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Fission yeast Swi2 designates cell-type specific donor and stimulates Rad51-driven strand exchange for mating-type switching

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ABSTRACT

A haploid of the fission yeast Schizosaccharomyces pombe expresses either the P or M mating-type, determined by the active, euchromatic, mat1 cassette. Mating-type is switched by Rad51-driven gene conversion of mat1 using a heterochromatic donor cassette, mat2-P or mat3-M. The Swi2-Swi5 complex, a mating-type switching factor, is central to this process by designating a preferred donor in a cell-type-specific manner. Swi2-Swi5 selectively enables one of two cis-acting recombination enhancers, SRE2 adjacent to mat2-P or SRE3 adjacent to mat3-M. Here, we identified two functionally important motifs in Swi2, a Swi6 (HP1 homolog)-binding site and two DNA-binding AT-hooks. Genetic analysis demonstrated that the AT-hooks were required for Swi2 localization at SRE3 to select the mat3-M donor in P cells, while the Swi6-binding site was required for Swi2 localization at SRE2 to select mat2-P in M cells. In addition, the Swi2-Swi5 complex promoted Rad51-driven strand exchange in vitro. Taken together, our results show how the Swi2-Swi5 complex would localize to recombination enhancers through a cell-type specific binding mechanism and stimulate Rad51-driven gene conversion at the localization site.

INTRODUCTION

Homothallic strains of the fission yeast Schizosaccharomyces pombe (h⁹₀) are either of the P or M mating-type and switch their mating type during vegetative growth (mating-type switching: MTS). The mating type is determined by the transcriptionally active mat1 cassette at the mat locus and is switched by a gene conversion event of the mat1 cassette using a donor in the heterochromatic silent region, mat2-P or mat3-M. P cells (mat1-P) and M cells (mat1-M) preferentially utilize mat3-M and mat2-P as a donor for gene conversion, respectively (1).

The chromosome organization of the mat locus is shown in Figure 1. The mat1 cassette expresses either P or M information, Pi and Pc genes in P cells (mat1-P) or Mi and Mc genes in M cells (mat1-M), which determines the mating type of a haploid cell (2). The mat2 and mat3 cassettes are located in the 20 kb region between IR-L and IR-R, which is ~15 kb from mat1. The mat2 and mat3 cassettes contain P (mat2-P) and M (mat3-M) information, respectively, and are silenced by heterochromatin. Each mat cassette is flanked by short homology boxes, H1 and H2, which are used for Rad51-mediated homology-driven gene conversion during MTS (1). Two cis-acting Swi2-dependent recombination enhancers, SRE2 and SRE3, are located adjacent to the H1 box of mat2-P and mat3-M, respectively. These two SREs play an important role in the donor choice step in each cell type during MTS (3–5).

MTS is initiated by the introduction of an imprint at the junction of mat1 and the H1 box during lagging strand synthesis in DNA replication (6,7). This imprint has been suggested to be a nick or incorporation of two ribonucleotides (8–11), and a daughter cell that inherits the imprint is converted to a ‘switchable’ state. On the other hand, a daughter cell that inherits the de novo leading strand is unable to switch cell type and is referred to as ‘unswitchable’ (6,12,13). During leading strand synthesis in a granddaughter cell, the imprint is converted to a single-ended DNA double-strand break (DSB), which triggers the gene conversion event driven by the homologous recombination (HR) protein Rad51 (14,15).

The following model can be put forward to explain gene conversion for MTS driven by Rad51. The DSB end is

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but also to the heterochromatic region between M cells, the Swi2-Swi5 complex not only localizes to SRE3, but also to the heterochromatin region between IR-L and IR-R repeats and their expression is repressed. REII and REIII stand for Repressor Element II and III, respectively. The SRE2 and SRE3 elements are located near the mat2-P or mat3-M cassettes, respectively. Each cassette is flanked by the $H1$ and $H2$ homology boxes. The imprinting site at the mat1-1H1 junction is indicated by a red asterisk. Imprinting is converted to a DSB. The MTS factor Swi2-Swi5 localizes to SRE3 in P cells, but localizes over the mat heterochromatin locus in M cells.

Figure 1. MTS in S. pombe. Simple schematic representation of the mating-type region in the $h^{10}$ strain. The switchable mat1 cassette contains P (blue; Pc and Pi) or M (yellow; Mi and Mc) mating-type genes. The mat2-P and mat3-M donor cassettes are located in a heterochromatin region between IR-L and IR-R repeats and their expression is repressed. REII and REIII stand for Repressor Element II and III, respectively. The SRE2 and SRE3 elements are located near the mat2-P and mat3-M cassettes, respectively. Each cassette is flanked by the $H1$ and $H2$ homology boxes. The imprinting site at the mat1-H1 junction is indicated by a red asterisk. Imprinting is converted to a DSB. The MTS factor Swi2-Swi5 localizes to SRE3 in P cells, but localizes over the mat heterochromatin locus in M cells.

Resected to produce single-stranded DNA (ssDNA) of the $H1$ box in the mat1 cassette, onto which the Rad51 protein forms a nucleoprotein filament (14). Subsequently, the resultant Rad51 filament searches the $H1$ box of the mat2-P or mat3-M cassette, and then the ssDNA invades either the $H1$ box of mat2-P or mat3-M. This process does not occur randomly. There are highly regulated donor choice mechanisms involving SRE2 and SRE3 (3–5). The SRE2 element is required to select mat2-P as a donor in M cells, while the SRE3 element is required to select mat3-M as a donor in P cells (Figure 1) (4). After strand invasion, the DSB is repaired by a synthesis-dependent strand annealing mechanism using the $H2$ box (15).

Ten MTS genes named swi1–swi10 were initially identified (16). Among them, swi2, swi5 and swi6 are essential for the normal donor choice mechanism (17). Swi6 is a homolog of heterochromatin protein 1 (HP1) in other organisms and localizes to heterochromatic regions such as centromeres, telomeres and the mat locus by recognizing histone H3K9 methylation (18). At the mat locus, Swi6 is distributed between the inverted repeats IR-L and IR-R in both mating types (19,20). Interestingly, in the absence of Swi6, $h^{10}$ cells prefer to use the mat3-M donor over the mat2-P donor (3,4,21).

Yeast two-hybrid assays have suggested that Swi2 and Swi5 form a complex and that Swi2 also binds to Swi6 (22). In P cells, the Swi2-Swi5 complex localizes only to SRE3, independently of Swi6, which is believed to contribute to the mat3-M donor choice. On the other hand, in M cells, the Swi2-Swi5 complex not only localizes to SRE3, but also to the heterochromatin region between IR-L and IR-R dependent on Swi6 (3). The Swi2 and Swi5 protein levels are increased in M cells due to stimulation of swi2 and swi5 gene transcription by the M cell-specific factor mat1-Mc and the CENP-B homolog Abp1 (23). The localization mechanism of the Swi2-Swi5 complex in M cells has been suggested to start from SRE3 via the physical interaction between Swi2 and Swi6 (3), although Swi2 has a function at SRE2 regardless of SRE3 to facilitate the mat2-P donor choice (4). Swi2 interacts with Rad51 (22) and therefore it has been hypothesized that the Swi2-Swi5 complex recruits the Rad51 filament formed on the $H1$ box of the mat1 cassette to SRE2 or SRE3 in a cell-type-specific manner (17). In addition to heterochromatin formation and the Swi2-Swi5 complex, we recently found that the euchromatic factors Set1C and HULC also affect MTS (24). Based on genetic analysis, we proposed that Set1C and HULC in M cells inhibit the mat3-M donor choice at SRE3 (25).

Swi5 forms a stable complex with another partner, Sfr1. Cells deficient for sfr1 are defective in recombination repair but exhibit normal MTS. On the other hand, swi2 mutants have normal DNA repair activity. swi5-deficient mutants show defects in both MTS and DNA repair. The Swi5-Sfr1 complex stimulates Rad51-driven strand exchange activity in vitro by acting as a recombination activator (26–28). The C-terminal part of Swi2 shares sequence homology with the C-terminal part of Sfr1, which forms an interface with Rad51; therefore, the Swi2-Swi5 complex is also expected to activate Rad51.

In this study, we identified two functionally important regions, the Swi6-binding site and two tandem AT-hook motifs, in Swi2. Swi6-binding site and AT-hook mutants had an MTS-deficient phenotype: Swi6-binding site mutants were biased toward M cells, while AT-hook mutants were biased toward P cells. This result indicates that the interaction between Swi2 and Swi6 is important for the mat2-P donor choice in M cells, consistent with a previous proposal (17), while the AT-hook motifs play a different role in MTS. In addition, we demonstrated that the purified Swi2-Swi5 complex stimulates Rad51-driven strand invasion.

MATERIALS AND METHODS

Yeast strains and strain constructions and manipulations

The S. pombe strains used in this study are described in Supplementary Table S1. Standard techniques were
used to cultivate, sporulate, cross and genetically manipulate *S. pombe* (29). Strains were generated by transformations or genetic crosses. The swi2 mutant strains (TM366, TM511, TM519 and TM527) were generated from EM20 by the CRISPR/Cas9 method (25). EM20 was transformed with a gRNA expression plasmid (pEM59 derivative), a Cas9 expression plasmid and donor template DNA for HR. The target sequences were 5′-TCATGCTCTGAGTACATTCT-3′ for Swi2-5A, 5′-GATGAAAGCAGCTGGAGGA-3′ for Swi2-AT1 and -AT12- AA, and 5′-GATGATGGCCAGAAGAG-3′ for Swi2-AT2-A and -AT12- AA, each of which was inserted into the BbsI site of pEM59. The HR donor templates for Swi2-AT2-A and -AT12-AA, each of which was inserted into the BbsI site of pEM59. The HR donor templates for mutagenesis were amplified from the HR donor template DNA for HR. The target sequences for Swi2-5A: 5′-GGGGAAGACCTACAGGCTGG-3′ and 5′-CAGGGGAAGTCTTCATCCTCTTGG-3′; and Swi2-AT mutants: 5′-GGTTGGAACCAACGCTGTGTG-3′ and 5′-TGAGGTAACGGGCTCTG-3′.

**Iodine staining assay**

The efficiency of MTS was estimated by iodine staining as described previously (21). Cells were streaked on MSA (minimal sporulating agar) plates (30) and grown at 26°C for 3–4 days. Plates on which colonies were grown were exposed to iodine vapor for ~10 min at room temperature.

**Multiplex PCR assay**

Sample preparation and multiplex PCR were performed as previously described (24, 25). Isolated colonies of the strains were cultured in 2 ml YE5S liquid at 30°C until saturation. Each culture was harvested and genomic DNA was extracted using a Dr GenTLE (from Yeast) High Recovery Kit (9082, Takara Bio). The genomic DNA concentration was quantified using the Quantifluor® ONE dsDNA Dye System (E4971, Promega). Genomic DNA (5–20 ng) was added to the PCR reaction mixture (total 20 μl). The common forward primer FAM-MT1 (5′-AAATAGTGGGTAGCCGTGAAGG-3′), the *mat1*-P-specific reverse primer MP1 (5′-ATCTATCAGGAGATGGCCAGGT-3′), and the *mat1*-M-specific reverse primer MM1 (5′-GGGAAACCCTGTTAATTCTTG-3′) were used at concentrations of 400, 200 and 200 nM, respectively. The 5′ end of FAM-MT1 was labeled with 6-carboxyfluorescein (FAM). To reduce non-specific PCR products, 400 nM heat-stable RecA protein from *Thermus thermophilus* and 400 μM ATP were included in the PCR reaction buffer (10 mM Tris–HCl [pH 8.3], 50 mM KCl and 2.5 mM MgCl₂) (31). The amplification program was 2 min at 94°C, followed by 27 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, and a final extension of 5 min at 72°C. The PCR fragments corresponding to the *mat1*-P and *mat1*-M alleles were resolved on 5% polyacrylamide gels. Fluorescence was detected and quantified using Typhoon FLA9500 (Cytiva) and ImageQuant (Cytiva).

**Yeast two-hybrid assay**

The yeast two-hybrid assay was performed as described previously (22). pGADT7 plasmids were generated using pGADT7-Swi2 full-length from our laboratory stock by inverse PCR or the quick-change method using the primer sets listed in Supplementary Table S2.

**Protein purification**

The *swi2N* (1–391 aa), *swi2*+ (1–722 aa), *swi2S* (205–722 aa), *swi2C* (392–722 aa) and *swi2CS* (607–722 aa) genes were cloned into a His-SUMO fusion protein expression vector (32) using an In-Fusion Cloning Kit (639648, Takara Bio). The glycine-arginine-proline (GRP) tripeptides in the AT-hooks of Swi2N were mutated by the quick-change method. The DNA fragments for cloning were amplified using PrimeStar Max DNA Polymerase (R045A, Takara Bio) and the primer sets listed in Supplementary Table S2.

His-SUMO-Swi2N proteins (both wild-type and AT-hook mutants) were expressed in *Excherichia coli* (BL21-CodonPlus (DE3) and purified as follows. Cells carrying the *swi2N* expression plasmid were grown at 37°C until OD₆₀₀ reached 0.5 in LB medium containing ampicillin. IPTG was added to a final concentration of 0.2 mM, and the culture was further incubated for ~16 h at 18°C. The cells were collected by centrifugation and the pellet was suspended in R buffer (20 mM Tris–HCl [pH 8.0], 1 mM EDTA, 1 mM DTT and 5% glycerol) containing 200 mM NaCl. The cells were disrupted by sonication and centrifuged at 40 000 × g for 30 min. The supernatant was loaded onto a Ni-NTA Superflow Cartridge (30761, Qiagen). The protein was washed with 100 ml R buffer containing 200 mM NaCl. Proteins were eluted with R buffer containing 300 mM imidazole and 200 mM NaCl. The sample was cleaved by His-Ulp1 protein overnight at 4°C in dialysis buffer 1 (20 mM Tris–HCl [pH 7.5], 100 mM NaCl, 5% glycerol and 1 mM DTT). The flow-through sample of Ni-NTA Superflow Cartridge was loaded onto a 5 ml HiTrap Heparin column (17040701, Cytiva). Proteins were eluted with a linear gradient of 0.1–0.6 M NaCl. Peak fractions of Swi2N protein appeared at 250 mM NaCl. The fractions were diluted three times with R buffer and loaded onto a Resource Q column (17117701, Cytiva). Swi2N eluted at ~250 mM NaCl. The eluted Swi2N was dialyzed against R buffer containing 200 mM NaCl, and concentrated using an Amicon Ultra-4 (30 k) centrifugal filter (UFC803008, Merck Millipore).

Swi2, Swi2S, Swi2C and Swi2CS proteins were purified in complex with Swi5 (Swi2-Swi5, Swi2S-Swi5, Swi2C-Swi5 and Swi2CS-Swi5). BL21-CodonPlus (DE3) carrying the His-SUMO-Swi2, -Swi2S, -Swi2C or -Swi2CS expression plasmid and the Swi5 expression plasmid were grown at 37°C until OD₆₀₀ reached 0.5 in LB medium containing ampicillin and kanamycin. IPTG was added to a final concentration of 0.2 mM, and the culture was further incubated for ~16 h at 18°C. The cells were collected by centrifugation and the pellet was suspended in R buffer containing 200 mM NaCl. The cells were disrupted by sonication and centrifuged at 40 000 × g for 30 min. The supernatant of the cell extract was loaded onto a Ni-NTA Superflow Cartridge (30761, Qiagen). The cartridge...
was washed with 100 ml R buffer containing 200 mM NaCl. Proteins were eluted with R buffer containing 300 mM imidazole and 200 mM NaCl. The eluted proteins were cleaved by His-UBp1 protein at 4°C for 4 h to overnight in dialysis buffer 2 (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 5% glycerol and 1 mM DTT). After cleavage of the SUMO tag, each protein complex underwent a different purification procedure as detailed in the following paragraphs.

For Swi2-Swi5 purification, after cleavage of the SUMO tag, the Swi2-Swi5 complex was applied to a 5 ml HiTrap Q column (17115401, Cytiva). Proteins were eluted with a linear gradient (100 ml of 0.2–0.6 M NaCl). Peak fractions were diluted with two volumes of R buffer and loaded onto a 5 ml HiTrap SP column (17115201, Cytiva). The Swi2-Swi5 complex was eluted with a linear gradient (100 ml of 0.15–0.55 M NaCl). Peak fractions were diluted with an equal volume of R buffer. The sample was loaded onto a 5 ml HiTrap Heparin column (17040701, Cytiva). The Swi2-Swi5 complex was eluted with a linear gradient (30 ml 0.25–0.65 M NaCl). The peak fraction was applied onto a Superdex 200 10/300 GL gel filtration column (17517501, Cytiva) in R buffer containing 200 mM NaCl. Peak fractions containing the Swi2-Swi5 complex were concentrated using an Amicon Ultra-4 (50 k) centrifugal filter (UFC805008, Merck Millipore).

For Swi2-Swi5 purification, after cleavage of the SUMO tag, the Swi2-Swi5 complex was applied to a 5 ml HiTrap Q column (17115401, Cytiva). Proteins were eluted with a linear gradient (100 ml of 0.2–0.6 M NaCl). The flow-through of the HiTrap Q column was loaded onto a 5 ml HiTrap SP column (17115201, Cytiva). The Swi2-Swi5 complex was eluted with a linear gradient (100 ml of 0.2–0.6 M NaCl). Peak fractions were diluted with two volumes of R buffer and loaded onto a 5 ml HiTrap Heparin column (17040701, Cytiva). The Swi2-Swi5 complex was eluted with a linear gradient (30 ml 0.2–0.8 M NaCl). The peak fraction was developed in a Superdex 200 10/300 GL gel filtration column (17517501, Cytiva) in R buffer containing 200 mM NaCl. The Swi2-Swi5 complex was concentrated using an Amicon Ultra-4 (50 k) centrifugal filter (UFC805008, Merck Millipore).

For Swi2C-Swi5 purification, after cleavage of the SUMO tag, the Swi2C-Swi5 complex was applied to a 5 ml HiTrap Q column and was loaded with R buffer containing 300 mM NaCl. The flow-through of the HiTrap Q column (17115401, Cytiva) was loaded onto a 5 ml HiTrap SP column (17115201, Cytiva). The Swi2C-Swi5 complex was eluted with a linear gradient (100 ml of 0.1–0.5 M NaCl). Peak fractions were diluted with four volumes of R buffer. The sample was loaded onto a 5 ml HiTrap Q column (17115401, Cytiva). The Swi2C-Swi5 complex was eluted with a linear gradient (50 ml of 0–0.5 M NaCl). The peak fraction was developed in a Superdex 200 10/300 GL gel filtration column (17517501, Cytiva) in R buffer containing 200 mM NaCl. The Swi2C-Swi5 complex was concentrated using an Amicon Ultra-4 (30 k) centrifugal filter (UFC803008, Merck Millipore).

For Swi2CS-Swi5 purification, after cleavage of the SUMO tag, the Swi2CS-Swi5 complex was diluted with four volumes of R buffer. The sample was loaded onto a 5 ml HiTrap Q column (17115401, Cytiva). The Swi2CS-Swi5 complex was eluted with a linear gradient (100 ml of 0–0.4 M NaCl). The peak fraction was developed in a Superdex 200 10/300 GL gel filtration column (17517501, Cytiva) in R buffer containing 200 mM NaCl. The Swi2CS-Swi5 complex was concentrated using an Amicon Ultra-4 (10 k) centrifugal filter (UFC801008, Merck Millipore).

S. pombe Rad51, Swi5-Sfr1, Swi5-Sfr1C and RPA proteins were purified as described previously (27,33). S. pombe Rad54 protein was purified as described by Afshar et al. (34) with the following modifications. Rad54 was expressed in a 4 L culture of E. coli BL21 (DE3) Star cells carrying the T7-based plasmid pBA110 (pET15b-6xHis-Fh8-PreScission-1xFLAG-rad54) as described previously (34). The cells were collected by centrifugation and the pellet was suspended in 120 ml R buffer containing 200 mM NaCl. The cells were disrupted by sonication and centrifuged at 40 000 × g for 30 min. The supernatant was loaded onto a 10 ml Ni-NTA Superflow cartridge (connected two 5 ml cartridges) (30761, Qiagen). The cartridge was washed with 100 ml R buffer containing 200 mM NaCl. Proteins were eluted with R buffer containing 300 mM imidazole and 200 mM NaCl. The sample was cleaved by PreScission Protease (27084301, Cytiva) overnight at 4°C in dialysis buffer 1. The protein sample was applied to a 5 ml HiTrap Q column (17115401, Cytiva). The flow-through of the HiTrap Q column was loaded onto a 5 ml HiTrap SP column (17115201, Cytiva). Proteins were eluted with a linear gradient (100 ml of 0.2–0.6 M NaCl). Peak fractions were diluted with two volumes of R buffer. The sample was loaded onto a 5 ml HiTrap Heparin column (17040701, Cytiva). Proteins were eluted with a linear gradient (50 ml of 0.2–0.6 M NaCl). Rad54 was concentrated using an Amicon Ultra-4 (50 k) centrifugal filter (UFC805008, Merck Millipore).

Electrophoresis mobility shift assay (EMSA)

The target DNA fragments mat1, SRE2 and SRE3 (400 bp each) were amplified by PCR using a laboratory stock plasmid containing the mat locus as a template and primer sets (SRE3: 5'-TATCCAAATATGTTTGTGTT TGGCGGAT-3' and 5' -GGGTAAAGAGAACCTTTTAAT TTATTTATGGCC-3'; SRE2: 5' -GTTTTGTGTATGTG TGTTACAGCATTTG-3' and 5' -GGGAAAGCATATTCT CTTATTATTCTTTTTT-3'; and mat1 region: 5' -TTTCCAT TATGTCGTTGCCTTATCTCAGCGAGAATT-3' and 5' -AGATGCT CTATGGTTGAGAAGT-3'). The 5' end FAM-labeled ssDNA of the H1 box (59 nucleotides) was purchased from Eurofin Genomics. One of the target DNAs (5 ng/ml final concentration) and Swi2N protein were mixed in buffer (20 mM Tris–HCl [pH7.5], 100 mM NaCl, 5% glycerol, 1 mM DTT) and incubated at room temperature for 10 min. The reactions were analyzed by 1% agarose gel electrophoresis at 50 V for 90 min with TAE buffer. Double-stranded DNA (dsDNA) was stained with GelRed (41002, Biotium) and detected using a Typhoon FLA9500 scanner (Cytiva).
Chromatin immunoprecipitation (ChIP)-quantitative PCR (qPCR) assay

The protein of interest (wild-type or mutant forms of Swi2) was tagged with 9xV5, repeating the IPNPLLGLD V5 epitope tag nine times (35). A DNA fragment coding the V5 epitope tag and the adjacent kanMX marker was amplified by PCR with a plasmid carrying V5 and KanMX as a template (36) and a primer set listed in Supplementary Table S2, and it was integrated at the 3′ end of the *swi2* ORF by homologous recombination. ChIP was performed essentially as described previously (25). EMM2 medium (50 ml) containing 0.1 g/l each of leucine, adenine, histidine, uracil and arginine was used for cell culture. The cultures were grown to a density of 1.0 × 10^7 cells/ml at 30°C and then shifted to 18°C for 2 h. A total of 5.0 × 10^8 cells were cross-linked with 1% formaldehyde for 15 min at 25°C and then incubated in 125 mM glycine for 5 min. Cross-linked cell lysates were solubilized using a multi-beads shucker (Yasui Kikai, Osaka, Japan) and sonicated using a Bioruptor UCD-200 system (Diagenode). The sheared samples were centrifuged at 20000 g for 10 min at 4°C. The supernatants were centri fuged at 20 000 g for 10 min at 4°C. The supernatants were incubated with 30 μl Dynabeads Protein A (DB10002, Thermo Fisher) preloaded with 2.5 μl anti-V5 antibody (015–23594, FUJIFILM Wako) for 6 h at 4°C. After washing the beads, materials coprecipitated with the beads were eluted with elution buffer (50 mM Tris–HCl [pH 7.6], 10 mM EDTA and 1% SDS) for 20 min at 65°C. The eluates were incubated overnight at 65°C to reverse cross-links and then treated with 10 μg/ml RNase A for 1 h at 37°C followed by 20 μg/ml proteinase K for 3 h at 50°C. DNA was purified with a MonoFas DNA Purification Kit I (A01-0004, ANIMOS). qPCR was performed with SYBR Green Premix (Tli RNaseH Plus, TaKaRa Bio) or TB Green Premix Ex Taq II (RR820A, TaKaRa Bio) or TB Green Premix DimerEraser (RR091A, Takara Bio) on a My3000P qPCR system (Agilent).

Three-strand DNA exchange assay

The three-strand assay was performed with reference to a previous study (37). In total, 5 μM Rad51 and 10 μM (nucleotide) ϕX174 circular single-stranded DNA (ccsDNA) (N3023L, NEB) were mixed in reaction buffer (25 mM Tris–HCl [pH 7.5], 30 mM KCl, 50 mM NaCl, 2 mM MgCl2, 1 mM DTT, 5% glycerol, 2 mM ATP, 8 mM creatine phosphate and 8 U/ml creatine kinase), and the mixture was incubated at 30°C. After 5 min, the indicated concentration of Swi2-Swi5 or Swi5-Sfr1 was added and incubated for 5 min, followed by addition of 1 μM RPA. After 10 min, 10 μM (nucleotide) linear ϕX174 dsDNA (N3021L, NEB) was added to the mixture to initiate the three-strand exchange reaction. After 120 min, 200 μg psoralen was added and the reaction mixtures were exposed to ultraviolet (UV) light to cross-link DNA. After DNA cross-linking, 1.8 μl reaction stop solution containing 5.3% SDS and 6.6 mg/ml proteinase K (9034, Takara) was added and the sample was incubated for 60 min at 37°C. Substrates and reaction products were separated by agarose gel electrophoresis and the gel was stained with SYBR-Gold (S11494, Thermo Fisher).

D-loop assay

A super-coiled plasmid DNA of pBluescript II SK containing *mat3* sequences (REIII-mat3-H1-SRE3), which was first prepared by using a Qiagen Plasmid Maxi Kit (12963, Qiagen) and further purified by CsCl equilibrium ultracentrifugation with ethidium bromide (38) was used as a substrate super-coiled DNA (scDNA).

The 5′ end FAM-labeled ssDNA of the *H1* box (59 bases) was purchased from Eurofin Genomics. All components used in the reactions are described in terms of their final concentrations. DNA concentrations are described in terms of total nucleotides. Reactions were performed in 30 mM Tris–HCl [pH 7.5], 40 mM NaCl, 20 mM KCl, 5 mM MgCl2, 2% (w/v) glycerol, 2 mM ATP and 1 mM DTT. Rad51 (1.5 μM) was mixed with FAM-labeled *H1* ssDNA (FAM-H1) (30 nM) and incubated for 5 min at 30°C. A mixture of scDNA (30 nM) and Swi2-Swi5 or Swi5-Sfr1 (concentration described in the figure) was pre-incubated for 15 min at room temperature and then added to the mixture containing Rad51 and ssDNA to initiate D-loop reaction. After incubation for 10 min at 30°C, the reaction was terminated by adding one-fifth of the volume of stop solution (1% (w/v) SDS and 1 mg/ml protease K). The product was separated by 1% agarose gel electrophoresis at 50 V for 90 min with TAE buffer. FAM-H1 was detected and quantified using Typhoon FLA9500 (Cytiva) and ImageQuant (Cytiva).

RESULTS

Amino acids of Swi2 that are important for interaction with Swi6

Previously, we roughly mapped a Swi6-binding interface to the 210–365 aa region of Swi2 by performing two-hybrid assays in *Saccharomyces cerevisiae* (22). Here, to identify amino acids in Swi2 that are critically important for Swi6 binding, we first generated a series of C-terminal truncations of Swi2 to more precisely pinpoint the Swi6-binding region. The Swi2 (1–280) truncation mutant, but not the Swi2 (1–260) truncation mutant, interacted with full-length Swi6 in yeast two-hybrid assay, suggesting that 261–280 aa of Swi2 play an essential role in Swi6 binding (Figure 2A). The 270–279 aa are highly conserved among the three fission yeast species *S. pombe*, *S. octosporus* and *S. cryophilus* (Figure 2B and Supplementary Figure S1). We next generated a series of plasmids expressing full-length Swi2 in which each of the 270–279 aa, except for A273, was individually replaced by alanine (Figure 2C). Unlike full-length Swi2, *S. cerevisiae* cells expressing Swi2-V270A, -F271A, -V274A, -V275A, -I276A or -P277A did not grow on 4DO plates expressing Swi6 (Figure 2C). The 270–279 aa were highly conserved among the three fission yeast species. *S. pombe*, *S. octosporus* and *S. cryophilus* (Figure 2B and Supplementary Figure S1). We next generated a series of plasmids expressing full-length Swi2 in which each of the 270–279 aa, except for A273, was individually replaced by alanine (Figure 2C). Unlike full-length Swi2, *S. cerevisiae* cells expressing Swi2-V270A, -F271A, -V274A, -V275A, -I276A or -P277A did not grow on 4DO plates expressing Swi6 (Figure 2C). The 270–279 aa were highly conserved among the three fission yeast species. *S. pombe*, *S. octosporus* and *S. cryophilus* (Figure 2B and Supplementary Figure S1). We next generated a series of plasmids expressing full-length Swi2 in which each of the 270–279 aa, except for A273, was individually replaced by alanine (Figure 2C). Unlike full-length Swi2, *S. cerevisiae* cells expressing Swi2-V270A, -F271A, -V274A, -V275A, -I276A or -P277A did not grow on 4DO plates expressing Swi6 (Figure 2C).
Swi2 has two DNA-binding AT-hook motifs

Sequence analysis revealed that Swi2 proteins from three Schizosaccharomyces yeast species share two highly conserved AT-hook motifs located close to the Swi6-binding site (Figure 3A and Supplementary Figure S1). The AT-hook motif is a DNA-binding motif consisting of approximately 15 amino acids with a central GRP consensus sequence rich in positively charged arginine/lysine residues on either side of the core GRP (39,40). Its core sequence binds to the minor groove of AT-rich DNA (40).

Swi2 localizes to the AT-rich SRE3 element independently of Swi6 (3); therefore, we speculated that the AT-hook motifs of Swi2 might have a high affinity for the SRE3 sequence. To investigate this, we purified Swi2N (1–391 aa) and its AT-hook mutant forms, all of which lacked the C-terminal Sfr1 homology region predicted to possess DNA-binding activity (Figure 3B). In the AT1-A and AT2-A mutants, all residues in the first and second GRP tripeptides, respectively, were mutated to alanine. In the AT12-AA mutant, all residues in the GRP tripeptides in both AT-hooks were mutated to alanine (Figures 3A and B).

We examined binding of the AT-hooks to a linear 400 bp dsDNA encompassing the SRE3 element using EMSA (Figure 3C upper panel). The Swi2N-wild-type (WT) protein efficiently shifted SRE3 DNA in a protein concentration-dependent manner. Although Swi2N AT1-A and Swi2N AT2-A exhibited only slightly reduced mobility-shifts, Swi2N AT12-AA exhibited drastically reduced band-shifts and could not form stable protein-DNA complexes even at higher protein concentrations. On the other hand, Swi2N-WT did not exhibit a detectable band-shift with ssDNA (Figure 3D). These results clearly indicate that both AT-hooks of Swi2 are involved in dsDNA binding.

Next, we tested the specificity of DNA binding mediated by the AT-hook motifs. An EMSA with Swi2N-WT and SRE2 or a mat1 sequence as the target DNA yielded very similar results to those of an EMSA with Swi2N-WT and SRE3 (Figure 3C middle and lower panels). A similar trend was also observed in an EMSA using the three AT-hook mutant proteins. These results suggest that the AT-hook motifs of Swi2 do not have sequence specificity for SRE3.

The results described above were confirmed by performing another EMSA using a non-cognate DNA sequence of bacteriophage \( \Phi X174 \) (Supplementary Figure S2). When the replicative DNA of \( \Phi X174 \) (dsDNA) was used both Swi2N-AT1 and Swi2N-AT2 exhibited a similar or slightly
Figure 3. The two AT-hook motifs in Swi2. (A) Schematics of the two AT-hook motifs in Swi2. The consensus sequence, GRP tripeptides, in the two AT-hook motifs were substituted with alanine to generate AT1-A, AT2-A and AT12-AA. (B) An SDS-PAGE gel of the purified Swi2N derivative proteins (1–391 aa; 5 μg each). *M*, molecular mass markers. (C) EMSA of the Swi2N derivatives. Linear dsDNA (400 bp) was mixed with increasing concentrations of Swi2N, and the resultant DNA-protein complexes were analyzed by 1% agarose gel electrophoresis. The dsDNA sequences used were SRE3 (top), SRE2 (middle) and the region next to H1 of mat1 (bottom). (D) EMSA of Swi2N with ssDNA derived from the H1 homology box (59 nucleotides).
reduced band-shift compared with Swi2N-WT, and Swi2N-AT12-AA exhibited a reduced band-shift. When viral DNA (ssDNA) was used, Swi2N-WT and the three AT-hook mutants showed similar weak DNA binding. These results indicate that the AT-hooks of Swi2 are dsDNA-binding motifs that lack specificity for SRE3. The finding that Swi2N proteins exhibited a slight shift with viral DNA at higher concentrations may be due to the formation of a secondary structure in viral DNA.

The C-terminal region of Swi2 homologous to Sfr1 also has non-specific DNA-binding activity

The C-terminal half of Swi2 shares sequence homology with full-length Sfr1 (22). Sfr1 protein can be roughly divided into two domains: the N- and C-terminal half domains. The N-terminal half is the DNA- and Rad51-binding domain, and the C-terminal half is the Swi5-binding domain (22,27,33,41,42). The C-terminal domain cannot be recovered as a soluble protein from E. coli cells unless it is co-expressed with Swi5 (27,33,41).

To analyze the biochemical activities of the C-terminal domain of Swi2, we expressed Swi2C (392–722 aa) and Swi2CS (607–722 aa) together with Swi5 in E. coli and purified them. For this, we needed to co-express Swi5 as it is the case of Sfr1. Purified protein samples are shown in Figure 4A. An EMSA showed modest binding of Swi2C-Swi5 to DNAs cognate to the mating-type region, but without any preference (Figure 4B). An EMSA showed modest binding of Swi2C-Swi5 to DNAs cognate to the mating-type region, but without any preference (Figure 4B).

In contrast to Swi2N (Figure 3D and Supplementary Figure S2), Swi2C-Swi5 showed notable ssDNA binding in an EMSA (Figure 4C). However, Swi2CS-Swi5 did not show any detectable binding to ssDNA or dsDNA (Figure 4C), indicating that the region (392–607 aa) is responsible for DNA binding. This region corresponds to the DNA-binding domain of Sfr1 (41,42).

The Swi6-binding motif and AT-hook motifs of Swi2 are important for donor choice in MTS

We next examined whether the conserved Swi6-binding residues and DNA-binding AT-hook motifs of Swi2 are involved in MTS. We first performed a classical iodine staining assay (Figure 5A). Among MTS-proficient homothallic h90 cells grown on a minimal medium plate, P and M cells can conjugate. In the assay, spores generated on the plate are darkly stained by iodine vapor. In sharp contrast, MTS-deficient cells are poorly stained because of inefficient spore formation resulting from reduced conjugation. All five swi2 mutants (Δ: deletion, 5A: Swi6-binding site mutant, AT1-A: first AT-hook mutant, AT2-A: second AT-hook mutant, and AT12-AA: two AT-hooks mutant) formed poorly stained colonies (Figure 5A). We next analyzed the P cell population in saturated liquid cultures by multiplex PCR (Figure 5B and Supplementary Figure S3A) (24,25). WT h90 cells have a balanced P/M cell ratio. On the other hand, swi2Δ cells exhibit clonal differences that cause a bias toward P or M cells (4). Thus, we analyzed six independent clones of the swi2-5A strain and three independent clones of each Swi2 AT-hook mutant strain, AT1-A, AT2-A and AT12-AA (Figure 5B and Supplementary Figure S3A). None of these strains exhibited clonal differences, but the Swi6-binding site mutant Swi2-5A and the three AT-hook mutant strains showed an opposite bias in their P/M cell population. The Swi2-5A mutant exhibited a bias toward M cells (25 ± 2% P cells), while each of the AT-hook mutants, AT1-A, AT2-A and AT12-AA, exhibited a bias toward P cells (72 ± 3%, 68 ± 0.3% and 76 ± 3% P cells, respectively) (Figure 5B).

To perform epistasis analysis, we combined the mutations in swi2 with a deletion of swi6 (Figure 5C and Supplementary Figure S3B). The proportion of P cells was 18 ± 4% in the single swi6Δ mutant. The five independent clones of the swi2-5A swi6Δ double mutant strain showed a similar or slightly higher P cell proportion than the swi2-5A strain (28 ± 2% P cells). On the other hand, the five independent clones of swi2-AT12-AA swi6Δ showed various P cell proportions (31–60% P cells; mean ± standard deviation [SD] of 40 ± 15%).

These results suggest that the MTS defect of the Swi6-binding site mutant of Swi2 (Swi2-5A) is mainly due to a lack of functional interactions with Swi6, leading to a defect in mat2-P utilization. Moreover, they suggest that the AT-hook motifs are involved in mat3-M utilization and/or inhibition of mat2-P utilization. The finding that swi6Δ did not completely suppress the bias toward P cells in the swi2-AT12-AA mutant also suggests that the AT-hook mutation protein functions, at least partly, independently of Swi6.

The Swi6-binding and AT-hook motifs are involved in the differential localizations of Swi2 at the two SRE elements

The donor choice mechanism is regulated by the cell-type specific SRE utilization of Swi2 (3,5,43). Therefore, we hypothesized that the two interactions of Swi2 with SRE2 and/or SRE3 through the Swi6 binding and AT-hook motifs found in this study have a distinct impact on its localization at the mat locus. To examine the localization of wild-type and mutant Swi2, we performed a ChIP-qPCR assay using Swi2 proteins tagged with nine V5 epitopes at the C-terminal region of Swi2 (31–60% P cells; mean ± standard deviation [SD] of 40 ± 15%).

The finding that Swi6 is required for the localization of Swi2 not only to SRE2, but also to SRE3, in M cells. In sharp contrast, the Swi2-5A mutant defective in Swi6 binding exhibited deficient localization to SRE2 in M cells although a low level of association remained. Localization to SRE2 remained low in both P and M cells, suggesting that the AT-hook motifs are required for the localization of Swi2 not only to SRE3 but also to SRE2.

The AT-hook and Swi6-binding motifs are involved in the donor choice mechanism

To examine the impact of Swi2 motif mutation on the donor choice, we measured the P/M cell ratio in SRE deletion cells.
Figure 4. EMSA of the Swi2C-Swi5 and Swi2CS-Swi5 complexes. (A) An SDS-PAGE gel of Swi2N (1–391 aa; 5 μg), the Swi2C (392–722 aa)-Swi5 complex (5 μg), and the Swi2CS (607–722 aa)-Swi5 complex (3 μg). Mr, molecular mass markers. (B) EMSA of the Swi2C-Swi5 complex. Linear dsDNAs (mat1, SRE2 and SRE3; 400 bp each) were mixed with increasing concentrations of the Swi2C-Swi5 complex. (C) EMSA of the Swi2C-Swi5 and Swi2CS-Swi5 complexes with a cssDNA (ΦX174 viral DNA) and a circular covalently closed DNA (ΦX174 replicative form DNA).

by the multiplex PCR assay (Figure 6B and Supplementary Figure S3C).

In the presence of wild-type Swi2, SRE3Δ cultures are predominantly of the P mating-type with a uniform P/M cell ratio across cultures (75–82% P cells; mean ± SD of 78 ± 4%). In contrast, four independent clones of the Swi2-5A mutant in the SRE3Δ background showed varied cell-type compositions (41–76% P cells; mean ± SD of 57 ± 17%), thus differing both from swi2Δ SRE3Δ cultures, and from swi2-5A SRE3Δ cultures whose bias is uniformly towards M (22–27% P cells; mean ± SD of 25 ± 2%). These phenotypes are compatible with functionally distinct defects in the swi2-5A and SRE3Δ mutant, whose combination would lead to poor MTS and clonal variation. On the other hand, four independent clones of the Swi2-AT12-AA mutant in the SRE3Δ background showed uniform, high proportions of P cells. In spite of a tendency for the proportion of P cells to be slightly lower in the SRE3Δ (68 ± 5% P cells) than in the SRE3Δ background (76 ± 3% P cells), the strong bias towards P cells in both single and double mutants indicates that Swi2-AT12-AA might act at least in part by reducing the interaction of Swi2 with SRE3.

In the SRE2Δ background, cultures are predominantly and uniformly of the M mating-type when wild-type Swi2 is present (18 ± 1% P cells). Four independent clones of the Swi2-5A mutant strain in the SRE2Δ background had an even stronger bias with a lower proportion of P cells (10 ± 2% P cells) than the swi2Δ SRE2Δ (18 ± 1% P cells) and swi2-5A SRE2Δ (25 ± 2% P cells) strains. In sharp contrast, four independent clones of the Swi2-AT12-AA mutant in the SRE2Δ background showed a strong bias toward P cells with slightly elevated clonal variation (48–65% P cells; mean ± SD of 58 ± 8% P cells), indicating that they preferentially – albeit somewhat inefficiently – utilize mat2-P independently of SRE2. For comparison, the Swi2-AT12-AA mutant in the SRE2Δ background showed a greater bias toward P cells (76 ± 3% P cells), without detectable clonal variation.

Recently, we reported that two euchromatin factors, Set1C and HULC, inhibit the choice of mat3-M as a donor.
in M cells, probably by affecting chromatin structure at SRE3 (24,25). We analyzed the possible involvement of these euchromatin factors in the function of Swi2 (Figure 6C and Supplementary Figure S3D). Epistatic analysis showed that the swi2-5A set1Δ strain was slightly more biased toward M cells (20 ± 2% P cells) than the swi2-5A set1Δ strain (25 ± 2% P cells), while the swi2-2 set1Δ strain had 41 ± 0.3% P cells. On the other hand, the set1Δ mutation moderately alleviated the bias toward P cells in the Swi2-AT12-AA strain (reduced from 76 ± 3% to 61 ± 5% P cells) compared to set1Δ background, although clonal variation was still observed. These results show that Set1C inhibits the mat3-M donor choice even in these Swi2 mutants, independently of the functions of the Swi2 binding and AT-hook motifs.

The Swi2-Swi5 complex promotes the Rad51-driven DNA strand exchange reaction in vitro

The C-terminal region of Swi2 is well-conserved in fission yeasts and shares sequence similarity with Sfr1 protein (Figure 7A and Supplementary Figure S1) (22). Similar to Swi2, Sfr1 forms a tight complex with Swi5. The Swi5-Sfr1 complex is evolutionarily well-conserved in eukaryotes and promotes the Rad51-driven strand exchange reaction in vitro (27,44). Based on the sequence similarity of Sfr1 and Swi2 (22), we hypothesized that the Swi2-Swi5 complex would also have strand exchange activity. To test this hypothesis in vitro, we purified the Swi2-Swi5 complex from E. coli producing Swi2 and Swi5 under the control of the T7 expression system (Figure 7B). In M cells, Swi2 is expressed from a short mRNA (Swi2S: 205–722 aa, as a putative protein) and
Figure 6. Swi6-binding sites and AT-hook motifs play a crucial role in localization of Swi2 to SRE elements. (A) ChIP-qPCR analysis of the Swi2 protein enrichments at the SRE regions in P and M cells. The values are indicated as relatives at SREs to the act1 region. Error bars indicate SD (n = 3–5). The two-tailed paired Student’s t-test was used to compare the mean values obtained for each mutant with the WT of the same mating type. n.s, not significant. (B) Quantification of the mat1 contents of each Swi2 mutant strain combined with SRE3Δ or SRE2Δ by multiplex PCR. Mean values ± SD of the percentage of P cells in four independent clones of strains with mutated SRE element (SRE3Δ and SRE2Δ). (C) Quantification of the mat1 contents of each Swi2 mutant strain combined with set1Δ by multiplex PCR. Mean values ± SD of the percentage of the P cells in four independent clones with set1Δ.
Figure 7. The Swi2-Swi5 complex promotes the Rad51-driven DNA strand exchange reaction. (A) Schematic representation of Swi2, Swi2S, Swi2C and Sfr1 proteins. AT-hook motifs and the interfaces with Swi6 are shown. Swi2S is a putative short form (1–204 aa truncation) of Swi2 expressed in M cells (5). Swi2C indicated in magenta exhibits sequence similarity with Sfr1 protein. (B) An SDS-PAGE gel of the purified Swi2-Swi5 (8 μg) and Swi2S-Swi5 (5.5 μg) complexes. Mr, molecular mass markers. (C) Diagram of the three-strand exchange assay. (D-G) Three-strand exchange reactions containing Rad51 (5 μM) and the indicated concentrations of Swi2-Swi5 (D), Swi2S-Swi5 (E), Swi5-Sfr1 (F) or Swi2C-Swi5 (G) were incubated for 2 h at 30°C. DNA was separated by agarose gel electrophoresis to visualize substrates (cssDNA and ldsDNA), intermediates (JM) and products (NC). Sub, Substrate. The yields of the NC product (orange) and total yields of the JM intermediate plus NC product (gray) were quantified from the gels. Mean values of three independent experiments ± SD are shown.
a full-length mRNA (5); therefore, we also purified Swi2S as a complex with Swi5 (Figure 7A and B). Neither Swi2 nor Swi2S alone, without Swi5, could be expressed in E. coli, as reported previously for Sfr1 (27).

We first performed an in vitro three-strand DNA exchange assay with αX174 viral cssDNA and linearized double-stranded DNA (ldsDNA) as substrates. The pairing of cssDNA with ldsDNA produces DNA joint molecules (JMs) as intermediates and a nicked circular duplex (NC) as the final product of strand transfer over the length of the substrates (Figure 7C). In Rad51-only reactions, JMs formed (less than 10% yield of the input) and NC was not detected, as reported previously (27). The addition of the Swi2-Swi5 complex increased ~2-fold JM formation (~14% of the input) (Figure 7D). On the other hand, the addition of the Swi2S-Swi5 complex increased JM and NC formation (~42% and 6% of the input, respectively) (Figure 7E). The addition of the Swi5-Sfr1 complex increased not only JM (~40%) but also NC (~20%) formation (Figure 7F). Importantly, the addition of Swi2C-Swi5, in which Swi2C corresponds to the Sfr1 homology structure (Figure 7A Supplementary Figure S1), did not give any detectable impact on the Rad51-driven strand exchange reaction (Figure 7G). These results suggest that Swi2-Swi5 stimulates Rad51-driven strand-exchange reaction and that not only the C-terminal Sfr1 homology region (392–722 aa) but also half of the long N-terminal portion (205–392aa) of Swi2 is needed for this stimulation. In addition, this stimulation is suggested to be inhibited by the very N-terminal region (1–204 aa) of Swi2.

Next, we examined whether the Swi2-Swi5 complex promotes Rad51-driven strand invasion into the H1 homology box in vitro. To this end, we employed a D-loop assay using FAM-labeled ssDNA containing 59 bases of the H1 sequence and scDNA in which the REIII-mat3-M-H1-SRE3 region was inserted (Figure 8A) (45). In Rad51-only reactions, D-loop formation was undetectable (Figure 8B). Addition of Swi2-Swi5 or Swi2S-Swi5 complex did not markedly increase D-loop formation (less than 2% of the input) (Figure 8C). The Swi5-Sfr1 complex did not promote D-loop formation under the experimental condition (Figure 8C).

Similar to Rad51, Rad54 is reportedly essential for MTS (14). Rad54 proteins from S. cerevisiae and human cells promote Rad51-driven D-loop formation in vitro (46,47). Therefore, we investigated whether Rad54 stimulates Rad51-driven D-loop formation (Figure 8B). Addition of Rad54 to Rad51-only reactions resulted in very low D-loop formation (~2%) (Figure 8C). D-loop formation was slightly increased by addition of the Swi2-Swi5 complex to the Rad51-Rad54 reaction (~3.5%) and was increased more by addition of the Swi2S-Swi5 complex (~5.5%). D-loop formation greatly increased upon addition of the Swi5-Sfr1 complex (~8.7%). D-loop formation could not be detected when Rad51 was not added to the reactions.

These results indicate that the Swi2-Swi5 complex, independently of Rad54, promotes the Rad51-driven strand exchange reaction in vitro. The shorter Swi2S-Swi5 complex has higher activation potential for Rad51-driven strand invasion than the full-length Swi2-Swi5 complex. However, the promotion of Rad51-driven strand exchange by the Swi2S-Swi5 complex is not as high as that of the Swi5-Sfr1 complex.

The Swi2-Swi5 complex forms droplets in vitro

During the purification steps, we observed that turbidity developed in solutions of Swi2-Swi5 and Swi2S-Swi5 complexes at high protein concentrations, low salt concentrations and high temperatures. In the presence of 50 and 200 mM NaCl, optical microscopy revealed the presence of clumps in solutions of Swi2-Swi5 complex, and particularly in 50 mM salt solutions containing the Swi2S-Swi5 complex (Figure 9A). By contrast, clumps were not observed in solutions containing the Swi5-Sfr1 complex. We quantified the number of clumps by measuring the turbidity of the solutions at 600 nm (Figure 9B). The turbidity of the solution containing the Swi2-Swi5 complex was higher in the presence of 50 mM NaCl than in the presence of 200 mM NaCl. The salt concentration also influenced the turbidity of solutions containing the Swi2S-Swi5 complex. Consistent with the microscopic observations, the turbidity of solutions containing the Swi2S-Swi5 complex was higher than that of the solution containing the Swi2-Swi5 complex. The turbidity was detected, albeit at very low level, for the solution containing Swi2S-Swi5 complex in the presence of 50 mM NaCl. Turbidity was not detected in solutions containing Swi2N, under either salt conditions nor in solutions containing the Swi5-Sfr1 complex. These results, summarized in Figure 9C, suggest that the molecular characteristics of the Swi2-Swi5 and Swi5-Sfr1 complexes are different. In addition, it is possible that the N-terminus of Swi2 in the Swi2-Swi5 complex mediates self-association, resulting in the formation of droplets.

DISCUSSION

The donor choice mechanism in MTS is highly regulated by the MTS factor Swi2-Swi5, chromatin formation, and cis-acting SRE2 and SRE3 elements (17,48). The Swi2-Swi5 complex localizes to SRE3 independent on Swi6 and to SRE2 dependent on Swi6, and has been hypothesized to promote the strand exchange reaction for MTS (3,4).

Here, we identified two functionally important sites in Swi2, the Swi6-binding site and DNA-binding AT-hook motifs. These two sites were involved in different donor preferences in MTS: the Swi6-binding site was required for the mat2-P donor choice, while the AT-hook motifs were required for the mat2-M donor choice. In addition, genetic analysis revealed that the binding sites are involved in an SRE-mediated function. Moreover, we demonstrated that the Swi2-Swi5 complex promotes Rad51-driven strand invasion in vitro and Rad54 further stimulates the activation of strand invasion of Rad51 by the Swi2-Swi5 complex.

Features of the Swi6-binding site and two AT-hook motifs in Swi2

HP1 was reported to form a platform for various heterochromatin localization proteins that contain a consensus PxVxL sequence as a HP1-binding motif (49). More recently, the binding consensus was proposed to be a more...
relaxed $\Phi x(V/P)x\Phi$ sequence ($\Phi$ and $x$ indicate a hydrophobic and any amino acid, respectively) (50). In *S. pombe*, proteins containing $\Phi x(V/P)x\Phi$, such as Sgo1, Clr3 and Mit1, bind to Swi6 and its paralog Chp2 (51–53). In Mit1, the $xx(I/V)x(I/V)$ sequence is suggested to be the binding motif (53). Swi2 contains two overlapping relaxed consensuses, $273^{-\text{AVV1D}-277}$ and $274^{-\text{VVIPY}-278}$ (the underlined amino acids correspond to the consensus sequences), which correspond to the Swi6-binding site identified in this study (Figure 2). Further work is needed to determine which aa exactly are important for Swi6 binding.

We also located two AT-hook motifs near the Swi6-binding motif (Figure 3). The AT-hook motif preferentially binds to the minor groove of A/T-rich DNA (40). Therefore, because in *vivo* Swi2 constitutively associates with SRE3, which is an A/T-rich sequence, we tested whether Swi2 preferentially binds to SRE3 DNA *in vitro*. However, the results of EMSA demonstrated that the AT-hook motifs bound to the SRE3 element in basically the same way as they bind to the SRE2 element. The AT nucleotide contents of the used *mat1* region and SRE2 and SRE3 elements were 60%, 75% and 72%, respectively. The preferential localization of Swi2 to SRE3 *in vivo* may be dependent on other factors such as DNA topology and/or chromatin formation.

Considering that the AT-hook and Swi6-binding motifs are located close to each other in the N-terminal region of Swi2, the configuration of these two motifs may be involved in cooperative and concerted Swi2 bindings to DNA and heterochromatin at SREs *in vivo*, allowing Swi2 to sense the different chromatin contexts in P and M cells.

**Roles of the Swi6-binding and AT-hook motifs of Swi2 in donor designation**

The interaction between Swi2 and Swi6 is crucial for *mat2-P* donor choice; therefore, it is well accepted that
Figure 9. The Swi2-Swi5 and Swi2S-Swi5 complexes form droplets. (A) Optical microscopic images of solutions (10 mM HEPES [pH 7.5] and 0.5 mM DTT) containing 0.5 µM Swi2-Swi5, Swi2S-Swi5 or Swi5-Sfr1 at 25°C and the indicated salt concentrations. (B) The turbidity (A600) of solutions containing the indicated proteins and various NaCl concentrations at 25°C was measured using a NanoDrop instrument. The solution was the same as in (A), but each protein concentration was 5 µM. Mean values of three independent experiments ± SD are shown. (C) Summarized properties of purified Swi2-Swi5 and Swi5-Sfr1. Swi2, 1–722 aa; Swi2S, 205–722 aa; Swi2C, 392–722 aa; Sfr1, 1–299 aa. AT-hook motifs are shown in red, the Swi6-binding site in blue and Rad51-binding site in ochre. The Sfr1 homology region in Swi2 is shown in pink. Stimulation of Rad51-dependent strand-exchange activity and self-association are as follows: ++, very strong +, strong; +/−, weak or not detectable; −, not detectable.
Swi6-mediated localization of Swi2 to SRE2 in M cells leads to the selection of mat2-P as a donor (3,4,17). In this study, a multiplex PCR assay showed that the Swi6-binding motif mutant swi2-5A had a reduced proportion of P cells (25 ± 2% P cells) (Figure 5B), consistent with the proposed model. The ChIP-qPCR assay in M cells showed that accumulation of the Swi2-5A mutant protein was dramatically reduced at SRE2 but was only slightly decreased at SRE3. Moreover, the accumulation of WT and Swi2-5A mutant proteins at SRE2 and SRE3 did not significantly differ in P cells (Figure 6A). These results clearly indicate that the interaction with Swi6 is essential for the accumulation of Swi2 at SRE2 in M cells but is not required for the accumulation of Swi2 at SRE3 in either cell type.

On the other hand, the AT-hook motif proteins showed reduced association both with SRE2 and SRE3 in M cells and with SRE3 in P cells (Figure 6A), suggesting that the AT-hook motifs are involved in Swi2 localization to SRE2 in M cells and to SRE3 in P cells. Accordingly, the proportion of P cells was higher in the Swi6-binding mutant Swi2-5A (25 ± 2%) and SRE2Δ strain (18 ± 1%) than in the double mutant (10 ± 2%; Figure 6B). This result suggests that the mat2-P donor choice is governed by partially independent pathways, one involving the Swi2-Swihook interaction and the other involving SRE2. The Swi2-Swihook-independent and SRE2-dependent pathway may involve the AT-hook motifs of Swi2.

We previously showed that Set1C and HULC participate in the mat2-P donor choice by acting on SRE3 (24,25). The current study showed that the double mutant with Swi6-binding and Set1 mutations had a slightly more severe phenotype, while the Set1 mutation slightly suppressed the P cell-bias phenotype of the AT-hook mutant (Figure 6C). This suggests that Set1C and the AT-hook motifs of Swi2 function in different pathways for donor choice during MTS.

Based on the results obtained in the present and previous studies, we propose a model for the donor choice mechanism (Figure 10). In the model, Swi2 in P cells designates the mat3-M donor by localizing to SRE3 via the AT-hook motifs. On the other hand, the mat2-P donor choice in M cells involves a complex mechanism. Swi2 localization to SRE2 via the Swi6 interaction plays a crucial role. In addition, the AT-hook motifs might both foster interaction of Swi2 directly with SRE2, and act indirectly through SRE3 to increase Swi2 occupancy in the whole region. In M cells, we suggest that Set1C also works at SRE3 to modulate chromatin conformation (24,25). While further work is necessary to elucidate the molecular mechanisms underlying Swi2 localization, our in vitro investigations provide new clues for how Swi2 would stimulate recombination when localized at a donor locus and for its cell-type specific properties.

Effect of the Swi2-Swi5 complex on strand exchange driven by Rad51

We demonstrated that the Swi2-Swi5 complex promotes Rad51-driven strand exchange in vitro (Figure 7), suggesting that it can directly promote Rad51-driven strand invasion and subsequent strand exchange at the mat locus. Addition of Rad54 protein further stimulated the strand invasion activity of Rad51 (Figure 8). Rad54 physically interacts with Rad51 and is involved in DSB repair at the mat locus (14,34). We propose a model in which Rad51 and Rad54 bind to ssDNA generated from a one-ended DSB next to the H1 box of the mat1 cassette. This occurs in cells of both mating types (Figure 10). The Swi2-Swi5 complex localizes in a cell-type-specific manner (to the heterochromatic SRE3 region in P cells and to the whole heterochromatic region from mat2 to mat3 in M cells, respectively) in the absence of a DSB at the mat1-H1 junction (4). Therefore, the complex is assumed to localize to each locus before a DSB forms and then to recruit the Rad51 filament (17).

The N-terminal half of Swi2 (1–391aa) in addition to the Sfr1 homology region (392–722aa) plays also an important role in stimulation of Rad51-driven strand-exchange activity (Figure 7). Interestingly, the N-terminal truncated Swi2-Swi5 complex (lacking 1–204 aa of Swi2) had much higher stimulation activity than the full-length Swi2-Swi5 complex (Figures 7 and 8) and the Swi2-Swi5 complex formed more clumps in solution than the full-length Swi2-Swi5 complex (Figure 9). This suggests that a portion of Swi2 (205–391aa) within the N-terminal half self-associates to facilitate clump formation by the complex and that the very N-terminus (1–204 aa) inhibits both stimulations of Rad51 activity and self-association. Although in this study we could not elicit a rigid conclusion that the observed clump formation was related to liquid-liquid phase separation, the self-association of Swi2-Swi5 and Rad51 stimulation by the complex were closely related to each other. Rad52 in S. cerevisiae has been reported to assemble in liquid droplets to promote genome stability (54). The effect of the Swi2-Swi5 clump formation on the strand exchange driven by Rad51 is very interesting to be studied in near future. Swi6 has also been shown to form droplets in the presence of DNA (55). We have shown that the direct interaction of Swi2 with Swi6 is important for mat2-P donor choice in M cells. Interestingly, evidence has been provided that Swi2S is produced specifically in M cells (5) and our results show that the Swi2S-Swi5 complex had higher clumping and Rad51 stimulation activities than the Swi2-Swi5 complex. The different characteristics of Swi2-Swi5 and Swi2S-Swi5 may affect the mat2-M or mat3-P donor choice and strand-exchange activities by Rad51 (Figure 10). This is also a very interesting question to be resolved in the future.

In this study, we found that the functions of the Swi2-Swi5 complex in donor directionality can be separated between the Swi6-binding motif and the DNA-binding AT-hook motifs of Swi2. Moreover, we provide the first biochemical evidence that the Swi2-Swi5 complex stimulates Rad51-driven strand exchange activity in vitro. The uncovered properties of Swi2 - to recognize the chromatin state, bind DNA and stimulate DNA recombination - are central to accurately executing MTS through the spatiotemporal regulation of donor choice. These properties provide a rare glimpse into events occurring at donor sites rather than the more studied events occurring at sites of DNA damage.
Figure 10. Model for donor choice in MTS. (A) Overall and enlarged views of the *mat* locus in P cells. The Swi2-Swi5 complex localizes to the SRE3 element via the AT-hook motifs of Swi2 (shown in red). The complex binds to the Rad51 filament formed at *mat1* following resection of *H1* (shown in brown) and stimulates Rad51 strand invasion activity at the *H1* box of the *mat3-M* donor, helped by the mediator of recombination Rad54 (shown in purple). Swi6 is shown as a dimer in green (chromo domain in green and chromo shadow domain in light green). The Swi6-binding motif of Swi2 is in blue, Rad51-binding site in ochre, the Sfr1 homology region in pink and Swi5 is shown in gray. (B) Overall and enlarged views of the *mat* locus in M cells. Swi2, which is in greater abundance in M cells than in P cells (and the shorter Swi2S form of the protein present in M cells) localizes to the SRE2 element via the Swi6-binding motif, stimulated by the AT-hooks. The Swi2-Swi5 complex on the SRE2 element binds to Rad51 filament and stimulates strand invasion activity to choose the *mat2-P* donor. The Swi2-Swi5 complex also localizes to the SRE3 element via the AT-hook motifs; however, Set1C inhibits *mat3-M* donor choice at the SRE3 element.
DATA AVAILABILITY
The data of this study are available within the article and/or its supplementary data, or available upon reasonable request.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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