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The heterochromatin protein Swi6/HP1 activates replication origins at the pericentromeric region and silent mating-type locus

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Heterochromatin is a structurally compacted region of chromosomes in which transcription and recombination are inactivated[1,2]. DNA replication is temporally regulated in heterochromatin, but the molecular mechanism for regulation has not been elucidated. Among heterochromatin loci in *Schizosaccharomyces pombe*, the pericentromeric region and the silent mating-type (*mat*) locus replicate in early S phase, whereas the sub-telomeric region does not[3–5], suggesting complex mechanisms for regulation of replication in heterochromatic regions. Here, we show that Swi6, an *S. pombe* counterpart of heterochromatin protein 1 (HP1), is required for early replication of the pericentromeric region and the *mat* locus. Origin-loading of Sld3, which depends on Dfp1/Dbf4-dependent kinase Cdc7 (DDK), is stimulated by Swi6. An HP1-binding motif within Dfp1 is required for interaction with Swi6 *in vitro* and for early replication of the pericentromeric region and *mat* locus. Tethering of Dfp1 to the pericentromeric region and *mat* locus in *swi6*-deficient cells restores early replication of these loci. Our results show that a heterochromatic protein positively regulates initiation of replication in silenced chromatin by interacting with an essential kinase.

The condensed chromatin structure in the heterochromatic region is thought to delay initiation of replication, probably by restricting access to replication factors[6,7]. Formation of heterochromatin involves methylation of histone H3 at Lys 9 (H3K9me) and binding of HP1, a highly conserved heterochromatin protein[8]. HP1 consists of a chromodomain (CD) and chromoshadow-domain, which are responsible for binding to H3K9me and other transfactors, respectively. Recent studies in the fission yeast *S. pombe* show that Swi6, a fission yeast HP1 homologue, is not only the molecular foundation for the heterochromatin structure that suppresses transcription and recombination[9,10], but also serves as a platform to recruit various effectors involved in transcriptional silencing, modifications of histones, chromosome segregation and even DNA replication[8]. Thus, we considered Swi6 as a candidate regulator of replication in heterochromatic regions. Swi6 has been shown to interact with replication factors, such as DNA polymerase α, a primase at the replication fork[11,12], and Dfp1 (ref. 13). However, these interactions have been thought to represent participation in heterochromatin maintenance and cohesin localization, and involvement of Swi6 in regulation of replication has remained unclear.

To elucidate the role of Swi6 in heterochromatin replication, we examined the replication kinetics of heterochromatin loci in wild-type and *swi6*-deficient (*swi6Δ*) cells. The heavy analogue 5-bromo-2′-deoxyuridine (BrdU) was incorporated into newly synthesized DNA for various periods of the synchronous cell cycle and replicated DNA with heavy-light density was separated from non-replicated DNA by CsCl density gradient centrifugation[3]. Replication efficiencies were calculated by real-time PCR for replication origins in the pericentromeric region[14], the silent *mat* locus (*matK*)[15] and the sub-telomeric region, as well as an early replication origin in the euchromatic region (*ars2004*) and a non-origin locus (*nonARS*) located about 30 kb from the *ars2004* (Fig. 1a). These origins, including the sub-telomeric origin, have autonomously replicating activity (ARS activity) on a plasmid (Supplementary Information, Fig. S1). In the wild-type, *ars2004* replicated about 10 min earlier than the *nonARS* locus (Fig. 1b). The replication kinetics of the pericentromeric region and *matK* were similar to those of *ars2004*, showing early replication of these heterochromatic loci, whereas the sub-telomeric origin replicated much later than the *nonARS* locus. In *swi6Δ* cells, the profiles of the *ars2004* and the *nonARS* loci were similar to those of the wild-type (Fig. 1b). The replication kinetics of the sub-telomeric region did not change (Fig. 1b), suggesting that the sub-telomeric origin does not fire in early S phase. Surprisingly, however, replication of the pericentromeric region and *matK* was delayed by as much as that of the *nonARS* locus (Fig. 1b), indicating that Swi6 is required for early replication of these heterochromatic loci. We further confirmed that a CD mutation, $swi6^{W104A}$, which impairs the interaction of *Drosophila melanogaster* HP1 with H3K9me[16], caused a delay in replication of the pericentromeric region and *matK* (Supplementary Information,

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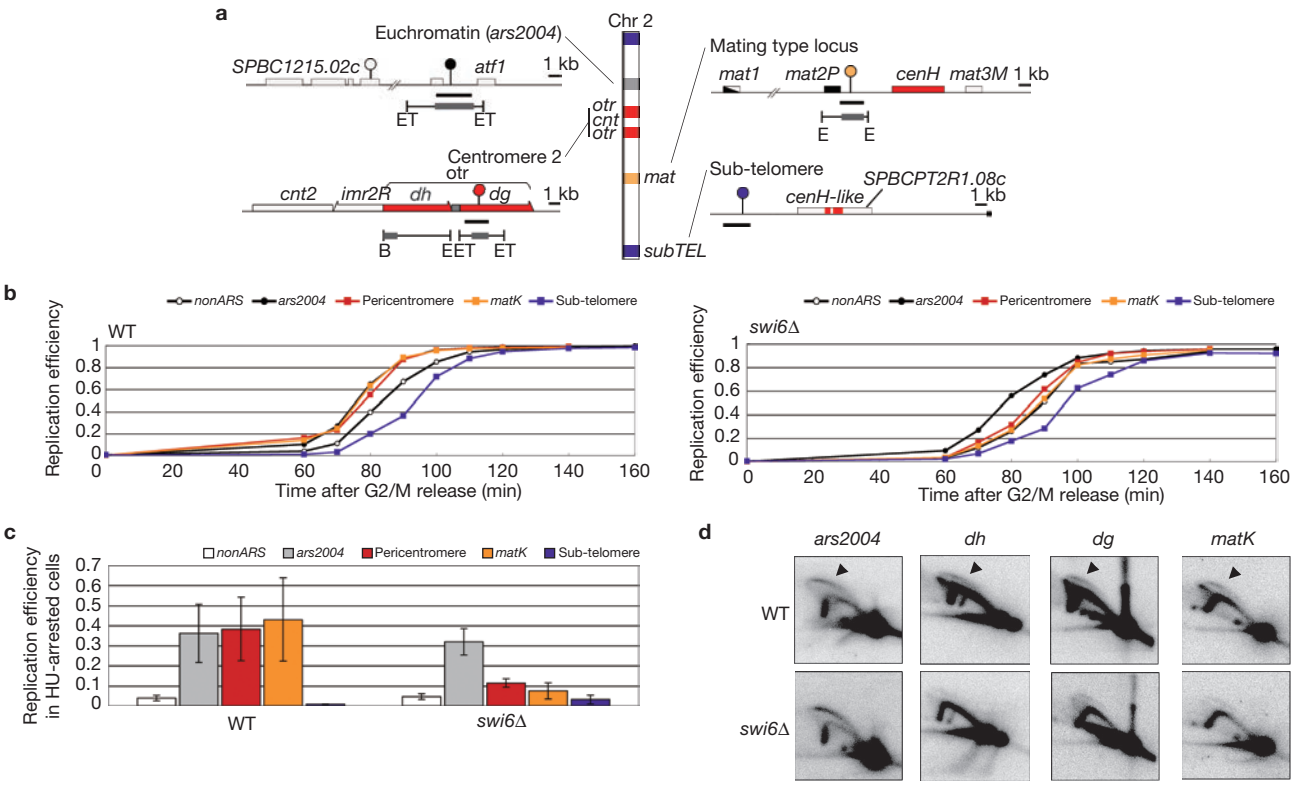

Figure 1 Swi6 promotes firing of origins in early S phase specifically at the pericentromeric region and *matK*. (**a**) Schematic representation of *S. pombe* euchromatic (*ars2004*) and heterochromatic (centromere, the mating type locus and sub-telomeric region) loci. The central vertical box represents a whole view of chromosome 2 along with the locations of *ars2004* (grey); *otr*, pericentromeric heterochromatin (red) flanking the central core region (*cnt*); *mat*, the mating type locus (yellow); and *subTEL*, sub-telomeric heterochromatin (purple). Maps of relevant regions are shown. The *dh* and *dg* repeats at the pericentromeric region, *cenH* in the silent mating type locus, and *cenH*-like in the sub-telomeric region contain sequences involved in the establishment of heterochromatin through an RNAi-dependent mechanism (red boxes on each map). A black bar below the map denotes the fragment used for ARS analysis (Supplementary Information, Fig. S1). Relevant restriction fragments (ET, *Eco*T22I; B, *Bam*HI; E, *Eco*RI) and probes (grey box) used for two-dimensional gel electrophoresis and Southern hybridization are shown below the maps. 'Lollipops' represent positions of the segment analysed by quantitative real-time PCR. (**b**) Early replication at the pericentromeric region and *matK* depends on Swi6. Wild-type and *swi6Δ* cells carrying *cdc25–22* were arrested in G2/M phase at 36 °C for 3 h and then released at 25 °C in the presence of BrdU. At the indicated time points, newly replicated DNA with heavy-light (HL) density was separated from unreplicated light-light (LL) DNA by CsCl density gradient centrifugation. Replication efficiency was determined by quantitative real-time PCR. (**c**) Replication efficiency was determined in wild-type and *swi6Δ* cells in the presence of hydroxyurea (10 mM) at 100 min after release from G2/M synchronization. Data are mean ± s.d. ($n = 3$). (**d**) Swi6 promotes initiation of replication at the pericentromeric region and *matK*. Replication intermediates prepared from wild-type or *swi6Δ* cells with hydroxyurea at 80 min after release from G2/M arrest were analysed by neutral-neutral 2D gel electrophoresis. Arrowheads indicate bubble arcs in wild-type samples.

Fig. S2), indicating that H3K9me-dependent localization of Swi6 is required for early replication of these loci.

To examine whether the delay in replication of the pericentromeric region and *matK* in *swi6Δ* cells is caused by impaired initiation of replication, replication efficiency was measured in the presence of hydroxyurea, which depletes dNTPs and allows replication only around active replication origins[3]. In the wild-type, *ars2004*, the pericentromeric region and *matK* replicated at efficiencies about ten times higher than the *nonARS* and the sub-telomeric region (Fig. 1c). In contrast, in *swi6Δ*, the replication efficiencies at the pericentromeric region and *matK* were reduced to the level of the *nonARS* locus, whereas *ars2004* replicated as efficiently as in the wild-type (Fig. 1c). These results suggest that Swi6 is required for initiation of replication at the pericentromeric region and *matK*. To examine more directly whether absence of Swi6 decreases initiation of replication at these loci, replication intermediates were analysed by two-dimensional (2D) gel electrophoresis. In the wild-type, the presence of bubble arcs at *ars2004*, the *dh* and *dg* repeats of the pericentromeric region, and *matK* locus indicates initiation of replication at these loci (Fig. 1d, arrowheads). Strong Y arcs at the *dh* and *dg* repeats probably represent passive replication from neighbouring repeats. In *swi6Δ* cells, the bubble arcs were reduced at the *dh* and *dg* repeats and *matK* locus but not at *ars2004* (Fig. 1d). These results confirm that Swi6 is required for efficient initiation of replication, specifically at the pericentromeric region and *matK*.

The initiation process consists of assembling initiation factors at replication origins. In *S. pombe*, the factors assemble in a distinct order[17] (Fig. 2a). ORC and MCM complexes bind to replication origins to form pre-replicative complexes (pre-RCs) in G1 phase. At the onset of S phase, Sld3 binds to origins in a DDK-dependent manner, and then the GINS complex is recruited in a CDK-dependent manner, followed by loading of Cdc45 before the single-strand DNA-binding protein (RPA) binds. To determine the mechanism by which Swi6 stimulates initiation of replication, we examined localization of replication factors at origins using chromatin immunoprecipitation (ChIP) analysis. We first examined Orc4 and Mcm6 in wild-type and *swi6Δ* cells arrested in early S phase by hydroxyurea. ChIP analysis of Orc4 in the wild-type revealed that the *ars2004*, pericentromeric region and *matK* fragments were enriched relative to the

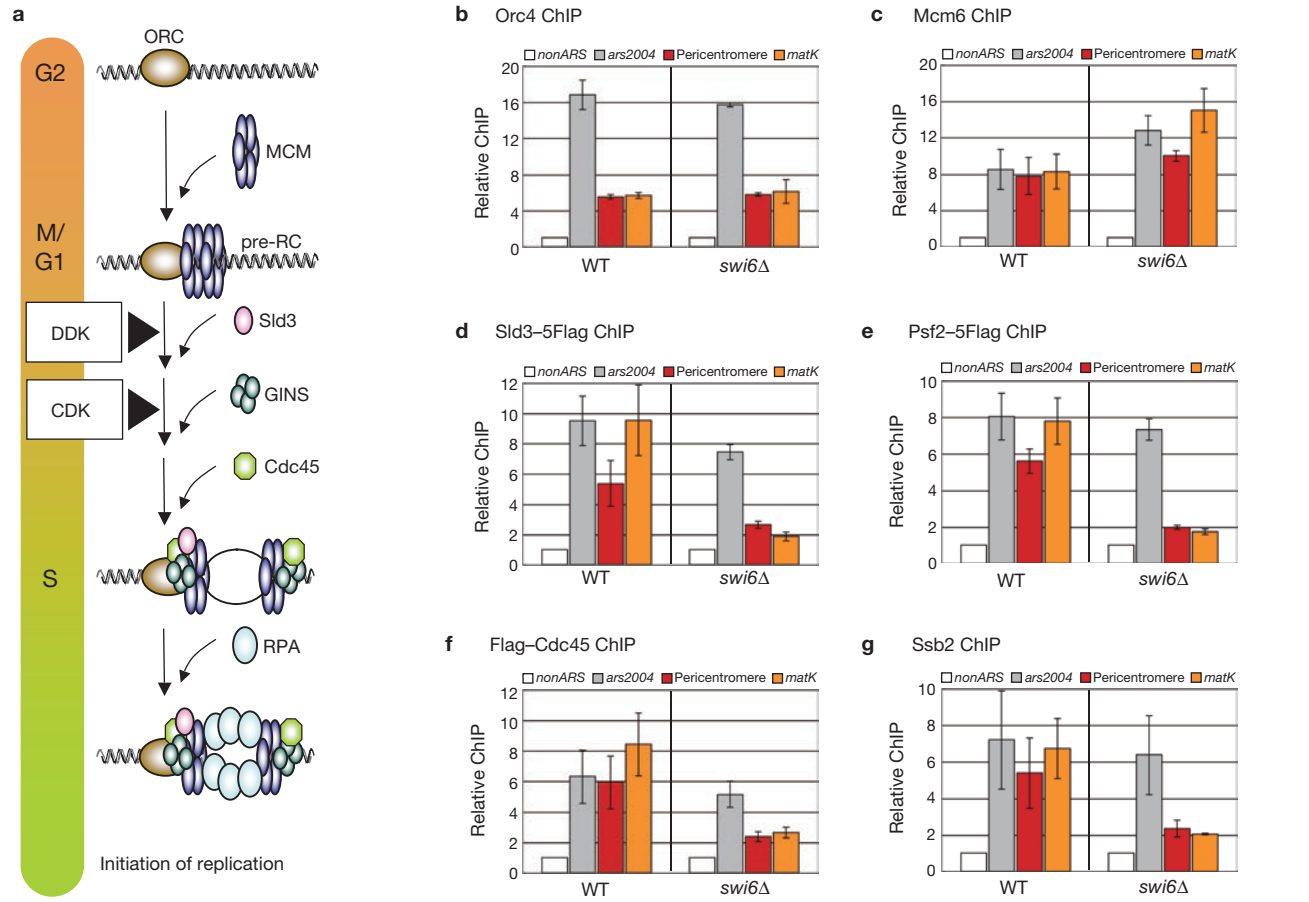

Figure 2 Swi6 stimulates loading of Sld3 at the pericentromeric region and *matK*. (**a**) A model for replication initiation in *S. pombe*. Details are described in the text. (**b–g**) Localization of Sld3 to the pericentromeric region and *matK* is impaired in *swi6Δ* cells. Localization of Orc4 (**b**), Mcm6 (**c**), Sld3–5Flag (**d**), Psf2–5Flag (**e**), Flag–Cdc45 (**f**) and Ssb2 (**g**) at euchromatic and heterochromatic loci was examined by ChIP using quantitative real-time PCR. Wild-type and *swi6Δ* cells, carrying *psf2–5Flag* or *Flag–cdc45*, were arrested in early S phase by hydroxyurea for 80 min after G2/M synchronization (**b**, **c**, **e–g**). Wild-type and *swi6Δ* cells, carrying *sld3–5Flag*, were arrested in early S phase using the *mcm5* mutant[18] after G2/M synchronization (**d**). Recovery ratios of immunoprecipitated DNA to total DNA at the indicated loci were normalized to the value of nonARS locus. Data are mean ± s.d. ($n = 3$).

nonARS locus, indicating localization of ORC at these origins (Fig. 2b). Absence of Swi6 did not affect localization of Orc4 (Fig. 2b). Similarly, the preferential localization of Mcm6 at *ars2004*, pericentromeric region and *matK* was not decreased in *swi6Δ* cells (Fig. 2c), indicating that Swi6 is not required for pre-RC formation. In contrast, localization of Sld3 was decreased in *swi6Δ* cells at the pericentromeric region and *matK* but not at *ars2004* (Fig. 2d). Consistent with this observation, localization of Psf2 (a component of GINS), Cdc45 and Ssb2 (a component of RPA) was reduced at these heterochromatic loci in *swi6Δ* (Fig. 2e–g). These results strongly suggest that Swi6 stimulates loading of Sld3 onto origins in the pericentromeric region and *matK*.

Next we investigated the molecular mechanism by which Swi6 stimulates Sld3 loading. It has been reported that the carboxy-terminal region of Dfp1, which contains a putative chromoshadow-domain-binding (HP1-binding) motif (PxVxL/I/V), is involved in the interaction with Swi6 (ref. 13). This interaction was thought to be separable from the initiation activity of DDK, because overall DNA replication seemed to be normal in a *dfp1* mutant lacking the C-terminal region[13]. However, as DDK is required for Sld3 loading onto replication origins[18], we examined the possibility that the interaction is required for initiation of replication specifically at the pericentromeric region and *matK*. To verify that Swi6 interacts with Dfp1 *in vitro*, S-peptide-tagged Dfp1 was mixed with GST–Swi6 or GST and pulled down with glutathione beads. Dfp1 was recovered with GST–Swi6 but not with GST, indicating that Dfp1 interacts directly with Swi6 (Fig. 3b). Introducing amino acid changes in the HP1-binding motif (Fig. 3a) markedly reduced the recovery of Dfp1 in pulldown assays (Fig. 3b), indicating that the motif is required for the interaction. To examine the effects of substitutions on DNA replication *in vivo*, the endogenous *dfp1*$^{+}$ gene was replaced with *dfp1-3A*. Replication of the pericentromeric region and *matK* in *dfp1-3A* cells was delayed to a similar extent as that of the *nonARS* locus (Fig. 3c). The *dfp1-2E* mutation caused a similar delay (Supplementary Information, Fig. S3). Localization of Swi6 at heterochromatin was not significantly altered by the *dfp1-3A* mutation (Fig. 3d), suggesting that DDK functions downstream of Swi6 localization. These results show that the interaction of Swi6 with Dfp1 is required for early replication at the pericentromeric region and *matK*. Although we attempted to assess whether recruitment of DDK to the pericentromeric region and *matK* depends on Swi6, we could not locate Flag–Dfp1 or Flag–Hsk1, a catalytic subunit of DDK at the pericentromeric region, *matK* or even at the euchromatic origin *ars2004*, because of the low signal-to-noise ratio in ChIP analysis (data not shown).

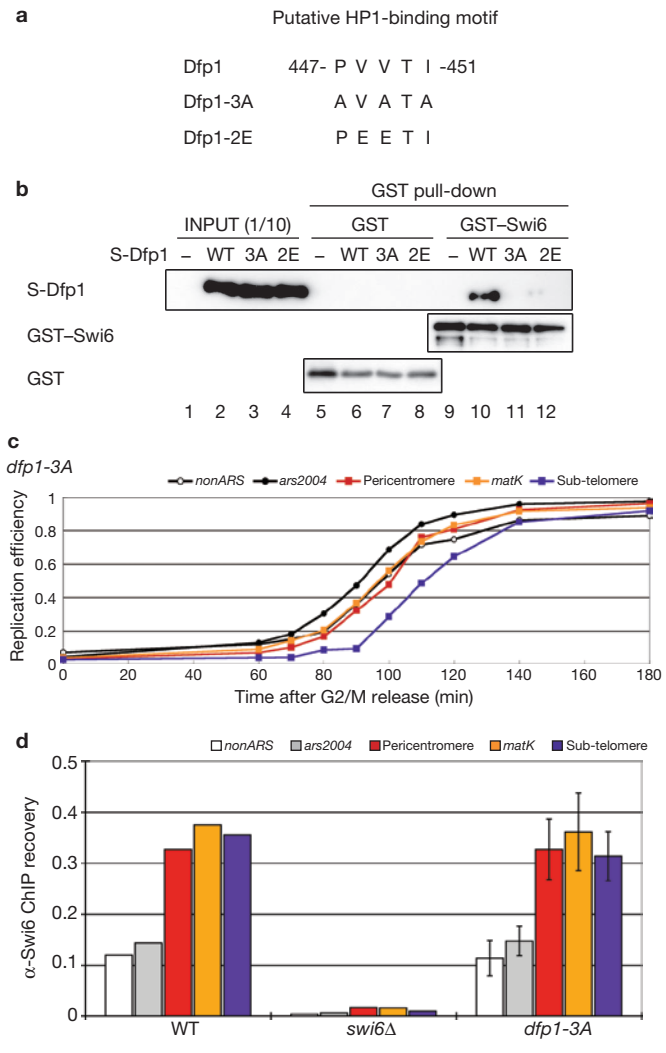

Figure 3 Point-mutations in the HP1-binding motif of Dfp1 decrease its *in vitro* interaction with Swi6 and delay replication at the pericentromeric region and *matK in vivo*. (**a**) The amino acid sequence of the HP1-binding motif in the C-terminal region of Dfp1 and substitutions in the motif (Dfp1-3A and Dfp1-2E) are shown. (**b**) The HP1-binding motif in Dfp1 is required for interaction with Swi6. S-peptide tagged Dfp1, Dfp1-3A and Dfp1-2E expressed *in vitro* were incubated with GST or GST–Swi6 in the presence of glutathione beads, and bead-bound proteins were analysed by western blotting with anti-S and anti-GST antibodies. (**c**) Early replication of the pericentromeric region and *matK* depends on the HP1-binding motif of Dfp1. Replication kinetics in *dfp1-3A* cells were analysed as described in Fig. 1b. (**d**) Swi6 localization at heterochromatin is maintained in the *dfp1-3A* mutant. The localization of Swi6 was analysed by ChIP in wild-type, *swi6Δ* and *dfp1-3A* cells arrested in early S phase by hydroxyurea. Recovery ratios of immunoprecipitated DNA to total DNA at the indicated loci are shown. Data are mean, $n = 2$ for wild-type and *swi6Δ*; and mean ± s.d., $n = 3$ for *dfp1-3A*).

The results described above prompted us to examine whether forced localization of DDK at the pericentromeric region and *matK* would restore early replication in the absence of Swi6. To localize Dfp1 to these loci in *swi6Δ* cells, Dfp1 fused at the C terminus with two copies of the CD of Swi6 was expressed from the native *dfp1* promoter because H3K9me remains in the pericentromeric region and at specific sites within the silent *mat* region, independently of Swi6 (refs 19–21). Early replication of the pericentromeric region and *matK* was restored in *swi6Δdfp1–CFP–2CD* cells, but not in *swi6ΔCFP–2CD*, without fusion to Dfp1 (Fig. 4a). Consistent with these results, we confirmed that bubble arcs were restored at the pericentromeric region and *matK* in *swi6Δ* cells by expressing Dfp1–CFP–2CD, but not by expressing CFP–2CD (Fig. 4b). We also showed that tethering of Dfp1–3A–CFP–2CD to the pericentromeric region and *matK* in *swi6Δ* cells restored early replication (Supplementary Information, Fig. S4), confirming that delayed replication of these loci in *dfp1-3A* cells was caused by inefficient localization of DDK at these sites. From these results, we conclude that Swi6 activates replication origins by recruiting DDK to heterochromatic loci. It should be noted that replication of the sub-telomeric region was not significantly altered in *swi6Δdfp1–CFP–2CD* cells (Fig. 4a). ChIP analysis showed that Dfp1–CFP–2CD is localized at the pericentromeric region and *matK*, but not at the sub-telomeric region (Fig. 4c). The absence of Dfp1–CFP–2CD from the sub-telomeric region in *swi6Δ* is probably a result of decreased maintenance and sub-telomeric spreading of H3K9me in the absence of Swi6 (refs 22, 23).

Our results suggest that access of DDK to the pericentromeric region and *matK* is hindered in *swi6Δ* cells. To test the possibility that remaining heterochromatin components that bind to H3K9me in the absence of Swi6 are responsible for the delay in replication, we examined the effects of deleting *clr4*$^{+}$, a fission yeast homologue of Su(var)3–9 histone methyltransferase, on replication in the *swi6Δ* background, as localization of all heterochromatin components was abrogated in *clr4Δ* cells[22]. In *swi6Δclr4Δ* cells, the pericentromeric region replicated in early S phase, supporting the notion that the remaining heterochromatin structures are responsible for the delay in replication (Supplementary Information, Fig. S5). However, replication timing at *matK* remained as late as the *nonARS* locus. Therefore, in the absence of Swi6, H3K9me-dependent localization of other heterochromatin components suppresses activation of replication at the pericentromeric region, whereas some other mechanism(s) may be responsible for the suppression at *matK*. Both suppressive mechanisms are overcome by localization of Swi6, which recruits Dfp1 to the loci. In contrast to these loci, replication of the sub-telomeric regions was very late in wild-type, *swi6Δ* and *swi6Δclr4Δ* (Figs 1b; Supplementary Information, Fig. S5). There seems to be a supressing mechanism(s) specific for the sub-telomeric region-specific that counteracts the positive effect of Swi6. These results suggest that replication origins are suppressed by different mechanisms at three heterochromatic loci.

We questioned why the sub-telomeric region replicates in late S phase, regardless of Swi6 localization. Orc4 and Mcm6 were localized at the sub-telomeric ARS in early S phase in wild-type and *swi6Δ* cells, whereas Sld3, Psf2, Cdc45 or Ssb2 were not efficiently enriched in either cell type (Supplementary Information, Fig. S6). Given that DDK has a pivotal role in replication of the pericentromeric region and *matK*, it is possible that the sub-telomeric region is highly inaccessible to DDK. We examined whether forced recruitment of DDK would accelerate replication in sub-telomeric heterochromatic protein by expressing Dfp1–CFP–2CD or CFP–2CD in wild-type cells. Expression of CFP–2CD did not alter the replication kinetics of the sub-telomeric region, relative to *ars2004* and the *nonARS* locus, although replication of the pericentromeric region and *matK* was slightly delayed (Fig. 4d), probably because CFP–2CD competes with Swi6 for binding to H3K9me. In contrast, expression of Dfp1–CFP–2CD accelerated replication of the sub-telomeric region to a rate similar to that of the *nonARS* locus, whereas the replication kinetics of the pericentromeric region and *matK* were not significantly altered (Fig. 4d). These results suggest that sub-telomeric origins are activated, at least partially, by tethering of DDK.

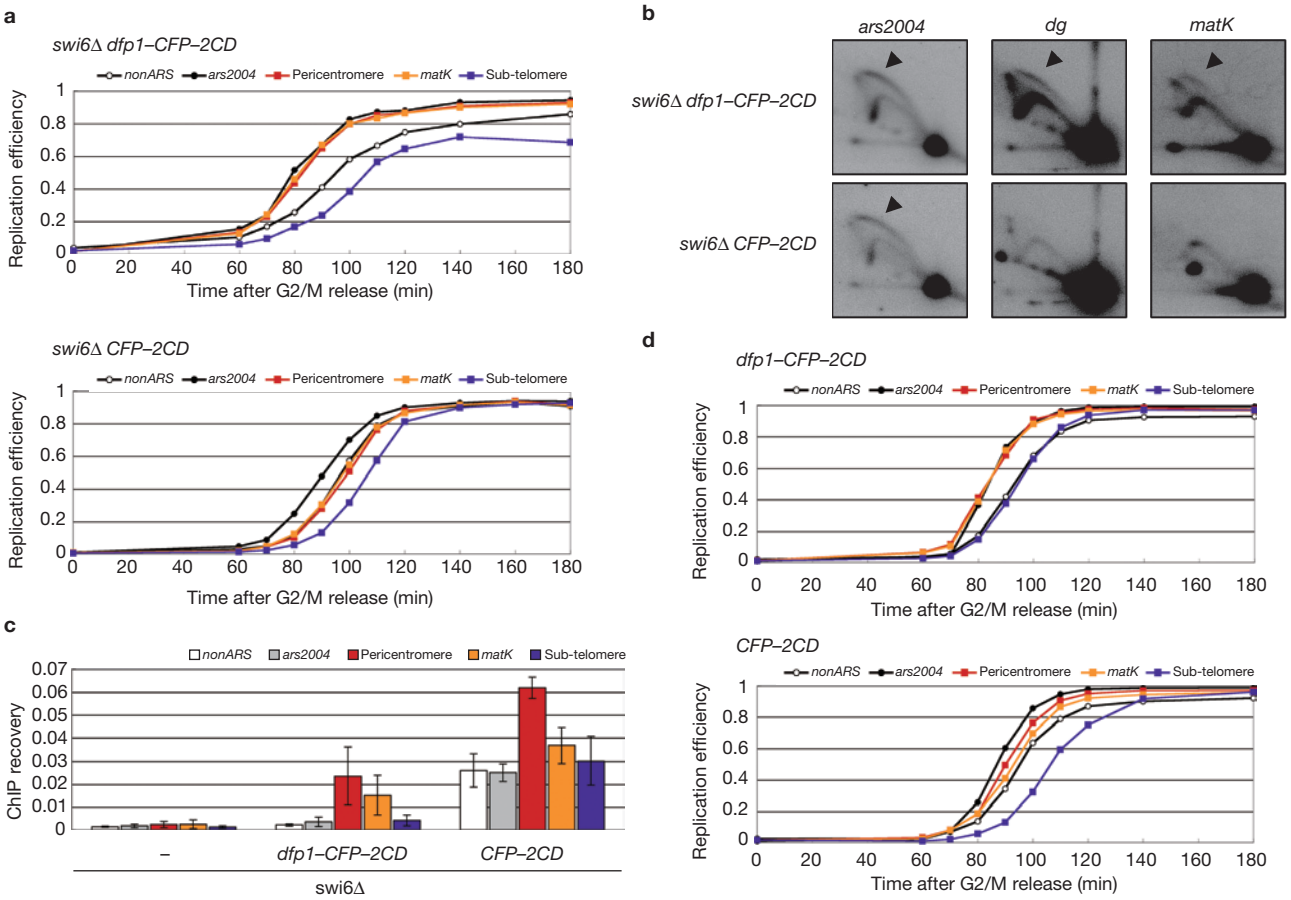

Figure 4 Tethering Dfp1 to heterochromatin accelerates replication timing independently of Swi6 localization. (**a**) Dfp1–CFP–2CD restores early replication timing at the pericentromeric region and *matK* in the absence of Swi6. Replication kinetics in *swi6Δ dfp1–CFP–2CD* and *swi6Δ CFP–2CD* cells were analysed as in Fig. 1b. (**b**) Expression of Dfp1–CFP–2CD restores initiation of replication at the pericentromeric region and *matK* in *swi6Δ* cells. Replication intermediates prepared from *swi6Δ* cells expressing Dfp1–CFP–2CD or CFP–2CD were analysed as described in Fig. 1d. Arrowheads indicate bubble arcs. (**c**) Dfp1–CFP–2CD localizes to the pericentromeric region and *matK* in *swi6Δ* cells. ChIP analysis using anti-GFP antibody for CFP-tag was performed in *swi6Δ dfp1–CFP–2CD* and *swi6Δ CFP–2CD* cells as described in Fig. 3d. Data are mean ± s.d. ($n = 3$) (**d**) Expression of Dfp1–CFP–2CD in wild-type cells accelerates replication timing at the sub-telomeric region. Replication kinetics in *dfp1–CFP–2CD* and *CFP–2CD* cells were analysed as in Fig. 1b.

The results presented here shed light on the dynamic nature of heterochromatin, providing the first example of a heterochromatic protein actively regulating initiation of DNA replication. The underlying mechanism is the physical interaction between Swi6 and DDK through the HP1-binding motif in Dfp1. Swi6 is likely to recruit DDK to the heterochromatic pericentromeric region and *matK* and stimulate DDK-dependent assembly of the replication factor Sld3 (Fig. 5). Our results showing that replication is delayed in *dfp1-3A* and *swi6Δ* cells lacking the interaction of Dfp1 with Swi6 (Fig. 5), and that replication of the pericentromeric region was not delayed in *swi6Δclr4Δ* cells, are consistent with the notion heterochromatin structures themselves are inaccessible to trans-acting factors. The HP1-binding motif in Dfp1 does not seem to be conserved in Dbf4 homologues in other eukaryotes. However, their direct interaction needs to be examined experimentally, because it is still possible that heterochromatin proteins participate in DNA replication by recruiting the regulatory kinase to heterochromatin in higher eukaryotes, in which global regions of chromosomes form heterochromatin. Controlling the timing of heterochromatin replication may be important for the regulation of gene expression during differentiation and development[7].

Our results also suggest that the sub-telomeric region may possess another mechanism to suppress origin activation despite localization of Swi6 (Fig. 5). Although the molecular mechanism for this suppression is unknown, tethering of DDK to the sub-telomeric region partially overcomes the inhibitory effect. Therefore, DDK seems to be an important target even in the regulation of sub-telomeric replication origins. Origins in euchromatic loci are classified into two categories: early origins that replicate in early S phase and late/potential origins that cannot initiate replication in early S phase[3]. Early origins in euchromatic region are probably accessible to DDK, or alternatively, euchromatic origins may have distinct mechanism(s) to recruit DDK at a specific period in S phase. Interestingly, euchromatic late/potential origins are suppressed, at least in early S phase, at the step of Sld3 loading (H. Yabuuchi and H.M., unpublished results). Thus, we propose that recruitment of DDK is a crucial step in the regulation of replication timing at all replication origins in fission yeast, although the mechanisms of recruitment may differ between chromatin regions. □

METHODS

Yeast strains and genetics. All *S. pombe* strains used in this study are listed in Supplementary Information, Table S1. To express Dfp1-3A or Dfp1-2E mutant protein from the endogenous *dfp1*$^{+}$ promoter, the *dfp1-3A* or *dfp1-2E* mutation was introduced by site-directed mutagenesis and cloned into a pFA6a-kanMX6 carrying the downstream region of the *dfp1* locus (pFA-K-dw), followed by replacement of the C-terminal 13-Myc cassette and *ADH1* terminator with a potential

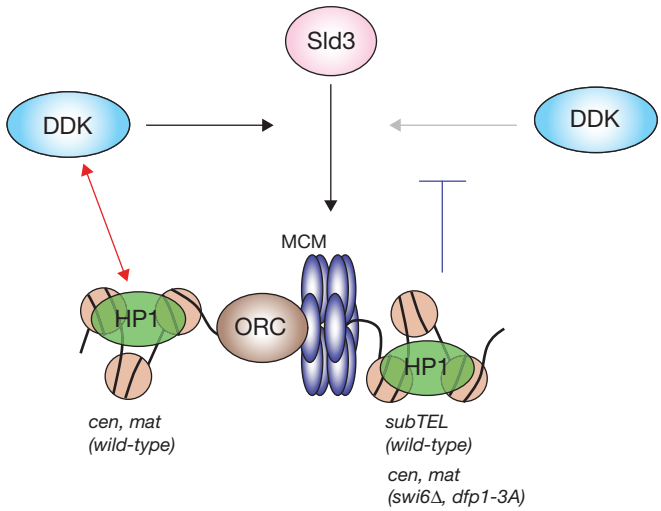

Figure 5 A model for the regulation of replication origins in heterochromatic loci. Swi6/HP1 recruits DDK to the pericentromere and the *matK* through physical interaction, which promotes loading of Sld3 onto pre-RCs and early replication of the loci (left). The positive effect of Swi6 seems to be cancelled in the sub-telomeric region by unknown mechanism(s) (right). In *swi6Δ* and *dfp1-3A* mutant cells, remaining heterochromatin components and/or unknown mechanism(s) may prevent DDK localization in heterochromatin, resulting in late replication of the loci (right).

terminator region of *dfp1*$^+$. The resulting plasmid (pFA-dfp1-3A-K-dw or pFA-dfp1-2E-K-dw) was digested at *Not*I sites for introduction into the *dfp1*$^+$ locus and the transformants were isolated using medium containing G418. For construction of Dfp1–CFP–2CD or CFP–2CD, the *CFP* open reading frame and a sequence encoding two copies of the chromodomain of Swi6 (ref. 24) were fused to the C terminus of *dfp1*$^+$ with its native promoter or immediately adjacent to the *dfp1*$^+$ native promoter, respectively, on a plasmid with a *hyg*^{r} cassette. The resulting plasmid carrying *dfp1–CFP–2CD* or *CFP–2CD* was integrated at the *lys1*$^+$ locus of *dfp1*$^+$ cells, and the transformants were isolated using medium containing hygromycin.

BrdU incorporation. Labelling of newly synthesized DNA with BrdU, separation of the heavy-light (HL) DNA from light-light (LL) DNA by CsCl density gradient centrifugation, and real-time PCR analysis were carried out as described previously[3]. Primers used for real-time PCR are listed in Supplementary Information, Table S2.

***In vitro* pulldown assay.** The binding of Dfp1 to Swi6 was analysed essentially as described previously[11]. The N-terminally S-tagged Dfp1, Dfp1-3A and Dfp1-2E proteins expressed by an *in vitro* translation system (Promega) were incubated with *Escherichia coli* expressed GST-tagged Swi6 or GST together with glutathione-Sepharose beads (GE Healthcare) in immunoprecipitation buffer-N50 (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1% N-P40, 1 mM EDTA, pH 8.0) for 2 h at 4 °C. The beads were washed three times with immunoprecipitation buffer-N50 and the bound proteins were analysed by SDS–PAGE followed by immunoblotting. S-Dfp1 and GST–Swi6 were detected by fluorography using rabbit anti-S antibodies (1:5,000; Bethyl) and goat anti-GST antibodies (1:2,000; GE Healthcare), respectively, followed by reactions with HRP-conjugated antibodies (1:10,000; Jackson).

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was performed essentially as described previously[3] with rabbit anti-Orc4 (1:267), rabbit anti-Mcm6 (1:200), rabbit anti-Ssb2 (1:400), mouse anti-Flag (1:333; M2 monoclonal, Sigma), rabbit anti-Swi6 (1:200) and rabbit anti-GFP (1:400; Living Colors full-length A.v. polyclonal antibody, Clontech) antibodies. DNA prepared from whole-cell extracts or immunoprecipitated fractions was analysed by real-time PCR using SYBR green I in a 7300 real-time PCR System (Applied Biosystems).

Two-dimensional gel electrophoresis. Neutral-neutral 2D gel electrophoresis analysis of replicating DNA was performed as described previously[3]. Relevant ARSs were used for Southern hybridization probes after random prime labelling. For detection of pericentromeric origins, portions of the ARSs in the pericentromeric region (ars3.0K for *dg* and arsL for *dh*)[14] were cloned by PCR to prevent cross hybridization between pericentromeric repeats and the cenH in *matK*.

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS
M.T.H. and H.M. designed the project and wrote the paper; M.T.H. performed the experiments; J.N. provided important experimental materials and advice. All authors contributed to the interpretation of the results and edited the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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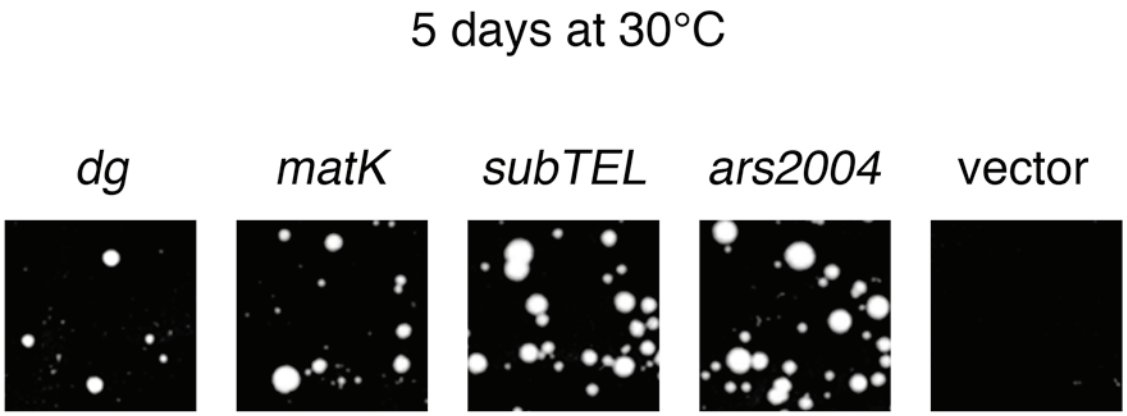

Figure S1 All heterochromatic loci contain autonomously replicating sequence (ARS). To examine the ARS activity of heterochromatic replication origins, fragments corresponding to ars3.0K[1] in the pericentromere (*dg*) and ars2PR[2] in the *mat* locus, which had been previously described, were PCR-cloned into pYC11 carrying *LEU2* gene. For the subtelomeric replication origin, a fragment that contains multiple AT-stretches characteristic in fission yeast replication origins at 21 kb from the right telomere of chromosome 2 was PCR-cloned into pYC11. Each plasmid was introduced into HM123 (h^- *leu1-32*) followed by 5 days incubation at 30°C and pictures were taken. *Ars2004* and vector serve as positive and negative controls, respectively.

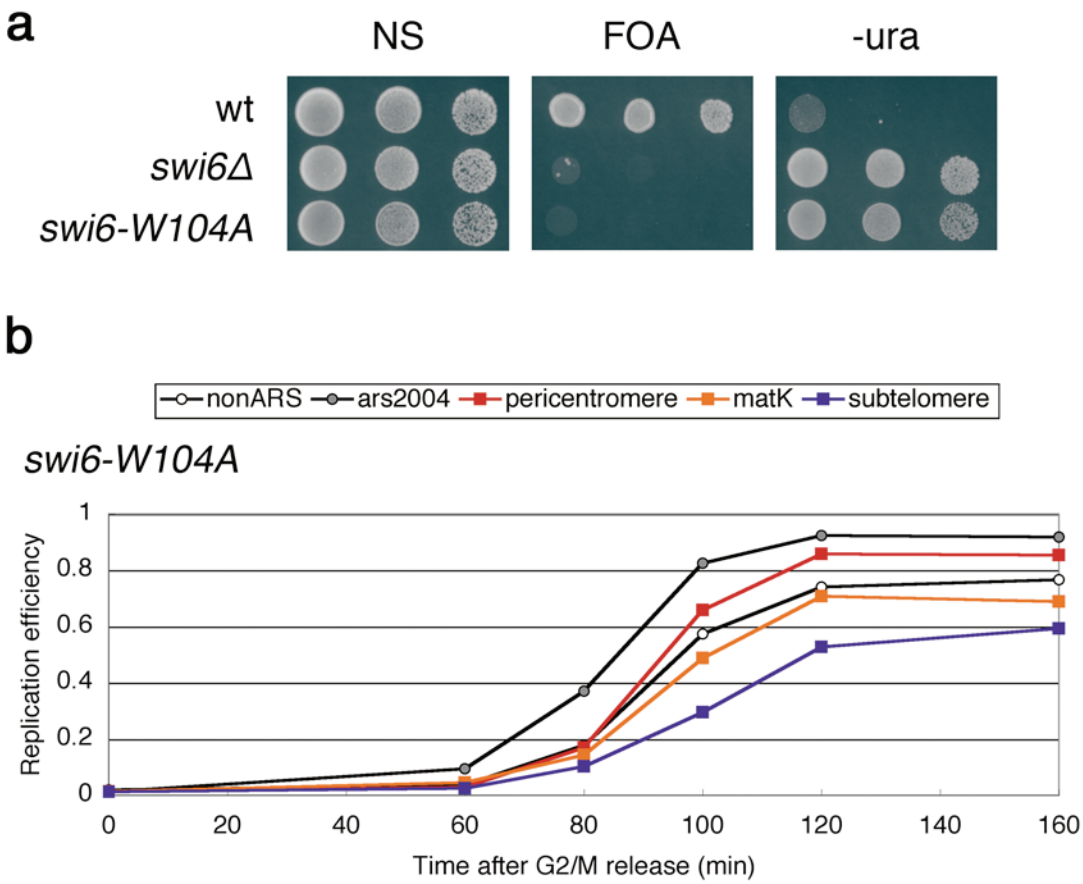

Figure S2 Swi6 promotes early replication at the pericentromere and the *mat* locus in a chromo-domain dependent manner. To express Swi6-W104A mutant protein from the endogenous *swi6*$^{+}$ promoter, the *swi6*$^{+}$ coding sequence with its potential promoter and terminator regions was first cloned into pBluescript (pAL2pBK), and a *hyg*^{r} cassette was introduced into the pAL2pBK plasmid (pAL2pBK-H). The W104A mutation was introduced using site-directed mutagenesis and sequence was confirmed. The resultant plasmid (pAL2W104ApBK-H) was cleaved with *Hpa*I for introduction into downstream of the *swi6* locus in *swi6Δ* cells, and the transformants were isolated using medium containing hygromycin. The expression of Swi6-W104A was confirmed by western blotting with anti-Swi6 antibody (data not shown). (**a**) The point mutation *swi6-W104A* impairs the silencing at the silent *mat* locus. Silencing of a *ura4*$^{+}$ marker inserted at the silent *mat* locus was examined by growth on selective media. Ten-fold-diluted cultures of indicated strains were plated onto nonselective medium (NS), medium containing 5-FOA (FOA) and medium lacking uracil (-ura). (**b**) Chromo-domain of Swi6 is required for early replication at the pericentromere and the *mat* locus. Replication kinetics in *swi6-W104A* cells was analyzed as in Fig. 1**b**.

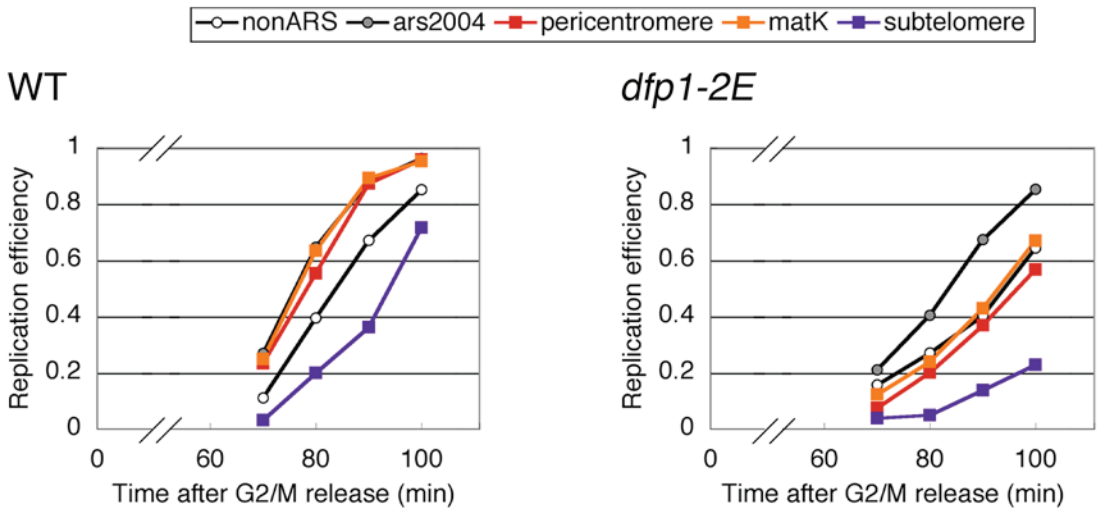

Figure S3 Replication delays at the pericentromere and the *mat* locus in *dfp1-2E* cells. The endogenous *dfp1*$^{+}$ gene was replaced with *dfp1-2E* and replication kinetics of indicated loci was examined as described in Fig. 1**b**.

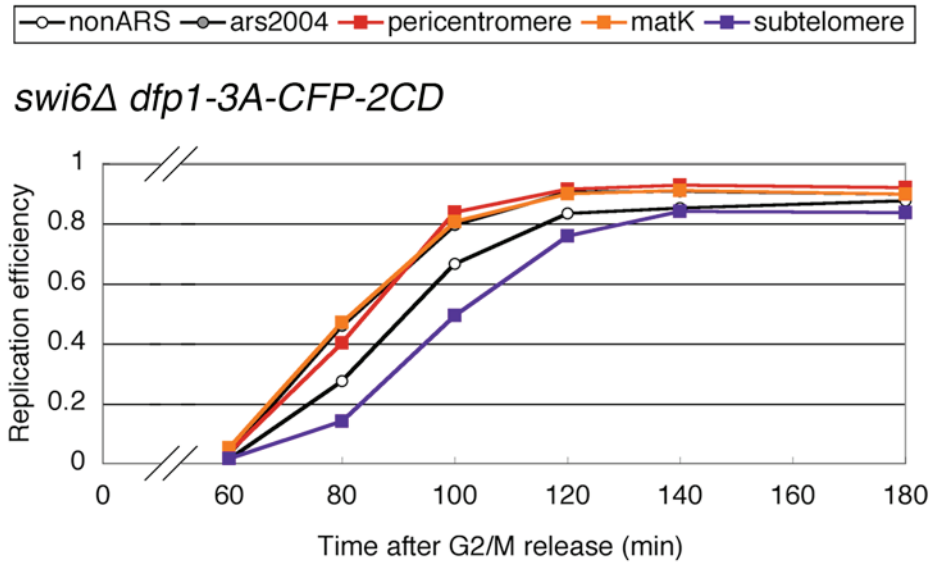

Figure S4 Tethering of Dfp1-3A restores early replication at the pericentromere and the *mat* locus in *swi6Δ* cells. Dfp1-3A was fused with CFP and two tandem copies of chromo-domain (CD) of Swi6 and expressed from the native *dfp1*$^{+}$ promoter in *swi6Δ* cells. Replication kinetics of indicated loci were analyzed as described in Fig. 1**b**.

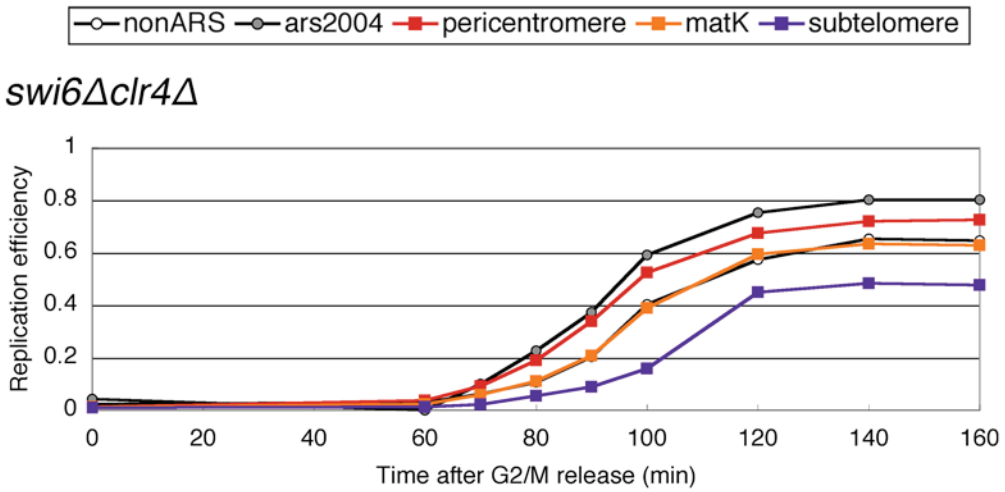

Figure S5 Deletion of the *clr4*$^{+}$ in *swi6Δ* background restores early replication at the pericentromere but not at the *mat* locus. Replication kinetics of indicated loci in *swi6Δclr4Δ* double mutant cells were analyzed as described in Fig. 1**b.**

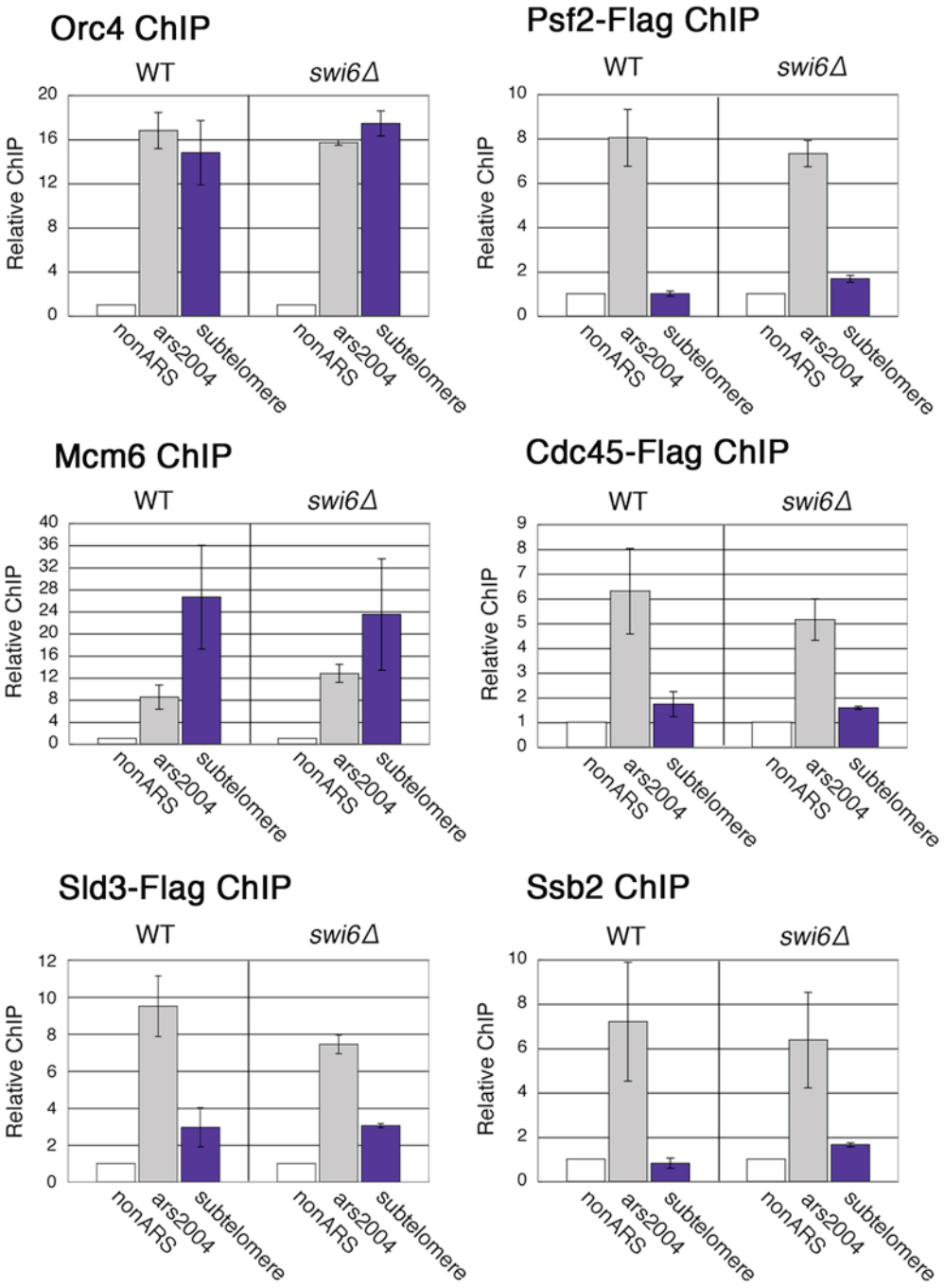

Figure S6 Initiation of replication at the subtelomeric ARS is inhibited after pre-RC formation in both wild type and *swi6Δ* cells. ChIP samples in Fig. 2 **b**-**g** were examined by quantitative real-time PCR using the subtelomeric primers shown in Fig. 1**a**. Error bars represent standard deviations (n=3).

Supplementary References

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Supplementary Table 1 *S. pombe* strains used in this study

Strain	Genotype		Figures and Supplementary figures
HM123	h^-	*leu1-32*	S1
HM664	h^-	*ura4-D18::ura4$^+$nmt1-TK$^+$*	used for transformation
HM683	h^+	*ura4-D18::ura4$^+$nmt1-TK$^+$*	used for transformation
HM1182	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ his2 Kint2::ura4$^+$*	1**b**, **c**, **d**, 3**d**, S3
HM1183	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ his2 Kint2::ura4$^+$ swi6Δ::kanMX6*	1**b**, **c**, **d**, 3**d**, 4**c**
HM1418	h^-	*ura4-D18::ura4$^+$nmt1-TK$^+$ lys1Δ::(dfp1$^+$-CFP-2CD hphMX6)*	transformant
HM1420	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ his2 Kint2::ura4$^+$ swi6Δ::kanMX6 lys1Δ::(dfp1$^+$-CFP-2CD hphMX6)*	4**a**, **b**, **c**
HM1423	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ his2 Kint2::ura4$^+$ lys1Δ::(dfp1$^+$-CFP-2CD hphMX6)*	4**d**
HM1460	h^-	*ura4-D18::ura4$^+$nmt1-TK$^+$ lys1Δ::(CFP-2CD hphMX6)*	transformant
HM1467	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ his2 Kint2::ura4$^+$ swi6Δ::kanMX6 lys1Δ::(CFP-2CD hphMX6)*	4**a**, **b**, **c**
HM1471	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ his2 Kint2::ura4$^+$ lys1Δ::(CFP-2CD hphMX6)*	4**d**
HM1482	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ his2 Kint2::ura4$^+$ swi6Δ::kanMX6 clr4Δ::kanMX6*	S5
HM1588	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ Flag-cdc45::kanMX6 his2 Kint2::ura4$^+$*	2**c**, **f**, **g**, S6
HM1589	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ Flag-cdc45::kanMX6 his2 Kint2::ura4$^+$ swi6Δ::kanMX6*	2**c**, **f**, **g**, S6
HM1590	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ psf2-5Flag::kanMX6 his2 Kint2::ura4$^+$*	2**b**, **e**, S6
HM1591	h^{90}	*cdc25-22 ura4-D18:: ura4$^+$nmt1-TK$^+$ psf2-5Flag::kanMX6 his2 Kint2::ura4$^+$ swi6Δ::kanMX6*	2**b**, **e**, S6
HM1826	h^+	*ura4-D18::ura4$^+$nmt1-TK$^+$ dfp1-3A::kanMX6*	transformant
HM1828	h^+	*ura4-D18::ura4$^+$nmt1-TK$^+$ dfp1-2E::kanMX6*	transformant
HM1841	h^{90}	*cdc25-22 nda4-108 ura4-D18::ura4$^+$nmt1-TK$^+$ sld3-5Flag::kanMX6 his2 Kint2::ura4$^+$*	2**d**, S6
HM1843	h^{90}	*cdc25-22 nda4-108 ura4-D18::ura4$^+$nmt1-TK$^+$ sld3-5Flag::kanMX6 his2 Kint2::ura4$^+$ swi6Δ::kanMX6*	2**d**, S6
HM1853	h^{90}	*cdc25-22 ura4-D18::ura4$^+$nmt1-TK$^+$ dfp1-3A::kanMX6 his2 Kint2::ura4$^+$*	3**c**, **d**
HM1857	h^{90}	*cdc25-22 ura4-D18::ura4$^+$nmt1-TK$^+$ dfp1-2E::kanMX6 his2 Kint2::ura4$^+$*	S3
HM1899	h^+	*ura4-D18::ura4$^+$nmt1-TK$^+$ lys1Δ::(dfp1-3A-CFP-2CD hphMX6)*	transformant
HM1934	h^{90}	*cdc25-22 ura4-D18::ura4+nmt1-TK$^+$ his2 Kint2::ura4$^+$ swi6Δ::kanMX6 lys1Δ::(dfp1-3A-CFP-2CD hphMX6)*	S4
HM1994	h^{90}	*ura4-DS/E Kint2::ura4$^+$ swi6Δ::kanMX6*	S2**a**
HM2608	h^{90}	*cdc25-22 ura4-D18::ura4+nmt1-TK$^+$ his2 Kint2::ura4$^+$ swi6Δ::kanMX6::(swi6-W104A hphMX6)*	S2**b**
HM2613	h^{90}	*ura4-DS/E Kint2::ura4$^+$ swi6Δ::kanMX6::(swi6-W104A hphMX6)*	S2**a**
TNF2518	h^{90}	*ura4-DS/E Kint2::ura4$^+$*	S2**a**

Supplementary Table 2 Primers used in this study

Locus	Name	Sequence	Source
ars2004	ars2004-66-F	5'-CGGATCCGTAATCCCAACAA-3'	Hayashi *et al*., 2007
	ars2004-66-R	5'-TTTGCTTACATTTTCGGGAACTTA-3'	
nonARS	nonARS-70-F	5'-TACGCGACGAACCTTGCATAT-3'	Hayashi *et al*., 2007
	nonARS-70-R	5'-TTATCAGACCATGGAGCCCATT-3'	
dg (pericentromere)	dg-108-F	5'-TCCAAATGTCGCATGAACACTC-3'	Hayashi *et al*., 2007
	dg-108-R	5'-CTTTTTTGGGAATACATTGGGTTT-3'	
mat K locus	matK-108-F	5'-TCTTCCCTGCGTTGGACTTC-3'	This study
	matK-108-R	5'-CACCCTACCATCCGTGTTACCT-3'	
subtelomere	TEL-59-F	5'-CAGAAGAGACTACAGAGGCGGTTT-3'	This study
	TEL-59-R	5'-GGATGCCTTATCTGCGACCA-3'	