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A role for DNA polymerase α in epigenetic control of transcriptional silencing in fission yeast

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In the fission yeast *Schizosaccharomyces pombe*, transcriptional silencing at the mating-type region, centromeres and telomeres is epigenetically controlled, and results from the assembly of higher order chromatin structures. Chromatin proteins associated with these silenced loci are believed to serve as molecular bookmarks that help promote inheritance of the silenced state during cell division. Specifically, a chromodomain protein Swi6 is believed to be an important determinant of the epigenetic imprint. Here, we show that a mutation in DNA polymerase α (*polα*) affects Swi6 localization at the mating-type region and causes a 45-fold increase in spontaneous transition from the silenced epigenetic state to the expressed state. We also demonstrate that *polα* mutant cells are defective in Swi6 localization at centromeres and telomeres. Genetic analysis suggests that Polα and Swi6 are part of the same silencing pathway. Interestingly, we found that Swi6 directly binds to Polα *in vitro*. Moreover, silencing-defective mutant Polα displays reduced binding to Swi6 protein. This work indicates involvement of a DNA replication protein, Polα, in heterochromatin assembly and inheritance of epigenetic chromatin structures.

Keywords: DNA polymerase/epigenetic/fission yeast/heterochromatin/silencing

Introduction

During cellular differentiation, the fate of individual cells is determined by specifying unique patterns of gene expression. Once established, these expression patterns are faithfully maintained through multiple rounds of cell division. It is believed that differentiated cells rely on an epigenetic cellular memory system, whereby non-mutational reversible alterations of the chromosomes promote stable transmission of determined states to progeny cells. Self-templating chromatin structures and/or DNA methylation-mediated mechanisms have been suggested to be important for the inheritance of epigenetic states through DNA replication and chromosome duplication (reviewed in Riggs and Porter, 1996; Grewal, 2000).

Epigenetic control of higher order chromatin assembly has been observed in fission yeast at the mating-type (*mat2/3*) region, centromeres and telomeres (Allshire, 1996; Grewal, 2000). Prototrophic markers artificially inserted at these loci are subject to transcriptional silencing and exhibit variegated expression patterns (Thon *et al*., 1994; Allshire *et al*., 1995; Grewal and Klar, 1996; Ayoub *et al*., 1999). Several *trans*-acting factors that affect silencing at the 15 kb *mat2/3* interval and centromeres are either structural components of heterochromatin or histone-modifying enzymes. Among these factors, Clr3 and Clr6 belong to the family of histone deacetylases (HDACs; Grewal *et al*., 1998). Swi6, Clr4, Chp1 and Chp2 proteins contain a chromodomain, an evolutionarily conserved motif initially identified in heterochromatin-associated protein HP1 and polycomb in *Drosophila* (Lorentz *et al*., 1994; Ivanova *et al*., 1998; Eissenberg and Elgin, 2000; Thon and Verhein-Hansen, 2000). Clr4, which possesses intrinsic histone methyltransferase activity (Rea *et al*., 2000), recently was shown to methylate Lys9 preferentially on histone H3 tail at the *mat2/3* region and centromeres (Nakayama *et al*., 2001). It was also demonstrated that Clr4 cooperates with a histone H3-specific HDAC Clr3 to establish a 'histone code' that is essential for higher order chromatin assembly (Nakayama *et al*., 2001). Moreover, the localization of Swi6 to the silenced loci is mediated through its binding to the H3 Lys9-methyl mark (Bannister *et al*., 2001; Nakayama *et al*., 2001). The Swi6 protein is present throughout the silent *mat2/3* interval but its presence at centromeres is confined to the outer repeat sequences (Nakayama *et al*., 2000; Partridge *et al*., 2000).

How the silenced chromatin state is faithfully maintained during cell division remains unclear. Several lines of evidence have indicated a connection between the DNA replication machinery and silencing. For example, mutations in subunits of the origin recognition complex (ORC) cause derepression of silent mating-type loci in *Saccharomyces cerevisiae* (Micklem *et al*., 1993; Bell *et al*., 1995; Fox *et al*., 1995) and suppress position effect variegation (PEV) in *Drosophila* (Pak *et al*., 1997). Furthermore, chromatin assembly factor 1 (CAF-1), which physically associates with proliferating cell nuclear antigen (PCNA) and HP1 family members (Murzina *et al*., 1999; Shibahara and Stillman, 1999), participates in inheritance of epigenetic chromatin states (Enomoto and Berman, 1998; Zhang *et al*., 2000).

Previously, we showed that Swi6 is an integral component of the epigenetic cellular memory mechanism and might serve as a molecular bookmark to propagate the silenced chromatin state during cell division (Nakayama *et al*., 2000). Here, we show that a mutation in an essential gene, *swi7* (Singh and Klar, 1993), encoding the catalytic subunit of DNA polymerase α (Polα; Damagnez *et al*., 1991), adversely affects silencing and Swi6 localization at the mating-type region and centromeres. We also demonstrate that Swi6 directly interacts with Polα *in vitro*.

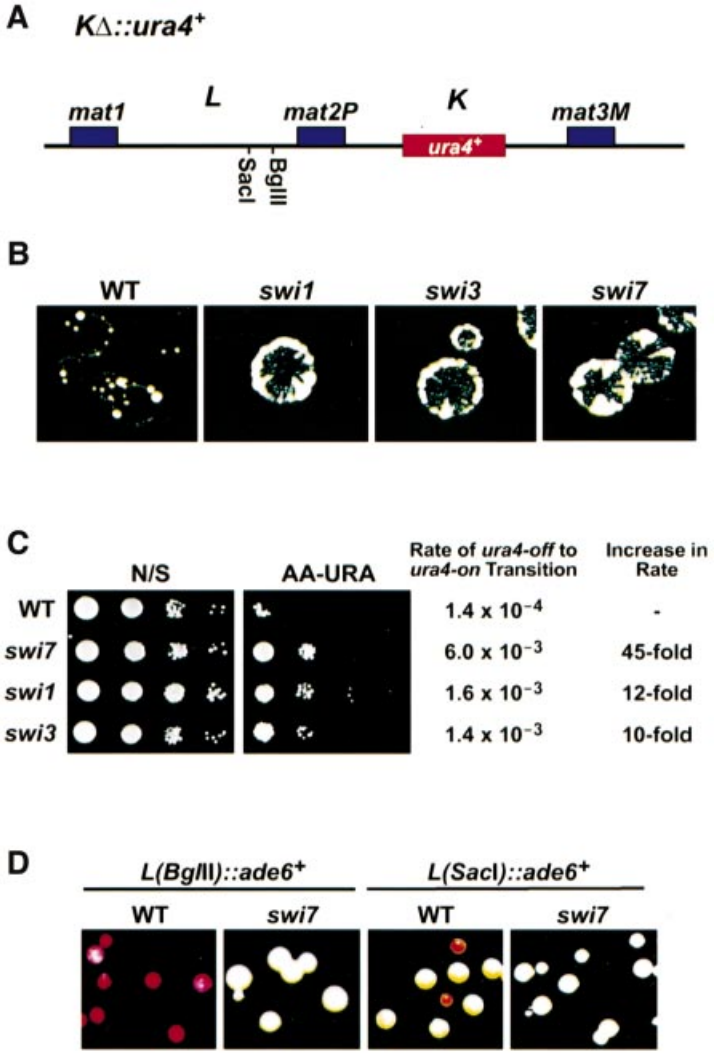

Fig. 1. Mutations in *swi1*, *swi3* and *swi7/polα* suppress PEV at the *mat2/3* locus and neighboring sequences. (**A**) The line drawing shows the physical map of the mating-type region in *KΔ::ura4*$^+$ cells (not drawn to scale). (**B**) Effect of *swi* mutations on *KΔ::ura4*$^+$ expression. The *ura4-off* derivatives of non-switching *mat1-Msmto KΔ::ura4*$^+$ strains with the wild-type (WT, SPG32), *swi7-1* (SPG106), *swi1-S28* (SPG112) or *swi3-146* (SPG114) mutant background were allowed to grow on YEA-rich medium. Colonies formed on YEA plates were then replicated onto AA-URA medium and incubated at 33°C for 72 h, except for the *swi7-1* mutant strain that was grown for only 24 h. (**C**) Serial dilution plating assay. Cells were suspended in water and then 10-fold serial dilutions were spotted onto non-selective (N/S), AA-URA or conterselective FOA medium and grown for 3 days before being photographed. The *KΔ::ura4*$^+$ strains used in (B) and (C) carried a mutation at the endogenous *ura4* locus. The Luria and Delbruck fluctuation test was employed to measure the transition rates quantitatively. (**D**) Mutation in *polα* suppresses PEV of *ade6*$^+$ expression at the *L* region. Cells were plated on adenine-limiting YE medium and incubated at 33°C for 3 days before being photographed. Representative colonies of wild-type (WT) or *swi7-1* (*swi7*) mutant strains with *ade6*$^+$ at the indicated sites in the *L* region are shown. The red or white colonies on YE medium imply *ade6-off* or *ade6-on* phenotypes, respectively. Strains used were: *L(Bgl*II*)::ade6*$^+$, SPG1218 (WT) and SPG1326 (*swi7*); *L(Sac*I*)::ade6*$^+$, SPG1217 (WT) and SPG1327 (*swi7*).

This study supports involvement of Polα in epigenetic control of higher order chromatin assembly.

Results

Mutation in polα suppresses PEV at the mating-type region

swi7/polα and two other genes (*swi1* and *swi3*), which genetically interact with *swi7*, were shown previously to be crucial for establishment of an imprint at *mat1* that is essential for mating-type switching (reviewed in Klar *et al*., 1998; Grewal, 2000). We tested whether mutations in these genes also affect transcriptional repression of the *KΔ::ura4*$^+$ marker gene at the *mat* locus. The *KΔ::ura4*$^+$ cells containing substitution of 7.5 kb of the *K* region with *ura4*$^+$ (see Figure 1A) exhibit variegation of *ura4*$^+$ expression through an epigenetic mechanism (Grewal and Klar, 1996). Furthermore, *ura4-off* and *ura4-on* epigenetic states representing 'closed' and 'open' chromatin structures, respectively, are inherited *in cis* during both mitosis and meiosis (Grewal and Klar, 1996; Nakayama *et al*., 2000). By genetic crosses, the mutations in *swi1*, *swi3* and *swi7/polα* were combined with the *ura4-off* derivative of a non-switching (*mat1-Msmto*; Styrkarsdottir *et al*., 1993) *KΔ::ura4*$^+$ allele. When replicated onto medium lacking uracil (AA-URA), we found that colonies formed by *ura4-off* cells carrying the *swi* mutations showed significantly higher levels of Ura$^+$

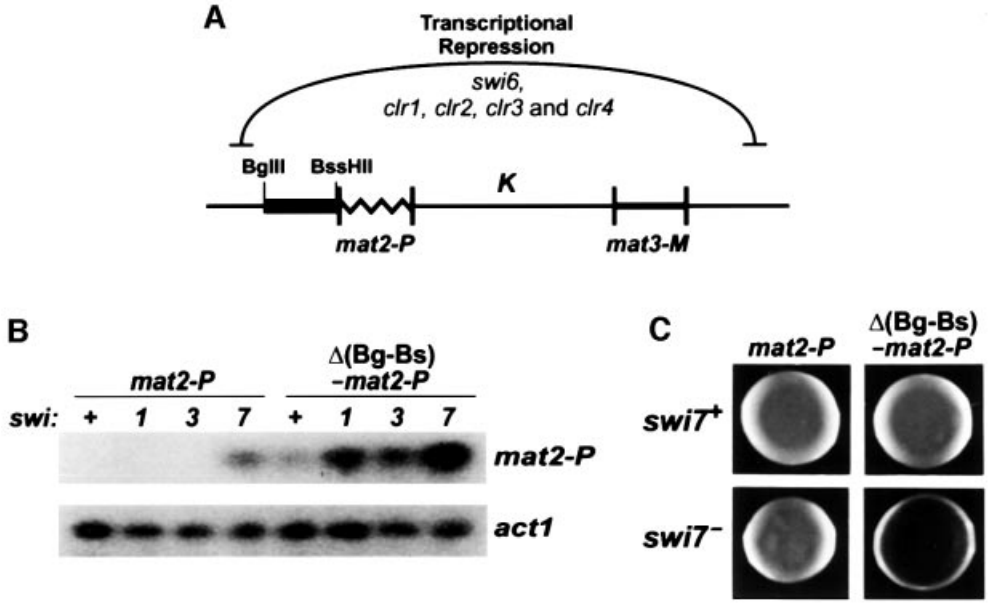

Fig. 2. Mutation in *swi7/polα* causes *mat2-P* derepression. (**A**) A schematic diagram of the *mat2/3* region. The *trans*-acting loci, *clr1-clr4* and *swi6*, affect silencing in the entire *mat2/3* interval and define one silencing pathway, while the *cis*-acting sequences between *Bgl*II and *Bss*HII sites, indicated by the black box, define a second pathway specific for *mat2-P* silencing. (**B**) Effect of *swi* mutations and Δ(Bg-Bs)-*mat2-P* deletion on *mat2-P* expression. RT–PCR analysis of *mat2-P* transcripts was performed as described in Materials and methods. Strains used were as follows: *mat1-Msmto mat2-P* derivatives, SP1125 (WT), SP814 (*swi1*) SP815 (*swi3*) and SPG151 (*swi7-1*); *mat1-Msmto* Δ(Bg-Bs)-*mat2-P* derivatives, SP1152 (WT), SPG153 (*swi1*), SPG154 (*swi3*) and SPG152 (*swi7-1*). (**C**) Analysis of *mat2-P* derepression by iodine staining. The colonies of *mat1-Msmto* or *mat1-Msmto* Δ(Bg-Bs)-*mat2-P* cells carrying the wild-type (*swi7*$^+$) or mutant *swi7* (*swi7*$^-$) allele were sporulated and exposed to iodine vapor before being photographed. Because *mat1-Msmto* strains fail to switch, their intensity of brown staining indicates the level of 'haploid meiosis', a phenotype caused by expression of both *P* and *M* information in haploid cells, thus reflecting the level of *mat2-P* derepression.

sectors as compared with their wild-type counterpart (Figure 1B), indicating an increase in *ura4-off* to *ura4-on* transition in mutant backgrounds. Serial dilution analysis and a fluctuation test to measure the effect of mutations quantitatively revealed that the *swi7/polα* mutant had the strongest (45-fold) effect (Figure 1C). Further genetic analysis revealed that the *swi7*-induced *ura4-on* state was segregating with the *mat* region (data not shown), suggesting that the mutation altered the imprint at the *mat* locus.

It has been shown that the silencing domain extends across the interval between *mat1* and *mat2*, called the *L* region (Ayoub *et al*., 1999). An *ade6*$^+$ reporter gene inserted on the left side of the *mat2* locus is subject to PEV. To test whether *swi7/polα* mutation globally affects silencing at the mating-type region, we also analyzed its effect on the expression of the *ade6*$^+$ marker integrated at two sites in the *L* region: *L(Bgl*II*)::ade6*$^+$ and *L(Sac*I*)::ade6*$^+$ located 1.5 and 2.5 kb from the *mat2* locus, respectively (Figure 1A). The expression of *ade6*$^+$ was monitored by plating cells on low-adenine (YE) medium. The occurrence of red or white colonies on this medium implies *ade6-off* and *ade6-on* phenotypes, respectively. In the wild-type background, expression of *L(Bgl*II*)::ade6*$^+$ and *L(Sac*I*)::ade6*$^+$ was variegated, resulting in a mixture of red and white colonies, as shown previously (Ayoub *et al*., 1999). Mutation in *swi7/polα* suppressed variegation of marker gene expression at both locations, resulting in formation of white colonies by the entire population of cells (Figure 1D). Based on these data, it was concluded that *swi7/polα* is essential for silencing at the *mat2/3* interval and in the neighboring regions.

The polα mutation affects donor mating-type loci silencing

We next tested whether mutations in *swi7/polα* as well as *swi1* and *swi3* also affect donor loci silencing. Previously, it has been shown that silencing of the *mat2-P* and *mat3-M* loci is controlled by at least two overlapping or redundant mechanisms. *Trans*-acting factors *clr1-clr4* and *swi6*, which have a weak effect on donor loci silencing but alleviate repression of markers at different locations, are involved in silencing the entire *mat2/3* region and neighboring sequences (see Figure 2A). The second pathway is defined by *cis*-acting sequences located near *mat2* (Thon *et al*., 1994) and *mat3* (Thon *et al*., 1999). Unlike the global effect of *clr1-clr4* and *swi6*, the effect of these repressor elements seems to be specific to sequences around *mat2* and *mat3*. Consistent with redundant silencing mechanisms, the chromosomal deletion of a 1.5 kb *Bgl*II–*Bss*HII fragment on the centromere-proximal side of *mat2-P* [Δ(Bg-Bs)-*mat2-P*], which removes *cis*-acting elements, does not derepress *mat2* noticeably. However, the same deletion in combination with a mutation in *clr1-clr4* or *swi6* causes cumulative derepression (Thon *et al*., 1994). To test whether *swi7/polα* is involved in specific or global silencing, or both, we constructed pairwise combinations of mutations in *clr1*, *clr2*, *clr3*, *clr4* and *swi6* or a Δ(Bg-Bs)-*mat2-P* deletion with the *swi7-1* mutant allele. RT–PCR analysis and/or an iodine staining assay (see Materials and methods for details) were used to measure

the level of *mat2-P* expression and haploid meiosis, a phenotype caused by concurrent expression of both *P* and *M* information in haploid cells. We found that mutation in *swi7/polα* did not show a cumulative effect in combination with *clr1-5*, *clr2-760*, *clr3-735*, *clr4-681* and *swi6-115* alleles (data not shown). However, when combined with the Δ(Bg-Bs)-*mat2-P* deletion, a significant cumulative derepression was seen (Figure 2B and C). Therefore, *swi7/polα* also affects *mat2-P* silencing and it appears to act in the same pathway as *clr1-clr4* and *swi6*. We also tested the effect of *swi1* and *swi3* mutations on the expression of *mat2-P*. Although mutations alone did not cause significant derepression, a cumulative derepression of *mat2-P* was observed when *swi3-146* and *swi1-S28* mutations were combined with the Δ(Bg-Bs)-*mat2-P* allele (Figure 2B).

Mutation in swi7/polα affects Swi6 localization at the mat2/3 region

Genetic and biochemical evidence suggests that Swi6 is an essential structural component of heterochromatin at the mating-type region (Lorentz *et al*., 1994; Grewal and Klar, 1997; Nakayama *et al*., 2000). Given that Polα and Swi6 seem to be part of the same silencing pathway, it was possible that *swi7/polα* mutant cells might be defective in Swi6 localization at the *mat* locus. We directly addressed this possibility by comparing Swi6 levels at the mating-type region of wild-type and mutant cells using the chromatin immunoprecipitation (CHIP) technique. Briefly, chromatin fractions prepared from formaldehyde-treated cells were fragmented and then immunoprecipitated (IPed) with affinity-purified anti-Swi6 antibodies (Nakayama *et al*., 2000). The DNA recovered from IPed fractions was quantitated using a competitive PCR strategy, whereby one primer pair amplifies 694 and 426 bp products from full-length *ura4*$^+$ inserted at the *mat* locus and the control *ura4DS/E* mini-gene at the endogenous location, respectively (Figure 3A). This approach allows the relative levels of the *ura4*$^+$ sequences, in comparison with the *ura4DS/E* DNA, in IPed fractions to be determined accurately. The levels of Swi6 present at *ura4*$^+$ inserted adjacent to *mat2-P* (*mat2-P::ura4*$^+$) were assayed. Data presented in Figure 3B revealed that *swi7/polα* mutant cells are defective in silencing as well as Swi6 localization at *mat2-P::ura4*$^+$. Thus, mutation in *polα* affects silencing by altering Swi6 localization and chromatin organization at the mating-type region.

We recently showed that the presence of multiple copies of *swi6*$^+$ results in efficient conversion of the *ura4-on* state to the *ura4-off* state in a *KΔ::ura4*$^+$ strain, and this change correlates with a stable increase in Swi6 levels at the *mat2/3* region (Nakayama *et al*., 2000). To investigate further the involvement of Polα in silencing and Swi6 localization, we tested whether Swi6-induced transition from the expressed state to the silenced state is affected by the *swi7/polα* mutation. Three copies of *swi6*$^+$ inserted at its endogenous chromosomal location (*swi6*$^+$*-333* allele; Nakayama *et al*., 2000) were combined with the *ade6-on* derivative of a *KΔ::ade6*$^+$ strain by using genetic crosses. As expected, the presence of the *swi6*$^+$*-333* allele resulted in efficient *ade6-on* to *ade6-off* transitions in *swi7*$^+$ cells but no transition was observed in *swi7*$^-$ cells (Figure 4A). These data were confirmed further by using *KΔ::ura4*$^+$ strains. Again, the presence of three copies of *swi6* caused a significant increase in *ura4-on* to *ura4-off* transition in *swi7*$^+$ cells but no change in state was observed in *swi7*$^-$ cells (Figure 4B, top panel). More importantly, CHIP analysis showed that the overexpression of Swi6 results in a notable increase in its levels at the mating-type region of *swi7*$^+$ cells, as observed previously, but no detectable increase in Swi6 levels was observed at the mating-type region of *swi7*$^-$ cells (Figure 4B). Therefore, the *swi6*$^+$*-333*-promoted increase in Swi6 levels at the *mat2/3* region requires a functional *swi7/polα* gene product. Taken together with the results described above, these data suggest that *swi7/polα* mutant cells are defective in Swi6 localization at the mating-type region.

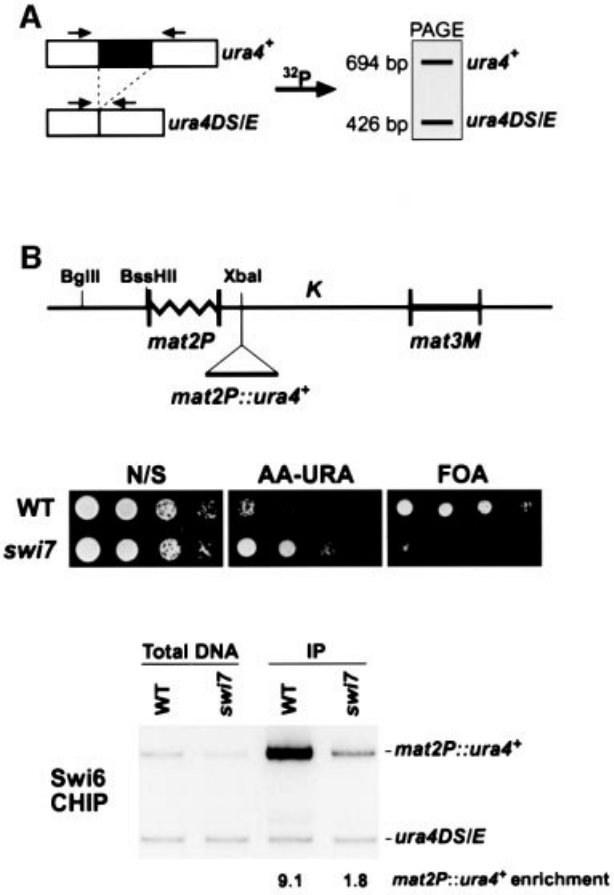

Fig. 3. Mutation in *polα* affects Swi6 localization at the *mat* locus. (**A**) Competitive PCR strategy for quantitating the amount of *ura4*$^+$ integrated at the mating-type region versus the mini-*ura4 (ura4DS/E)* allele at the endogenous locus. (**B**) The loss of silencing correlates with the decrease in Swi6 levels near *mat2-P*. A map of the *mat2/3* interval indicating the location of the *mat2-P::ura4*$^+$ insertion is shown (not drawn to scale). Serial dilution analysis on non-selective (N/S), AA-URA or FOA medium was used to study the effect of *swi7-1* mutation on *mat2-P::ura4*$^+$ expression (middle panel). Quantitative estimation of Swi6 levels was carried out using CHIP methodology. DNA isolated from either crude extracts or anti-Swi6 IPed fractions was subjected to competitive PCR assay. PCR products of 426 and 694 bp from *ura4DS/E* and *mat2-P::ura4*$^+$, respectively, were separated on a polyacrylamide gel and then quantified using a phosphoimager. The ratio of *ura4*$^+$ and *ura4DS/E* signals present in total DNA prepared from crude extract was normalized to one. Then the same correction factor was applied to the ratio of *ura4*$^+$ to the *ura4DS/E* signal in IPed sample lanes to calculate the relative enrichment. Strains used were SPG1228 (WT) and SPG1229 (*swi7*).

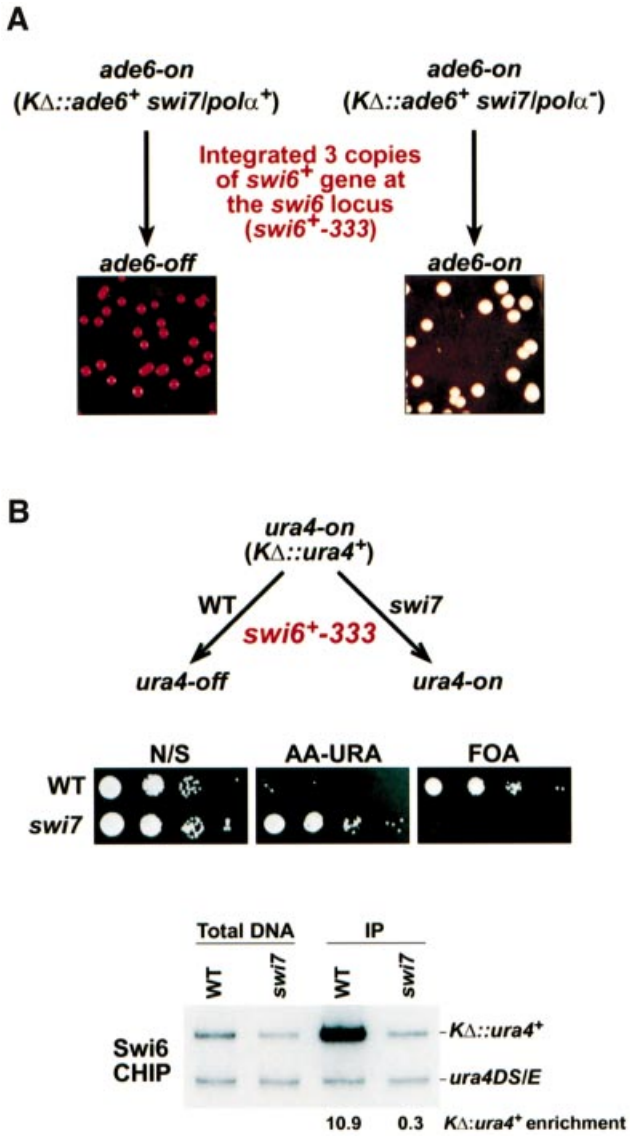

Fig. 4. *swi6⁺-333*-mediated 'on' to 'off' state transition and the corresponding increase in Swi6 levels at the mating-type region require *swi7/polα*. (**A**) Mutation in *polα* affects *ade6-on* to *ade6-off* transition. Three copies of *swi6⁺* inserted at its normal endogenous location (*swi6⁺-333* allele) were combined with *ade6-on* derivatives of *KΔ::ade6⁺ swi7⁺* or *KΔ::ade6⁺ swi7⁻* strains by using genetic crosses. The resulting strains were grown on adenine-limiting YE medium before being photographed. Formation of red colonies by *swi7⁺* cells indicated efficient *ade6-on* to *ade6-off* transition. In contrast, no change in *ade6⁺* expression state was observed in *swi7⁻* cells as suggested by formation of white colonies by all cells. Strains used were SPG1304 (WT) and SPG1305 (*swi7*). (**B**) The *KΔ::ura4⁺ ura4-on* derivatives of the *swi7⁺* and *swi7⁻* strains were combined with *swi6⁺-333*. The resultant cultures were subjected to CHIP analysis and serial dilution plating assay to assess the levels of Swi6 at the *mat* locus and *KΔ::ura4⁺* expression, respectively. Strains used were SPG1301 (*swi7*) and SPG1302 (WT).

The polα mutation also affects Swi6 localization at centromeres and telomeres

In addition to the *mat2/3* region, fission yeast centromeres are also assembled into silent heterochromatin-like structures. Moreover, factors affecting silencing at the mating-type region also affect centromeric silencing (Allshire *et al*., 1995; Grewal *et al*., 1998). We investigated whether *swi7/polα* mutation affects silencing of markers artificially inserted into centromeric sequences. For this purpose, the *swi7-1* allele was combined with *ura4⁺* integrated at three different locations within *cen1*: one each at outer repeat regions *otr* and *imr* and one at the central domain *cnt1* (Allshire *et al*., 1995; Figure 5A, top panel). Dilution analysis revealed that a mutation in *swi7/polα* caused derepression at all three locations tested though its effect at the *cnt* location was subtle (Figure 5A, middle panel).

We next used CHIPs to test whether the *swi7/polα* mutant cells are defective in Swi6 localization at *cen1*. An ~5-fold decrease in Swi6 levels at the *otr* and *imr* repeats was observed in *swi7⁻* cells, when compared with their *swi7⁺* counterparts (Figure 5A, bottom panel). In contrast to the *otr* and *imr* repeats, Swi6 levels at the *cnt* region were found to be almost negligible (Figure 5A; Partridge *et al*., 2000). Hence, the possible effect of *swi7/polα* mutation on Swi6 localization in this region could not be ascertained. We also analyzed the effect of *swi7/polα* mutation on Swi6 localization at telomeres. Data presented in Figure 5B indicate reduced levels of Swi6 at telomeres of *swi7⁻* cells when compared with *swi7⁺* cells. These results suggest that in addition to the *mat2/3* region, *swi7/polα* mutant cells are also deficient in Swi6 localization at the centromeres and telomeres.

Direct interaction between Polα and Swi6

Polα might affect Swi6 localization at heterochromatic loci by interacting with it directly. To test this, protein extracts from wild-type or *swi7/polα* mutant cells were loaded onto a Swi6 affinity column or a blank column as described in Materials and methods. Analysis of the eluted fractions showed that Polα binds to a His_6-Swi6 affinity column but not to a control blank column (Figure 6A). Significantly, the *swi7-1* mutation in Polα that adversely affects silencing and Swi6 localization at heterochromatic domains also affects interaction between these proteins (Figure 6A). To characterize further the interaction of Polα with Swi6 protein, we tested whether these proteins interact directly. *In vitro* translated deletion derivatives representing the N- (amino acids 1–635) or C-terminal (amino acids 1032–1405) part of Polα were incubated with glutathione *S*-transferase (GST)–Swi6 or GST alone, and then subjected to GST pull-down assays. As shown in Figure 6B, the C-terminal region of Polα bound directly to the GST–Swi6 fusion protein, whereas luciferase protein, used as a non-specific binding control, or the N-terminal region of Polα did not bind to Swi6. Therefore, the C-terminal region of Polα, the domain containing the G1116E change in *swi7-1* mutant cells (Singh and Klar, 1993), is sufficient for its interaction with Swi6 protein. As further confirmation of this result, we found that MBP–Polα, containing amino acids 635–1405 of Polα fused to maltose-binding protein, bound to His_6-Swi6 (data not shown). This interaction seems to be mediated through Polα as MBP alone did not bind to Swi6. In summary, our results argue that Polα physically interacts with Swi6 protein *in vitro* and that this interaction might be mediated, at least in part, through the C-terminal region of Polα.

The role of Polα in Swi6 localization was also investigated using immunofluorescence. As observed

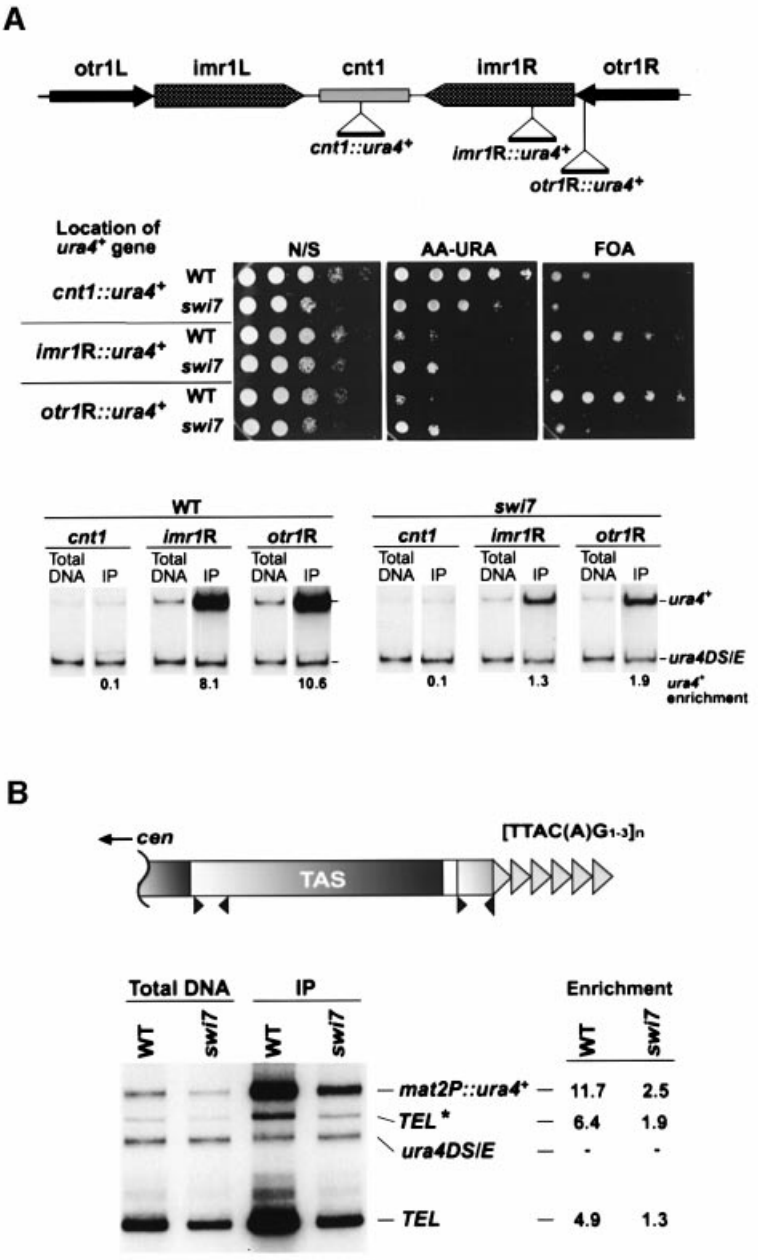

Fig. 5. Mutation in *swi7/polα* affects Swi6 localization at centromeres and telomeres. (**A**) A map of the *cen1* indicating marker gene insertion sites (not drawn to scale) and results from serial dilution plating analysis are shown (top and middle panel). CHIP analysis was used to quantitate Swi6 levels at *cnt*, *imr* and *otr* sites within *cen1* (bottom panel). Strains used were: *cnt1*::*ura4*$^+$, FY336 (WT) and SPG1060 (*swi7*); *imr1R*::*ura4*$^+$, FY498 (WT) and SPG1062 (*swi7*); *otr1R*::*ura4*$^+$, FY648 (WT) and SPG1067 (*swi7*). (**B**) Multiplex PCR was used to examine Swi6 levels at telomeres. DNA isolated from crude extracts or anti-Swi6 IPed fractions was analyzed for telomere-associated sequences (TAS) as well as *mat2-P::ura4*$^+$ and *ura4DS/E* sequences, used as controls. A diagram of a telomere indicating TAS, short repeats (gray triangles) and PCR primer-binding sites (black triangles) is shown (top panel). The PCR product marked with an asterisk resulted from primers binding to an additional site within TAS, as confirmed by its cloning and sequencing. Strains used were SPG1228 (WT) and SPG1229 (*swi7*).

previously (Ekwall *et al.*, 1996), wild-type cells showed normal Swi6 staining with 2–4 foci at the nuclear periphery (Figure 7). In comparison, Swi6 localization was disrupted in *swi7/polα* mutant cells, resulting in a diffuse/punctate pattern of nuclear staining in the majority of cells, while a small proportion of cells showed three or more faint spots. Taken together with the *in vitro* binding experiments, these data support our genetic and CHIP analysis showing a decrease in silencing and Swi6 levels at heterochromatic regions in *swi7/polα* mutant cells.

Discussion

Chromatin-based epigenetic imprints marking the mating-type region and centromeres contribute to maintenance of the higher order chromatin structure, which controls

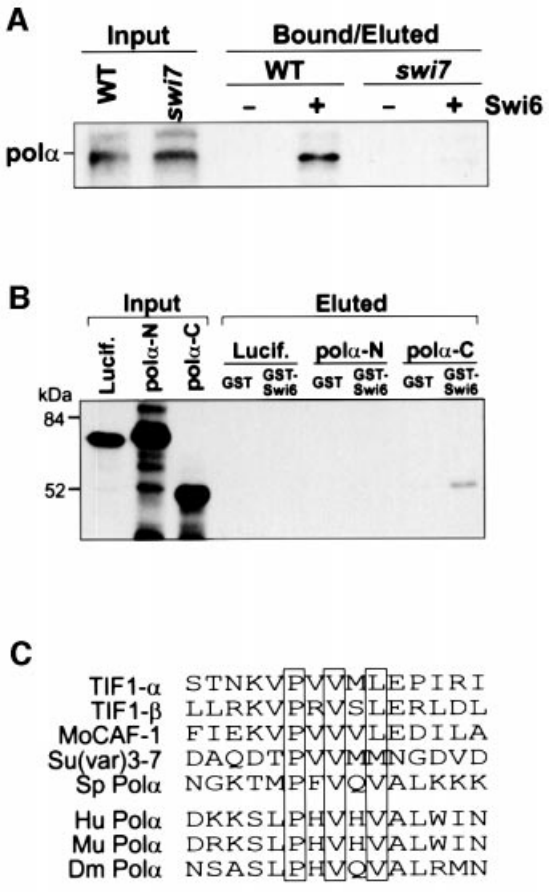

Fig. 6. Direct interaction between Polα and Swi6. (**A**) Binding of Polα to Swi6 protein. Yeast cell lysates from wild-type (WT; SP976) or *polα* mutant (*swi7*; SPJ5) cells were loaded onto a Swi6-coupled affinity column (+) or a mock-coupled blank column (–). The bound proteins were eluted and subjected to western blotting using affinity-purified anti-Polα antibodies. (**B**) The N- and C-terminal parts of Polα or luciferase protein were expressed *in vitro* and incubated with GST or GST–Swi6 beads. After extensive washes, the proteins that remained bound to beads were separated using SDS–PAGE and detected by fluorography. (**C**) The C-terminal domain of Polα contains the MIR consensus sequence found in mouse TIF1α, TIF1β and CAF-1, and in *Drosophila* Su(var)3–7. Corresponding regions of Polα in other species are also aligned (Hu, human; Mu, mouse; Dm, *Drosophila*). Conserved residues are indicated by boxes.

transcriptional silencing at these loci (Grewal and Klar, 1996; Ekwall *et al*., 1997; Nakayama *et al*., 2000). A key component of heterochromatin in fission yeast, Swi6, is believed to be an important determinant of the epigenetic imprint at the *mat* locus (Nakayama *et al*., 2000). We demonstrate that a mutation in DNA replication factor Polα suppresses variegation of marker gene expression at the *mat2/3* interval and centromeres. Moreover, biochemical analyses revealed that Polα interacts with Swi6 and that *swi7/polα* mutant cells are defective in Swi6 localization at the mating-type region, centromeres and telomeres. Taken together, these observations indicate that Polα might participate in higher order chromatin assembly at the silenced chromosomal domains.

Whether the requirement for Polα in silencing is coupled to its role in DNA replication in general remains to be explored. It seems unlikely, however, because the *swi7-1* mutation does not seem to increase UV sensitivity significantly as expected for reduced primase activity (Schmidt *et al*., 1989). Also, the mutation is not located in any of the regions conserved in the DNA polymerases of other species (Singh and Klar, 1993). In budding yeast, mutations in Polα cause lengthened telomeres (Smith *et al*., 1999; Adams Martin *et al*., 2000). It was therefore possible that variations in telomere length in the *swi7/polα* mutant might influence silencing by redistributing a limited pool of Swi6 protein to the telomere compartment, causing a decrease in Swi6 levels at the mating-type region and centromeres. However, we found that in addition to the *mat2/3* region and centromeres, Swi6 levels at the telomeres of *swi7/polα* mutant cells were also significantly decreased. We suggest a model whereby a distinct role for Polα, in conjunction with other factors including Clr4 and Rik1 that affect Swi6 localization (Ekwall *et al*., 1996; Bannister *et al*., 2001; Nakayama *et al*., 2001), may be to recruit and maintain Swi6 at the heterochromatic loci. That is, there may be different factors contributing to nucleation and/or maintenance of heterochromatin structures, but each may feed into a common pathway leading to heterochromatin assembly. SIR proteins in budding yeast were shown recently to move to new locations inside the nucleus in response to DNA damage (Martin *et al*., 1999; Mills *et al*., 1999). Similarly, it can be imagined that the delocalization of Swi6 in *swi7/polα* mutant cells might occur in response to signals sent by changes in DNA. Another possibility is that the changes in replication timing of the silenced chromosomal domains in mutant cells affect heterochromatin assembly. In this model, Swi6 protein, through its association with Polα, might control the timing of replication and, hence, epigenetic marking at *mat* and *cen* loci.

Epigenetic inheritance of silencing has several links to the DNA replication machinery in other organisms. Apart from components of the ORC complex (Micklem *et al*., 1993; Bell *et al*., 1995; Fox *et al*., 1995), mutations in PCNA, RF-C, Polε and Polα affect silencing in budding yeast (Ehrenhofer-Murray *et al*., 1999; Smith *et al*., 1999; Zhang *et al*., 2000). However, the precise role of the replication factors in silencing is not clear. The role of the ORC in silencing was shown to be independent of its role as a replication initiator (Fox *et al*., 1997), suggesting that some replication factors might have dual functions. Furthermore, recent evidence suggests that establishment of the silenced state can be uncoupled mechanistically from the passage of a replication fork (Kirchmaier and Rine, 2001; Li *et al*., 2001). A more direct connection between DNA replication and chromatin assembly is suggested by a recent study showing that the marking of newly replicated DNA molecules by PCNA helps promote nucleosome assembly by the chromatin assembly factor CAF-1 (Shibahara and Stillman, 1999). Interestingly, similarly to *polα*, mutations in CAF-1 or PCNA affect inheritance of repressed chromatin states in *S.cerevisiae* (Enomoto and Berman, 1998; Zhang *et al*., 2000) and the PCNA mutant allele *mus209* is a dominant suppressor of PEV in *Drosophila* (Henderson *et al*., 1994). Although our data suggest that Polα participates directly in heterochromatin assembly through its interaction with Swi6, it remains a possibility that mutation in *polα* impairs the

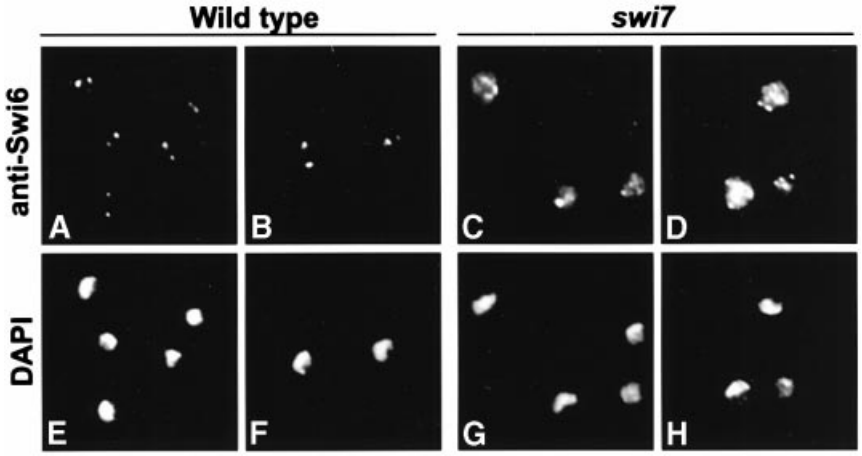

Fig. 7. Mutation in *swi7/polα* disrupts the Swi6 localization. Wild-type (**A** and **B**) or *swi7/polα* mutant cells (**C** and **D**) were stained with affinity-purified anti-Swi6 antibody. DAPI staining of each cell is also shown (**E**–**H**). Strains used were SP976 (WT) and SPJ5 (*swi7*).

CAF-1 interaction with other proteins at the replication fork.

Recent analyses showed that the *Drosophila* HP1 protein, which shares structural and functional similarities with Swi6, binds a consensus pentamer peptide sequence in which proline predominates at position 1, valine at position 3 and a hydrophobic residue at position 5 (Smothers and Henikoff, 2000). This consensus sequence, referred to as MIR peptide (MOD1-interacting region), is present in the HP1-interacting regions of TIF1-α, TIF1-β and CAF-1 (Murzina *et al*., 1999; Ryan *et al*., 1999). We found that a putative MIR peptide (residues 1129–1133) lies within the C-terminal part of Polα, 13 amino acids downstream from the *swi7-1* mutation site, in a region that binds to Swi6 *in vitro* (Figure 6C). Although further work is required, it is tempting to speculate that an evolutionarily conserved mechanism, mediated through MIR peptide, might promote interaction between Polα and Swi6.

In summary, the data presented here implicate the replication protein Polα in higher order chromatin assembly and Swi6 localization at the silenced chromosomal locations. Considering that proteins related to Swi6 are also found in higher eukaryotes, similar interactions in other species might be involved in the assembly of specific chromatin structures.

Materials and methods

Strains and culture conditions

Standard conditions were used for growth, sporulation, tetrad analysis and construction of diploids from haploid strains. The *swi6*$^+$*-333* allele was constructed by integrating two additional copies of *swi6*$^+$, marked with the *LEU2* gene, at the *swi6* locus. The proper integration and number of copies of *swi6* were confirmed by Southern blot analysis. The construction of *KΔ::ura4*$^+$, *KΔ::ade6*$^+$, Δ(Bg-Bs)*mat2-Pint::ura4*$^+$, *L(Bgl*II*)::ade6*$^+$, *L(Sac*I*)::ade6*$^+$ or insertions of *ura4*$^+$ at different *cen1* locations are described elsewhere (Thon *et al*., 1994; Allshire *et al*., 1995; Grewal and Klar, 1996; Ayoub *et al*., 1999). Genetic crosses were used to construct all other strains. The complete genotypes of strains are listed in Table I.

Iodine staining

The iodine staining assay was used to assay the derepression of donor loci. Individual colonies grown on sporulation (PMA$^+$) medium for 3 days were exposed to iodine vapors to stain a starch-like compound produced by sporulating cells. In mating-type switching-defective strains, such as *mat1-Msmto*, the intensity of brown/black staining indicates the level of 'haploid meiosis', a phenotype resulting from concurrent expression of both *P* and *M* information in haploid cells.

RT–PCR analysis

RNA prepared from the nitrogen-starved non-switching (*mat1-Msmto*) cells was treated with DNase to remove contaminating DNA. Reverse transcription was done using the Gibco Superscript™ Pre-amplification system, utilizing oligo$(dT)_{16}$ to prime cDNA synthesis. The cDNAs were then amplified in logarithmic phase, using primers 5′-GAATATAGTATGCGCTCTAACTTGGC-3′ and 5′-ATGGATCCAAGATTAAGAGCACC-3′ for *mat2-P* transcripts, and 5′-TCCTACGTTGGTGATGAAGC-3′ and 5′-TCCGATAGTGATAACTTGAC-3′ for *act1* control transcripts. The cDNA products were resolved by agarose gel (1.2%) electrophoresis and subjected to Southern analysis using the 0.9 kb *Hin*P1–*Mlu*I fragment of the *mat2-P* cassette and the 0.6 kb PCR product of *act1* as probes.

Plasmid constructions

All cloning steps were performed according to standard techniques. A 2.7 kb *Xba*I–*Pst*I fragment was excised from the plasmid containing the full-length *polα* gene (Singh and Klar, 1993) and cloned into pMALC2 (New England Biolabs) to produce recombinant C-terminal Polα protein (amino acids 635–1405) fused to MBP. Similarly, a 1.6 kb *Sph*I–*Pst*I fragment containing the 3′ end of the *polα* gene was cloned into pBluescript KS to construct pBK-*polα*-C. The PCR-amplified 5′-terminal fragment (450 bp) was cloned and ligated to a 1.5 kb *Eco*RI–*Xba*I fragment to construct pBK-*polα*-N. pBK-*polα*-N and pBK-*polα*-C plasmids were used for the T7 promoter-based *in vitro* translation–transcription reaction (Promega) to synthesize N- (amino acids 1–635) or C-terminal (amino acids 1032–1405) Polα protein. The Swi6 gene was amplified using primers swi6/FW 5′-GGGATCCAAATGAAGAAAGGAGGTGTTCG-3′ and swi6/RV 5′-AGAATTCTTATTCATTTTCACGGAACG-3′. The resultant PCR product was cloned into pRSETc (Invitrogen) to construct the pRSET/swi6 plasmid used to express His_6-tagged Swi6.

Formaldehyde cross-linked CHIP

The crude rabbit antisera raised against full-length Swi6 protein were affinity purified using His_6-tagged Swi6. Purified antibodies were used for CHIP and immunofluorescence as described previously (Ekwall and Partridge, 1999; Nakayama *et al*., 2000). The primer sets used were: for *ura4*$^+$*/ura4DS/E*, ura4#1 (5′-GAGGGGATGAAAAATCCCAT-3′) and ura4#2 (5′-TTCGACAACAGGATTACGACC-3′); and for telomeres, TEL-FW (5′-GGATGAAAAATTTGAAGTTCACC-3′) and TEL-RV (5′-CTCGCCTTACGGCTCGGCTGAC-3′).

Table I. *Schizosaccharomyces pombe* strains used in this study

Strain	*ura4*$^+$/*ade6*$^+$ insertion	*mat1*	Auxotrophic markers	*swi* loci
SP814		*mat1-Msmto*	*leu1-32 ade6-210*	*swi1-111*
SP815		*mat1-Msmto*	*leu1-32 ade6-216*	*swi3-146*
SP976		*h*^{90}	*leu1-32 ura4D18 ade6-210*	
SP1125	*mat2-Pint::ura4*$^+$	*mat1-Msmto*	*ura4D18 ade6-210*	
SP1152	Δ(Bg-Bs)*mat2-Pint::ura4*$^+$	*mat1-Msmto*	*ura4D18 ade6-210*	
SPG32	*KΔ::ura4*$^+$	*mat1-Msmto*	*leu1-32 ura4 ade6-210*	
SPG106	*KΔ::ura4*$^+$	*mat1-Msmto*	*leu1-32 ura4 ade6-216*	*swi7-1*
SPG112	*KΔ::ura4*$^+$	*mat1-Msmto*	*leu1-32 ura4-D18 ade6-216*	*swi1-s28*
SPG114	*KΔ::ura4*$^+$	*mat1-Msmto*	*leu1-32 ura4 ade6-210*	*swi3-146*
SPG151	*mat2-Pint::ura4*$^+$	*mat1-Msmto*	*ura4 ade6-210*	*swi7-1*
SPG152	Δ(Bg-Bs)*mat2-Pint::ura4*$^+$	*mat1-Msmto*	*ura4 ade6-216*	*swi7-1*
SPG153	Δ(Bg-Bs)*mat2-Pint::ura4*$^+$	*mat1-Msmto*	*ura4 ade6-216*	*swi1-s28*
SPG154	Δ(Bg-Bs)*mat2-Pint::ura4*$^+$	*mat1-Msmto*	*ura4 ade6-210*	*swi3-146*
SPG1060	*cnt1*/TM1(*Nco*I)*::ura4*$^+$	*h*$^+$	*leu1-32 ura4DS/E ade6-216*	*swi7-1*
SPG1062	*imr1*R(*Nco*I)*::ura4*$^+$	*h*$^+$	*leu1-32 ura4DS/E ade6-210*	*swi7-1*
SPG1067	*otr1*R(*Sph*I)*::ura4*$^+$	*h*$^+$	*leu1-32 ura4DS/E ade6-210*	*swi7-1*
SPG1217	*L(SacI)::ade6*$^+$	*mat1-Msmto*	*leu1-32 ura4D18 his2 ade6DN/N*	
SPG1218	*L(BglII)::ade6*$^+$	*mat1-Msmto*	*leu1-32 ura4D18 his2 ade6DN/N*	
SPG1228	Δ(Bg-Bs)*mat2-Pint::ura4*$^+$	*mat1-Msmto*	*leu1-32 ura4DS/E ade6-210*	
SPG1229	Δ(Bg-Bs)*mat2-Pint::ura4*$^+$	*mat1-Msmto*	*leu1-32 ura4DS/E ade6-216*	*swi7-1*
SPG1301	*KΔ::ura4*$^+$	*mat1-Msmto*	*leu1-32 ura4DS/E ade6-210*	*swi7-1 swi6*$^+$*-333*
SPG1302	*KΔ::ura4*$^+$	*mat1-Msmto*	*leu1-32 ura4DS/E ade6-210*	*swi6*$^+$*-333*
SPG1304	*KΔ::ade6*$^+$	*mat1-Msmto*	*leu1-32 ura4 his2 ade6-210*	*swi6*$^+$*-333*
SPG1305	*KΔ::ade6*$^+$	*mat1-Msmto*	*leu1-32 ura4 his2 ade6-210*	*swi7-1 swi6*$^+$*-333*
SPG1326	*L(Bgl*II*)::ade6*$^+$	*mat1-Msmt-o*	*leu1-32 ura4 his2 ade6DN/N*	*swi7-1*
SPG1327	*L(SacI)::ade6*$^+$	*mat1-Msmto*	*leu1-32 ura4 his2 ade6DN/N*	*swi7-1*
SPJ5		*h*^{90}	*leu1-32 ura4 ade6-216*	*swi7-1*
FY336	*cnt1*/TM1(*Nco*I)*::ura4*$^+$	*h*$^+$	*leu1-32 ura4DS/E ade6-210*	
FY498	*imr1*R(*Nco*I)*::ura4*$^+$	*h*$^+$	*leu1-32 ura4DS/E ade6-210*	
FY648	*otr1*R(*Sph*I)*::ura4*$^+$	*h*$^+$	*leu1-32 ura4DS/E ade6-210*	

Protein affinity chromatography

Purified His_6-tagged Swi6 protein was cross-linked to NHS-activated HiTrap (Amersham Pharmacia). The column was washed successively with 10 column volumes of phosphate-buffered saline (PBS), 10 column volumes of buffer E [50 mM Tris–HCl pH 7.5, 75 mM NaCl, 1 mM dithiothreitol (DTT), 2.5 M urea] and 10 column volumes of buffer A [50 mM Tris–HCl pH 7.5, 75 mM NaCl, 0.1% NP-40, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF) and proteinase inhibitor cocktail (Boehringer)]. A blank column cross-linked without ligand was used as negative control. One liter of yeast culture was grown to an A_{595} of 0.5, harvested by centrifugation, washed with ice-cold STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3 pH 8.0) and stored at –70°C. The frozen cell pellet (1×10^{10} cells) was resuspended in 1 ml of ice-cold HB buffer (25 mM MOPS pH 7.2, 60 mM β-glycerophosphate, 15 mM *p*-nitrophenylphosphate, 15 mM $MgCl_2$, 15 mM EGTA, 1 mM DTT, 0.1 mM sodium vanadate, 1% Triton X-100, 1 mM PMSF, supplemented with proteinase inhibitor cocktail) and disrupted by agitation with glass beads at 4°C. The glass beads were washed with 10 ml of ice-cold HB buffer and the cell lysate was centrifuged at 10 000 r.p.m. for 20 min at 4°C. The supernatant was diluted with 1 vol. of buffer A and loaded onto a Swi6 affinity column or blank column at a flow rate of ~10 ml/h. The columns were washed with 20 column volumes of buffer A, and the bound proteins were eluted using eight column volumes of buffer E. The peak fractions were precipitated by 2 vols of ice-cold ethanol, resolved on 6% SDS–polyacrylamide gels and subjected to western analysis with affinity-purified anti-Polα antibodies. The binding of Swi6 to Polα was performed essentially as described previously (Murzina *et al*., 1999). Polα protein samples were produced by *in vitro* translation in the rabbit reticulocyte lysate and incubated with recombinant GST-tagged Swi6 bound to glutathione–agarose beads. After extensive washes, the proteins that remained bound to GST–Swi6 were separated using SDS–PAGE and detected by fluorography.

Immunofluorescence analysis

Immunofluorescence was performed as previously described (Ekwall *et al*., 1996). Cells were grown to mid-log phase in YEA medium. An equal volume of YEA + 2.4 M sorbitol was added and the culture was incubated further at 18°C for 5 min. Cells were fixed by adding paraformaldehyde to a final concentration of 3.8% and incubating at 18°C for 30 min. After the fixation was stopped by adding 2.5 M glycine to 0.125 M, the cell pellet was washed twice with PEM (100 mM PIPES pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$) and once with PEMS (PEM + 1 M sorbitol). Cells were incubated in PEMS + 1 mg/ml of zymolyase 100T (ICN) for 60–120 min at 37°C to digest the cell wall, washed sequentially with PEMS, PEMS + 1% Triton X-100, PEMS and PEM, and then incubated with PEMBAL [PEM + 1% bovine serum albumin (Sigma A6738), 0.1% sodium azide, 0.1 M L-lysine] for 1 h. Cells were incubated overnight with affinity-purified Swi6 antibody in PEMBAL, washed three times in PEMBAL and incubated with Alexa-conjugated anti-rabbit immunoglobulin G (Molecular Probe) at a 1/2000 dilution in PEMBAL for 3 h. After extensive washing, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI), mounted in Vectashield mounting medium (Vector laboratories) and analyzed by a fluorescence microscope.

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