Supplemental information

Phosphorylation of Swi6/HP1 regulates transcriptional gene silencing at heterochromatin.

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Supplemental Figures and Tables

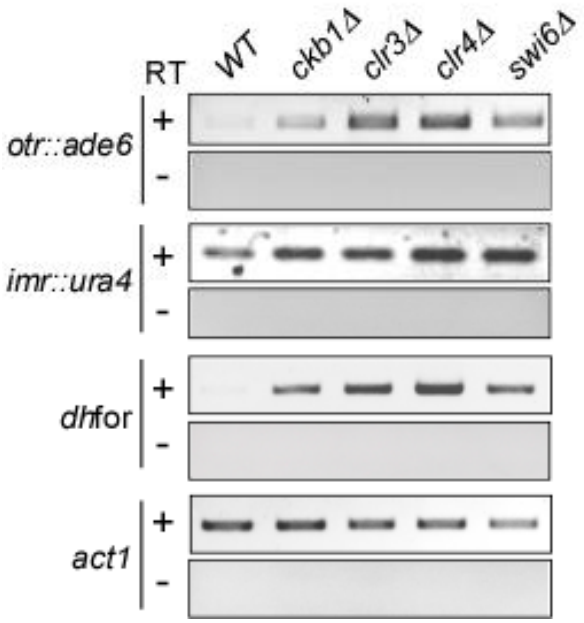


Figure S1. Effects of *ckb1*-deletion on the transcription of marker genes and non-coding RNA

The level of transcripts generated from marker genes (*otr::ade6* and *imr::ura4*) and centromeric non-coding RNA (*dh*for) in the indicated strains were analyzed by reverse-transcription PCR (RT-PCR) using total RNA prepared from the indicated strains in the presence (+) or absence (-) of reverse transcriptase (RT). *act1* RNA was analyzed as a control.

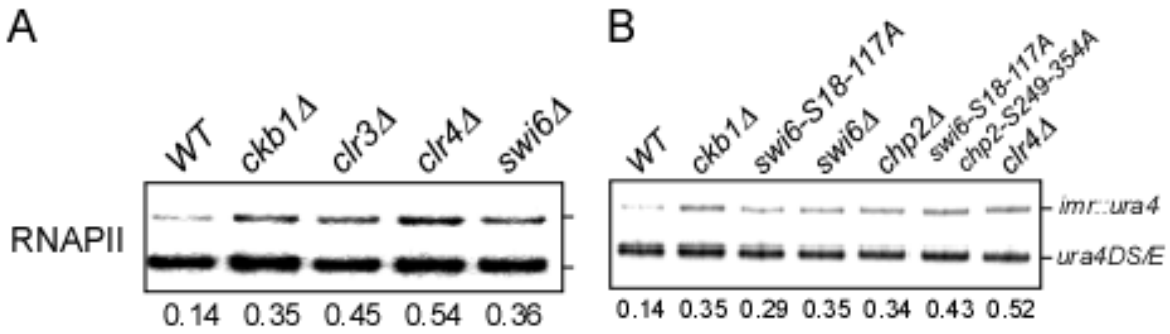


Figure S2. Locarization of RNAPII on marker gene inserted in heterochromatin.
The level of RNA polymerase II on *imr::ura4* in indicated various mutants was analyzed by ChIP assay. The relative enrichment at *imr::ura4* against the euchromatic *ura4DS/E* mini genes shown below each lane.

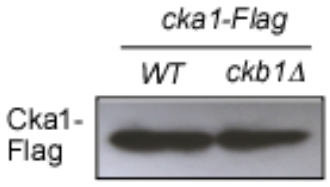


Figure S3. Amount of Cka1 prepared by immunoprecipitation
The amounts of Cka1 in the precipitated CK2 used in kinase reactions in Fig. 2A were analyzed by western blotting.

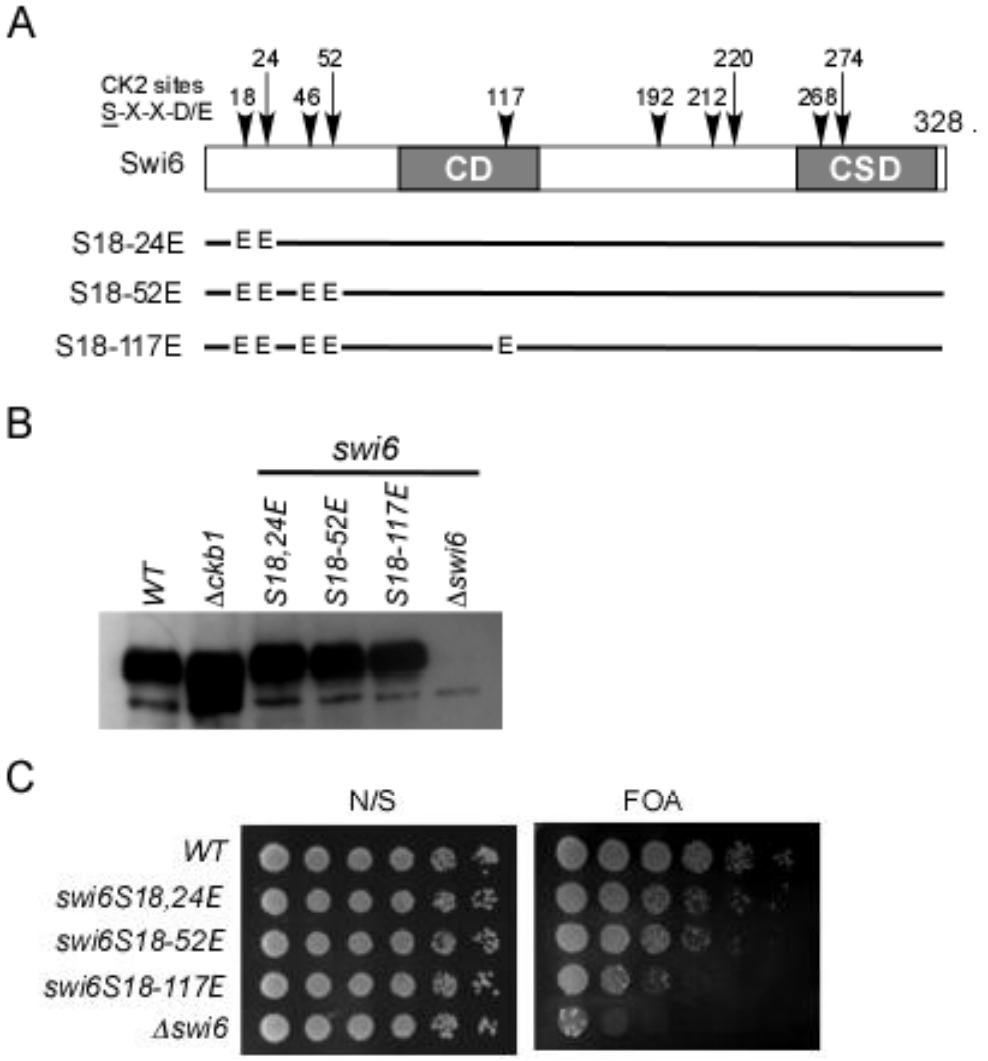
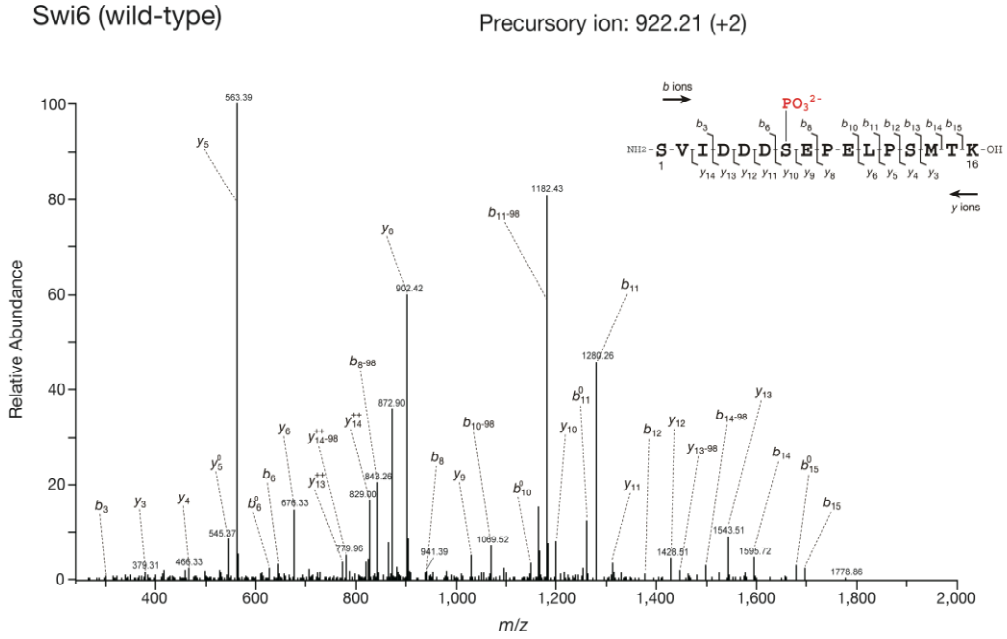


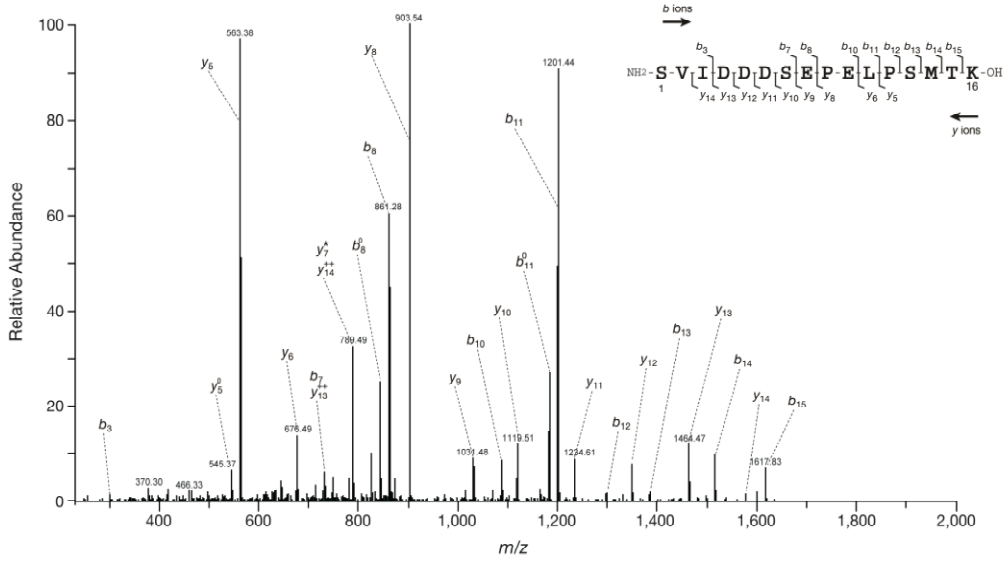
Figure S4. Effects of aspartic acid substitution mutations of Swi6 CK2-phosphorylation sites

(*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 aspartic acid (E) were introduced into genomic *swi6* gene by PCR mediated mutagenesis and homologous recombination (see supplemental methods). (*B*) Western blotting analysis of Swi6 in the extract prepared from the indicated strains. (*C*) Silencing at centromeric heterochromatin of the Swi6 phosphorylation mutants. Serial dilution (1:5) of exponentially growing cell culture was spotted onto non-selective medium (N/S) and medium containing 1.0 mg/ml of 5-fluorotic acid (FOA). Disruption of the silencing caused FOA sensitivity as observed in *swi6Δ* cells.

A


Swi6 (wild-type)
Precursory ion: 922.21 (+2)
b ions
y ions
NH2-S-V-I-D-D-D-S-E-P-E-L-P-S-M-T-K-OH
PO3 2-
Relative Abundance
m/z

B


Swi6 (Δckb1)
Precursory ion: 882.31 (+2)
b ions
y ions
NH2-S-V-I-D-D-D-S-E-P-E-L-P-S-M-T-K-OH
Relative Abundance
m/z

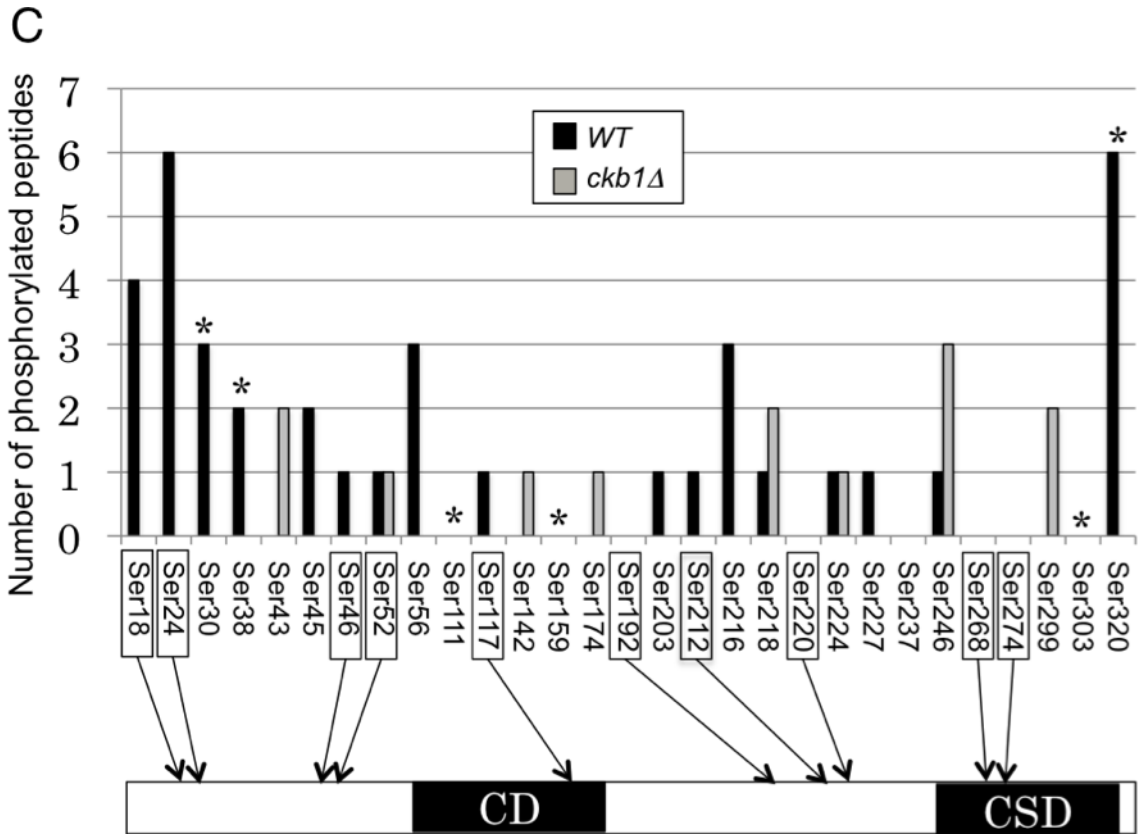


Figure S5. Identification of phosphorylation sites of Swi6 using LC-MS/MS.
Swi6 in wild-type or Δ*ckb1* mutant cells was isolated by affinity purification, digested with trypsin, and analyzed using a quadrapole ion trap spectrometer (Finnigan LTQ; Thermo Fisher Scientific). Representative MS/MS spectra of the peptide fragment spanning residues 18-33 for wild-type (A) and *ckb1*Δ strains (B) are depicted. The observed *y* and *b* ions and the fragment map are shown. (C) The histogram indicated the number of peptides harboring phopsphorylated serine at indicated sites. The y axis indicates the total number of peptides obtained from 4 independent analyses. The boxed Serines correspond with CK2 consensus sequence. Asterisk indicates the number that could be an error because the signal was obtained from the analysis using Swi6 produced in *E. coli*, which is supposed not to have any phospho-serine. Note that the number of the each peptide does not directly represents the extent of phosphorylation because the detection frequencies of phospho-peptides are not quantitative.

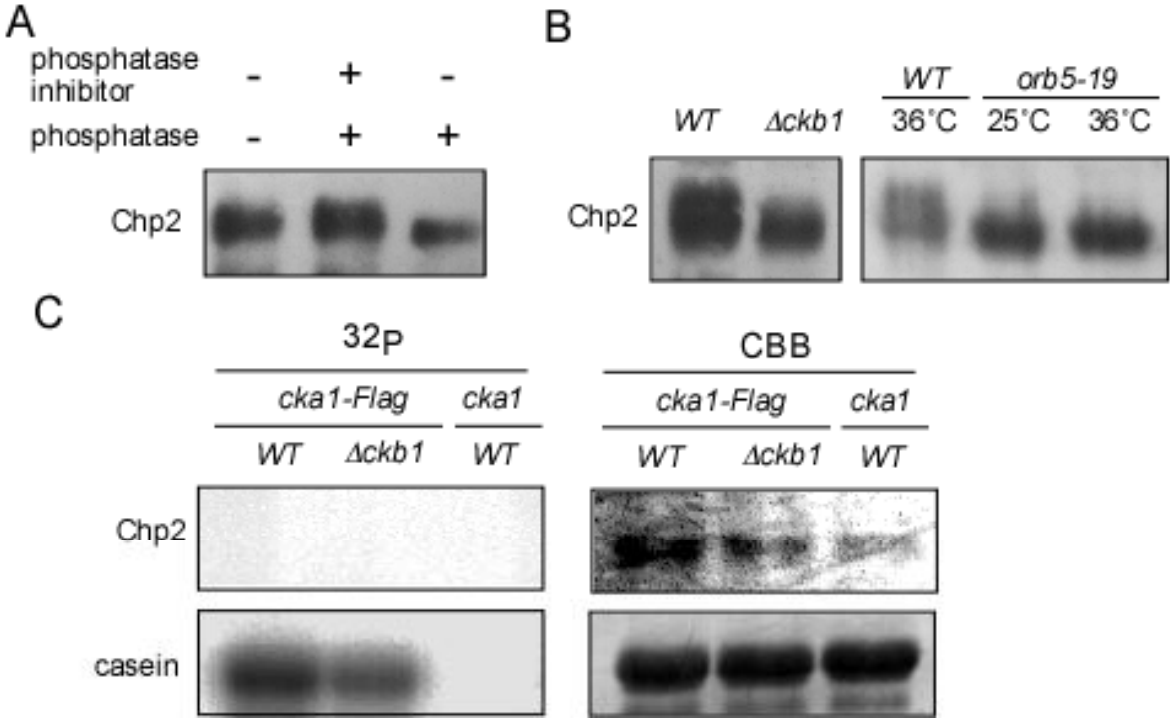


Figure S6. Chp2 was phosphorylated *in vivo* in a CK2-dependent manner but was not phoshphorylated by CK2 *in vitro*.

(*A*) Chp2 immunoprecipitated from wild type cell extracts were incubated with or without calf intestine phosphatase (CIP). Phsopatase inhibitors were added where indicated. Reaction mixtures were separated with SDS-PAGE and Chp2 was detected by Western blotting. (*B*) Cell extracts were prepared from wild type, *ckb1Δ* and *orb5-19* cells growing at indicated temperature. *orb5-19* is a temperature sensitive mutant of a catalytic subunit of CK2 (Cka1). Chp2 proteins in the extracts were analyzed by Western blotting using anti-Chp2 polyclonal antibodies (Sadaie et al. 2008a). (*C*) Bacterially produced Chp2 or dephosphorylated α-casein was incubaeted with CK2 that was immunoprecipitated from the indicated strains expressing Flag-tagged Cka1, a catalytic subunit of CK2, with anti-Flag antibody. As a control, imuunoprecipitates from cells does not have tagged-Cka1 were used. The reaction products were analyzed by SDS-polyacrylamide electrophoresis. Chp2 and α-casein was detected by autoradiography (^{32}P) and by Coomassie Brilliant Blue staining (CBB).

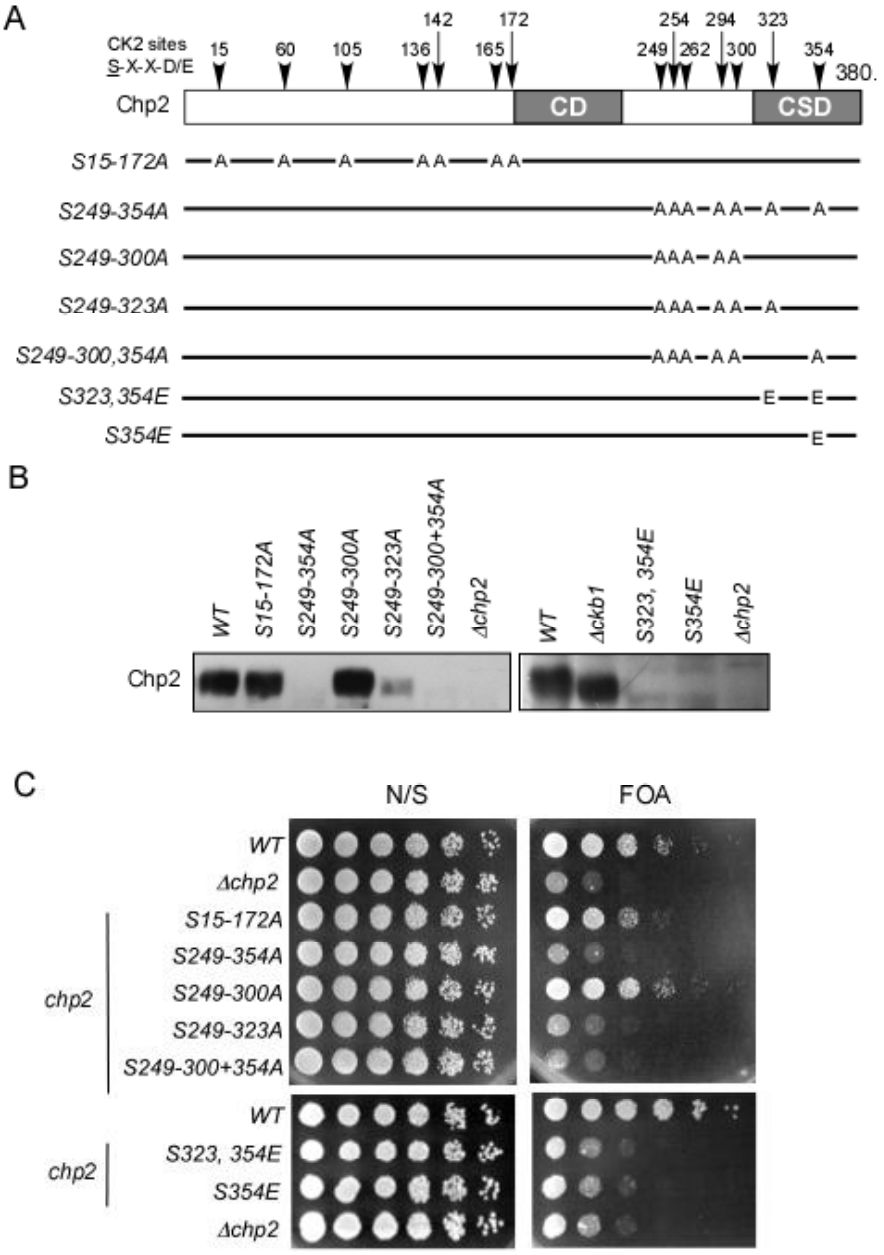


Figure S7. Amino acids-substitution mutants of CK2-phosphorylation sites of Chp2
(A) Schematic diagram of the structure of Chp2 and the location of mutations introduced at the putative CK2 phosphorylation sites (S-X-X-D/E) in each mutant. The amino acid substitutions from serine (S) to alanine (A) or aspartic acid (E) were introduced into genomic *chp2* gene by PCR mediated mutagenesis and homologous recombination (see supplemental Methods). (*B*) Western blotting analysis of Chp2 in the extract prepared from the indicated strains. (*C*) Silencing at centromeric heterochromatin of the Chp2 phosphorylation mutants were analyzed as described in Figure S1.

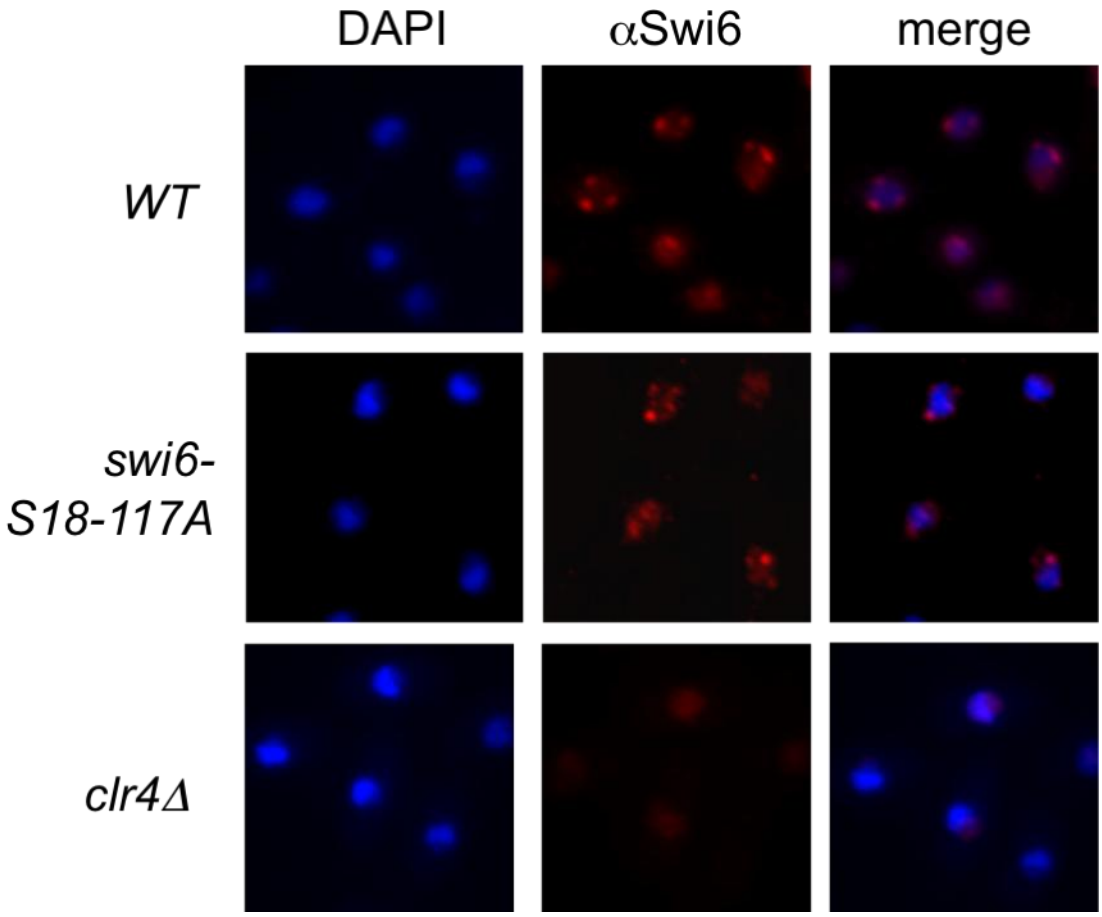


Figure S8. Localization of wild-type Swi6 and Swi6-S18-117A in the cells.
Localization of Swi6 was examined by immunofluorescence analysis in wild type (WT), *swi6-S18-117A* and *clr4Δ* cells using anti-Swi6 antibodies as described in supplemental methods. DAPI staining was simultaneously done to show nuclei. Note that we could not get clear staining of Swi6 foci in *ckb1Δ* cells because of abnormal cell shape of *ckb1Δ* cells.

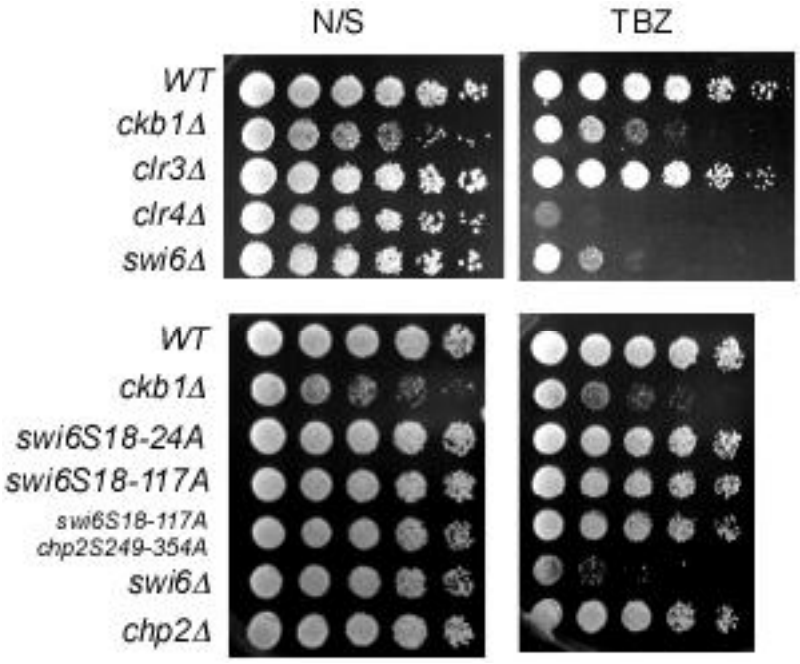


Figure S9. TBZ-sensitivity of *ckb1Δ* and Swi6 phosphorylation mutants

Serial dilution experiments for measuring TBZ sensitivity were performed by spotting serially diluted (1:5), exponentially growing cell cultures onto non-selective medium (N/S), and medium containing 15 μg/ml TBZ. Note that *ckb1Δ* cells were slightly sensitive to TBZ, probably because multiple defects observed in *ckb1Δ* cells (Roussou and Draetta 1994) indirectly affects TBZ sensitivity. However, considered the slow growth of cells on non-selective plate (N/S), *ckb1Δ* cells are less sensitive to TBZ than *swi6Δ* or *clr4Δ* cells.

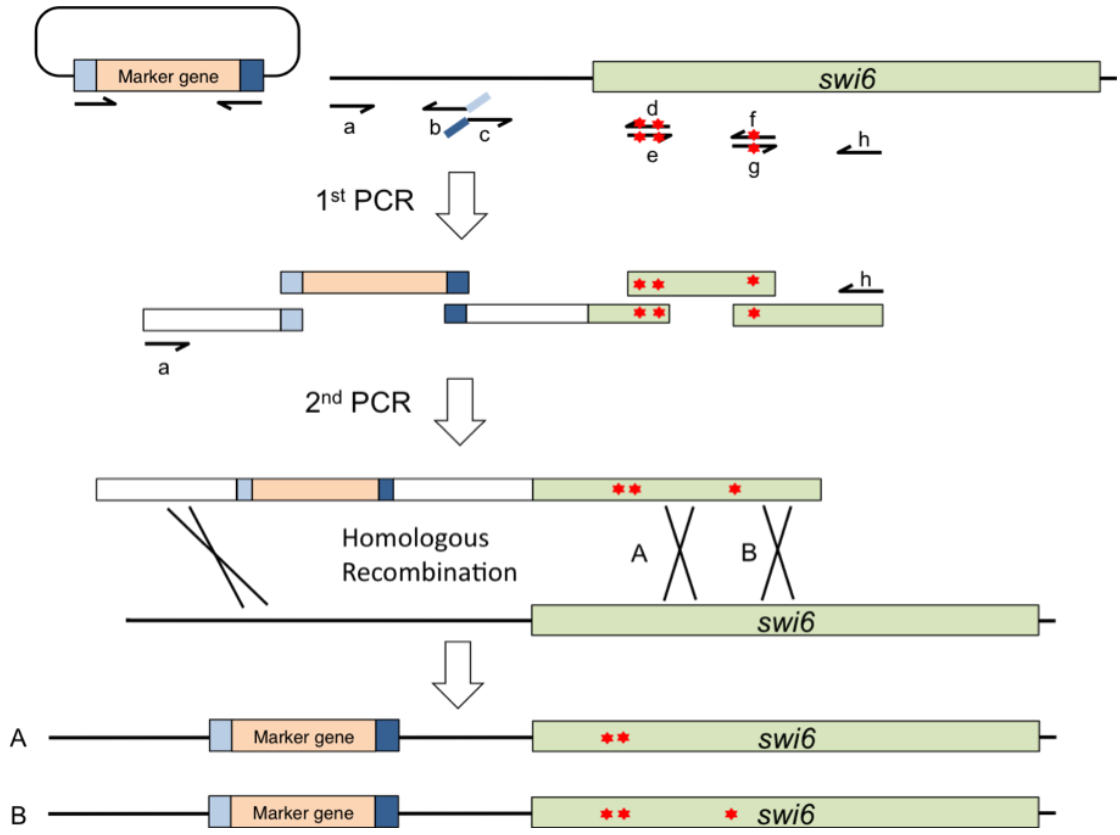


Figure S10. Schematic presentation of the method for introducing multiple mutations in *swi6*

For detail, see supplemental methods.

Table S1. Frequencies of cells showing lagging chromosome in anaphase

strains	Total counted cells in anaphase	cells showing lagging chromosome	% lagging chromosome
wild type	160	2	1.2
ckb1Δ	166	3	1.8
swi6 S18-117	141	2	1.4
swi6Δ	142	30	21.1
chp2Δ	144	2	1.4
swi6S18-117 chp2S249-354A	164	3	1.8
clr3Δ	152	2	1.3

Table S2. Strains used in this study

Strain	genotype
GAS3	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$
GAS114	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *ckb1Δ::kanMX6*$^+$
GAS280	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *clr3Δ::hphMX6*$^+$
GAS281	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *clr4Δ::kanMX6*$^+$
GAS282	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *swi6Δ::hphMX6*$^+$
GAS204	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *kanMX6*$^+$*-swi6(S18-117A)*
GAS169	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *chp2Δ::kanMX6*$^+$
GAS206	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *kanMX6*$^+$*-swi6(S18-117A) chp2(S249-354A)-hphMX6*$^+$
GAS205	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *kanMX6-swi6*$^+$
GAS202	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *kanMX6*$^+$*-Swi6(S18,24A)*
GAS210	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *swi6(S192-274A)-kanMX6*$^+$
GAS211	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *swi6-hphMX6*$^+$
GAS213	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *hphMX6*$^+$*-chp2(S15-175A)*
GAS217	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *chp2(S249-354A)-kanMX6*$^+$
GAS219	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *chp2(S249-300A)-kanMX6*$^+$
GAS224	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *chp2(S249-323A)-hphMX6*$^+$
GAS225	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *chp2(S249-300,354A)-hphMX6*$^+$
GAS200	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *cka1-5FLAG-kanMX6*$^+$ *ckb1Δ::hphMX6*$^+$
GAS201	*h+ ade6-DN/N leu1-32 ura4-DS/E imr1*L::*ura4*$^+$ *otr1*R::*ade6*$^+$ *cka1-5FLAG-kanMX6*$^+$

Table S2. continued

Strain		genotype
GAS265	*h-*	*orb5-19 ade6-m216 leu1-32*
GAS25	*h90*	*ade6-m210 leu1-32 ura4-DS/E kint2::ura4*$^+$
GAS283	*h90*	*ade6-m210 leu1-32 ura4-DS/E kint2::ura4*$^+$ *dcr1Δ::kanMX6*$^+$
GAS122	*h90*	*ade6-m210 leu1-32 ura4-DS/E kint2::ura4*$^+$ *ckb1Δ::kanMX6*$^+$
GAS153	*h90*	*ade6-m210 leu1-32 ura4-DS/E kint2::ura4*$^+$ *ckb1Δ::natMX6*$^+$ *dcr1Δ::kanMX6*$^+$
GAS152	*h90*	*ade6-m210 leu1-32 ura4-DS/E kint2::ura4*$^+$ *atf1Δ::natMX6*$^+$
GAS193	*h90*	*ade6-m210 leu1-32 ura4-DS/E kint2::ura4*$^+$ *ckb1Δ::hphMX6*$^+$ *atf1Δ::natMX6*$^+$
GAS284	*h90*	*ade6-m210 leu1-32 ura4-DS/E kint2::ura4*$^+$ *clr4Δ::kanMX6*$^+$
GAS240	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *rad21-13myc-natMX6*$^+$
GAS243	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *rad21-13myc-natMX6 ckb1Δ::kanMX6*$^+$
GAS256	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *rad21-13myc-natMX6*$^+$ *kanMX6*$^+$*-swi6(S18-117A)*
GAS248	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *rad21-13myc-natMX6*$^+$ *swi6Δ::hphMX6*$^+$
GAS251	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *rad21-13myc-natMX6 chp2Δ::kanMX6*$^+$
GAS259	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *rad21-13myc-natMX6*$^+$ *kanMX6*$^+$*-swi6(S18-117A) chp2(S249-354A)-hphMX6*$^+$
GAS235	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *rad21-13myc-natMX6*$^+$ *clr3Δ::hphMX6*$^+$
GAS222	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *epe1-5FLAG-kanMX6*$^+$
GAS269	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *epe1-5FLAG-kanMX6*$^+$ *ckb1Δ::hphMX6*$^+$
GAS277	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *epe1-5FLAG-kanMX6*$^+$ *natMX6*$^+$*-swi6(S18-117A)*
GAS268	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *epe1-5FLAG-kanMX6*$^+$ *swi6Δ::hphMX6*$^+$
GAS267	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *epe1-5FLAG-kanMX6*$^+$ *chp2Δ::hphMX6*$^+$

Table S2. continued

Strain		genotype
GAS278	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *epe1-5FLAG-kanMX6*$^+$ *natMX6*$^+$*-swi6(S18-117A) chp2(S249-354A)-hphMX6*$^+$
GAS285	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *epe1-5FLAG-kanMX6*$^+$ *clr3Δ::hphMX6*$^+$
GAS194	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *Clr3-5FLAG-kanMX6*$^+$
GAS195	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *clr3-5FLAG-kanMX6*$^+$ *ckb1Δ::hphMX6*$^+$
GAS198	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *clr3-5FLAG-kanMX6*$^+$ *natMX6*$^+$*-swi6(S18-117A)*
GAS196	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *clr3-5FLAG-kanMX6*$^+$ *swi6Δ::hphMX6*$^+$
GAS197	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *clr3-5FLAG-kanMX6*$^+$ *chp2Δ::hphMX6*$^+$
GAS199	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *clr3-5FLAG-kanMX6*$^+$ *natMX6*$^+$*-swi6(S18-117A) chp2(S249-354A)-hphMX6*$^+$
GAS172	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *chp2-13myc-kanMX6*$^+$
GAS142	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *chp2-13myc-kanMX6*$^+$ *ckb1Δ::hphMX6*$^+$
GAS298	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *kanMX6*$^+$*-swi6(S18-24E)*
GAS299	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *kanMX6*$^+$*-swi6(S18-52E)*
GAS300	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *kanMX6*$^+$*-swi6(S18-117E)*
GAS301	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *chp2(S354E)-hphMX6*$^+$
GAS302	*h+*	*ade6-DN/N leu1-32 ura4-DS/E imr1*L*::ura4*$^+$ *otr1*R*::ade6*$^+$ *chp2(S323-354E)-hphMX6*$^+$
SPM2072	*h+*	*ade6-m210 leu1-32 ura4-DS/E otr1R::ura4*$^+$ *swi6+:: hphMX6-3FLAG-swi6+*
SPM2091	*h+*	*ade6-m210 leu1-32 ura4-DS/E otr1R::ura4*$^+$ *swi6:: hphMX6-3FLAG-swi6*$^+$ *ckb1Δ::kanMX6*$^+$

Supplemental Methods

Schizosaccharomyces pombe genetic procedures

Serial dilution experiments for measuring silencing were performed by spotting serially diluted (1:5), exponentially growing cell cultures onto non-selective medium (N/S), medium containing 1.0 mg/ml of 5-fluorotic acid (FOA), medium containing low concentration of adenine (YE medium without adding adenine, low Ade). Since *ckb1Δ* cells grew poorly on minimal medium, we did not use medium lacking uracil to analyze silencing of *ura4* gene. Counting of lagging chromosome was performed as described previously (Kato et al. 2005).

Deletion or epitope-tagging of target genes were performed by a PCR-based module method (Krawchuk and Wahls 1999).

To construct Swi6- or Chp2- phosphorylation sites mutants, two step PCR method (Krawchuk and Wahls 1999) was used with modifications. As an example, the procedure to construct mutants harboring multiple mutations in the N-terminal half of swi6 is indicated in the Fig. S10. At first, the target region including swi6 N-terminal half was amplified by PCR as several independent fragments using primer sets indicated Fig. S10 (primers a-h) and accurate DNA polymerase (KOD plus, Toyobo). Primers in the coding region were designed to make overlapped-regions at the end of the fragments and have desired mutations at putative phosphorylation sites (primer sets d/e and f/g in Fig. S10). Note that we could introduce mutations in two neighboring sites with one primer set (primer set f/g in Fig. S10). A selectable marker gene (usually drag resistance gene) was also amplified by PCR. To insert the selectable marker gene, primers at the insertion sites have extra-sequences that overlapped with the sequences at the end of marker gene (primers b and c in Fig. S10). Each amplified fragment was gel-purified. The purified fragments were mixed and used as template for second PCR with primers corresponding to both end of the target region (primers a and h). In the second PCR, each fragment was annealed with next fragment with overlapped fragments and as a result the target DNA region harboring mutations and marker gene was amplified. The obtained DNA fragments were then purified and inserted to genome by homologous recombination. Depending on

the position of homlogous recombination, we could get a series of mutants (Fig. S10, A, B). The sequences of the obtained mutants were verified by sequencing.

Screening of heterochromatin-related genes

We noticed that many of gene products involved in heterochromatin formation or function localize to spindle pole body (SPB) in *S. pombe* post genome database (http://cgl.riken.go.jp/gene/login.html) probably due to clustering of centeromeres at SPB in fission yeast. Thus, to identify novel genes involved in centromeric heterochromatin formation, we systematically disrupted genes whose products are classified as SPB localization in the database. For the screening, we used a strain harboring the *ade6* gene and *ura4* gene inserted at *otr* (*otr::ade6*) and *imr* (*imr::ura4*) loci in the centromeric heterochromatin, respectively (Fig 1A). Both marker genes are repressed by the H3K9me/Swi6-dependent heterochromatin resulting in red-colony formation on low-adenine plates (repression of *ade6*) and resistance to 5-fluorotic acid (FOA) (repression of *ura4*). Six of 20 disruptants formed white or pink colonies and were not able to grow on a FOA plate, which showed the heterochromatic silencing was disturbed in these strains. One of the strongest phenotype among the six candidates had disruption of *ckb1*, which encodes a regulatory subunit of casein kinase II (CK2).

***In vitro* kinase assay**

Cka1-5FLAG was immunoprecipitated from the total extracts with anti-FLAG antibody (M2, Sigma). Immunoprecipitates were washed four times in lysis buffer (50 mM Hepes/KOH [pH 7.5], 1 mM EDTA, 140 mM NaCl and Na-Deoxycholate, and 1% Triton) and once in kinase assay buffer (25 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, and 1 mM dithiothreitol). Kinase assays were performed at 30°C for 10 min in kinase assay buffer in the presence of 50 μM ATP, 7.5 μCi of (γ-^{32}P)ATP, and substrates. The concentrations of substrates used in this assay were as follows: 1 mg/ml of dephosphorylated α-casein (Sigma), 100 μg/ml of bacterially-produced Swi6, and 50 μg/ml of bacterially-produced Chp2. The reactions were stopped with the addition of 2X laemmli buffer (4% SDS, 20% glycerol, 0.6 M β-mercaptoethanol, and 0.12 M Tris-HCl [pH 6.8]). The reaction mixture was resolved by SDS-PAGE and the labelled proteins were detected by a phospho-imager

(BAS2000, Fuji).

Phosphatase assay

Swi6 and Chp2-13myc were immunoprecipitated from the total extracts with anti-Swi6 and anti-myc antibodies, respectively, in the presence of a phosphatase inhibitor cocktail (conatins imidazole, EDTA, Sodium Orthovanadate, β-glycerophosphoric acid disodium salt, sodium(+)-tartrate dihydrate, sodium fluoride, and disodium molybdate dihydrate; Nacalai Tesque). Immunoprecipitates were washed 4 times in lysis buffer and once in phosphatase assay buffer (50 mM Tris-HCl [pH 7.5] and 1 mM $MgCl_2$). The immunoprecipitates were incubated at 37°C for 30 min in 50 μl of phosphatase assay buffer in the presence or absence of 12 units of CIP (TOYOBO). When necessary, phosphatase inhibitor cocktail (Nacalai Tesque) was added to the reaction mixture. The reactions were stopped by adding 2X laemmli buffer. The products were detected by immunodetection after Western blotting.

Identification of *in vivo* phosphorylation sites on Swi6 by mass spectrometer analysis

Strains expressing N-terminal-3x FLAG-tagged Swi6 (FLAG-Swi6) were grown in YEA medium to an A_{595} of 2~5. Cells (~1 x 10^{11}) were harvested and washed once with cold PBS, and each of 2 x 10^{10} cells were frozen by liquid nitrogen. The cell pellets (2 x 10^{10} cells) were resuspended in 500 μl of lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P40 (NP-40), 50 mM NaF, 1 mM DTT, 1 mM PMSF) containing protease inhibitor cocktail (Complete™ [Roche]), and lysed by vigorous agitation with zirconia beads (BioSpec). Twenty milliliter of lysis buffer was added, and the extract was spun at 12,000 *g* at 4°C for 20 min. The supernatant was saved, diluted with 1 volume of Q buffer (20 mM Tris-HCl [pH 8.0], 10% (v/v) glycerol, 1 mM DTT, 1 mM PMSF), and then loaded on HiPrep 16/10 Q XL column (GE Healthcare). Bound proteins were separated by 0-75% linear gradient with Q buffer containing 1M NaC and fractions containing FLAG-Swi6 were pooled. The pooled lysate were incubated with 250~500 μl of pre-washed M2-agarose (Sigma) for 2 hr or overnight at 4°C. The beads and immobilized FLAG-Swi6 protein were harvested by centrifugation at 700 *g* at 4°C, loaded on a BioRad polyprep column, and washed 2~3 times with Q buffer. Bound FLAG-Swi6 was eluted with

elution buffer (0.1 M Glycine [pH 2.5], 150 mM NaCl), precipitated with 2 volume of ethanol, and resolved by SDS-PAGE. The FLAG-Swi6 was excised from the gel, subjected to reduction with 10 mM DTT, alkylation with 55 mM iodoacetamide, and digestion with 10 μg/ml modified Trypsin (Promega) at 37 ˚C for 16 h. After in-gel digestion, the extracted peptides were subjected to mass spectrometer analysis as previously described (Sadaie et al. 2008b). The molecular masses of the resulting peptides were searched against the nonredundant NCBI database using the MASCOT program with an additional mass corresponding to the phosphorylation.

Detection of Swi6 by Immunogefluorescence

Indirect immunofluorescence was performed with anti-Swi6 antibody as described before (Dohke et al. 2008).

Oligo nucleotide sequences used in this study

Primers used for RT-PCR

ura4: 5' TTGCTTCTTGGGCTCATATC 3', 5' AGCCAAAAGCAAGAGACCAC 3'

ade6: 5' TTAGTATATGCCCCTGCTCG 3', 5' AAATGGTCTCACCATCTTGC 3'

dh forward: 5' CTCTCATCTCGACTCGTTTG 3', 5' GGCATTCACGAAACATAGCG 3'

act1: 5' TGCCGATCGTATGCAAAAGG3', 5' CCGCTCTCATCATACTCTTG 3'

primers used for ChIP analysis

imr::ura4, kint2::ura4, ura4 DS/E:

5' GAGGGGATGAAAAATCCCAT 3', 5' TTCGACAACAGGATTACGACC 3'

dg223: 5' TGGTAATACGTACTAGCTCTCG 3', 5' AACTAATTCATGGTGATTGATG 3'

act1: 5' GAAGTACCCCATTGAGCACGG 3', 5' CAATTTCACGTTCGGCGGTAG 3'

primers used for Swi6 point mutant construction

upstream point mutant (S18-117A,S18-117E):

5' CAGATGCCTCTAAGTTCATG 3'

5' TTAATTAACCCGGGGATCCGGCAATGCTGAGTCTCTCAAG 3'

5' GTTTAAACGAGCTCGAATTCATCAGCATGCCAACCGTAAC 3'

5’ TTCCGCATCGTCGTCAATAACTGCTCGTTTTG 3’

5’ CAAAACGAGCAGTTATTGACGACGATGCGGAACC 3’

5’ ATCGGCTTCAACTTCATTGTCCGCCGACCC 3’

5’ GGGTCGGCGGACAATGAAGTTGAAGCCGATC 3’

5’ ACAATCGGCTTCTGCACTCC 3’

5’ ATGGAGTGCAGAAGCCGATTGTAGC 3’

5’ CCTCTTGCGCTTTATCGGCG 3’

5’ TTCCTCATCGTCGTCAATAACTTCTCGTTTTG 3’

5’ ATCCTCTTCAACTTCATTGTCCTCCGACCC 3’

5’ CAAAACGAGAAGTTATTGACGACGATGAGGAACC 3’

5’ GGGTCGGAGGACAATGAAGTTGAAGAGGATC 3’

5’ ATGGAGTGAAGAAGCCGATTGTAGC 3’

5’ ACAATCGGCTTCTTCACTCC 3’

downstream point mutant (S192-274A):

5’ GGGACTGATGTCTTCATGTG 3’

5’ GTTTAAACGAGCTCGAATTCGAGGGTGCATGAATATCTGC 3’

5’ TTAATTAACCCGGGGATCCGGCATGAAGAGCAGTGCATGC 3’

5’ CTATGATGCTTGGGAAGACTTGGTAGCTAGCATC 3’

5’ GATGCTAGCTACCAAGTCTTCCCAAGCATCATAG 3’

5’ GAAGCTGACAATGAGTCTAAATCTCCTGCACAAAAAG 3’

5’ TTTTTGTGCAGGAGATTTAGACTCATTGTCAGCTTCAATG 3’

5’ CGCCGATAAAGCGCAAGAGG 3’

5’ CCTCTTGCGCTTTATCGGCG 3’

5’ ATGGAGTGCAGAAGCCGATTGTAGC 3’

primers used for Chp2 point mutant construction

upstream point mutant (S15-172A):

5’ AGCTGCAAAGCTGCTTTTGG 3’

5’ TTAATTAACCCGGGGATCCGGTTAAGGCAAGTAAGGGATG 3’

5’ GTTTAAACGAGCTCGAATTCGCATCCTAACCCTTTACATG 3’

5’ CATCACTTTCAGCGATTATAC 3’

5’ GTATAATCGCTGAAAGTGATG 3’

5’ CGGGAACAGCAAGACCGTAAAG 3’

5’ CTTTACGGTCTTGCTGTTCCCG 3’

5’ GAGTCTTCCATGTTAGCGATTGG 3’

5’ CCAATCGCTAACATGGAAGACTC 3’

5’ GAACCAGCAGTCTTTTCGAAATTTGCAGAAAC 3’

5’ GTTTCTGCAAATTTCGAAAAGACTGCTGGTTC 3’

5’ GAACCAGCAGTCTTTTCGAAATTTGCAGAAAC 3’

5’ CTAGTGCTGGTAGTGAAGATAAAAATGCAGATG 3’

5’ GTTTGCACTCTCATCTTTCTCAACATAGGCAGCTTCATTCGAAGCTCGTG 3’

downstream point mutant (S249-354A,S323-354E):

5’ ACAATCTCAACACGCTTGCG 3’

5’ GTTTAAACGAGCTCGAATTCACCAAAGCAGAAGGTAATGC 3’

5’ TTAATTAACCCGGGGATCCGTTGTAGGCACTGTAAAAGCG 3’

5’ GGTTACGTCGCAACTCACGATAAC 3’

5’ CCAAATCTTCCCAGGCTTTCTTCTG 3’

5’ CAGAAGAAAGCCTGGGAAGATTTGG 3’

5’ CCAAATCTTCCCAGGCTTTCTTCTG 3’

5’ GAATCTGCTATGAAAGAAAAGCAAGCCAAAATTG 3’

5’ CAATTTTGGCTTGCTTTTCTTTCATAGCAGATTC 3’

5’CACGAGCTTCGAATGAAGCTGCCTATGTTGAGAAAGATGAGAGTGCAAAC 3’

5’ GTTTGCACTCTCATCTTTCTCAACATAGGCAGCTTCATTCGAAGCTCGTG 3’

5’ CTAGTGCTGGTAGTGAAGATAAAAATGCAGATG 3’

5’ CAGAAGAAAGAGTGGGAAGATTTGG 3’

5’ CCAAATCTTCCCACTCTTTCTTCTG 3’

5’ GGTTACGTCGAAACTCACGATAAC 3’

5’ GTTATCGTGAGTTTCGACGTAACC 3’

References

Dohke, K., Miyazaki, S., Tanaka, K., Urano, T., Grewal, S.I., and Murakami, Y. 2008. Fission yeast chromatin assembly factor 1 assists in the replication-coupled maintenance of heterochromatin. *Genes Cells* **13**: 1027-1043.

Kato, H., Goto, D.B., Martienssen, R.A., Urano, T., Furukawa, K., and Murakami, Y. 2005. RNA polymerase II is required for RNAi-dependent heterochromatin assembly. *Science* **309**: 467-469.

Krawchuk, M.D. and Wahls, W.P. 1999. High-efficiency gene targeting in Schizosaccharomyces pombe using a modular, PCR-based approach with long tracts of flanking homology. *Yeast* **15**: 1419-1427.

Roussou, I. and Draetta, G. 1994. The Schizosaccharomyces pombe casein kinase II alpha and beta subunits: evolutionary conservation and positive role of the beta subunit. *Mol Cell Biol* **14**: 576-586.

Sadaie, M., Kawaguchi, R., Ohtani, Y., Arisaka, F., Tanaka, K., Shirahige, K., and Nakayama, J.I. 2008a. Balance between distinct HP1 proteins controls heterochromatin assembly in fission yeast. *Mol Cell Biol*.

Sadaie, M., Shinmyozu, K., and Nakayama, J. 2008b. A conserved SET domain methyltransferase, Set11, modifies ribosomal protein Rpl12 in fission yeast. *J Biol Chem* **283**: 7185-7195.