair (Loizou et al. 2004; Cheung et al. 2005; Ayoub et al. 2008). Therefore, the regulation of CK2-dependent Swi6 phosphorylation might be involved in the nuclear response to various cellular conditions. It was recently shown that human HP1β is released from chromatin in response to chromosome breakage by CK2-dependent phosphorylation at chromodomain Thr 51 (Ayoub et al. 2008). However, this DNA-damage response system does not seem to operate in fission yeast, as Thr 51 of HP1 is not conserved in Swi6, and loss of CK2-dependent phosphorylation does not affect the chromatin association of Swi6. Since Swi6 seems to be constitutively phosphorylated by CK2 in normally growing cells (A. Shimada and Y. Murakami, unpubl.), dephosphorylation would be one of the mechanisms to regulate the heterochromatin activity. For example, heterochromatin must be "open" to allow quick repair while keeping other heterochromatin functions intact. In this situation, local dephosphorylation of Swi6 at the site of damage may provide such an "open" state. We also want to point out the possibility that CK2 phosphorylates heterochromatin-related proteins to regulate heterochromatin activity in combination with Swi6 (and probably Chp2) phosphorylation.

Here, we show that HP1/Swi6-mediated silencing subcode specifically regulates the heterochromatic TGS, and suggest that other modifications of HP1 and probably modifications of other heterochromatic proteins differentially regulate multiple heterochromatin functions. Further analysis using fission yeast will contribute to the understanding of the dynamic nature of heterochromatin, which is important for epigenetic regulation of gene expression and the maintenance of genome integrity.

Materials and methods

Schizosaccharomyces pombe *strains, media, genetic procedures*

Strains used in this study are listed in Supplemental Table S2. Media and genetic procedures used in this study were essentially as described (Moreno et al. 1991). The genetic procedures for serial dilution experiments for measuring silencing and for generating deletion or epitope tagging of target genes are described in the Supplemental Material. The detail of the method for generating Swi6 or Chp2phosphorylation sites mutants are also described in the Supplemental Material.

Antibodies

The following antibodies were used in this study: anti-c-myc (9E11, Santa Cruz Biotechnologies), anti-Flag (M2, Sigma), anti-PolII (8WG16, Abcam), anti-H3-K9-2Me monoclonal antibodies (Kato et al. 2005), and anti-Swi6 (Sadaie et al. 2004) polyclonal antibodies.

ChIP assay

ChIP was performed as described previously (Nakayama et al. 2000), except that 1% paraformaldehyde instead of 3% was used for fixation of cells.

In vitro kinase assay and phsophatase assay

Information detailing in vitro kinase assay and phosphatase assay is provided in the Supplemental Material.

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