Going in the right direction: mating-type switching of S. pombe is controlled by judicious expression of two different swi2 transcripts
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Running title: Two swi2 mRNAs control switching directionality

Words: 5692
Abstract

*Schizosaccharomyces pombe*, the fission yeast, cells alternate between P and M mating type, controlled by the alternate alleles of the mating-type locus (*mat1*). The *mat1* switching occurs by replacing *mat1* with a copy derived from a silenced ‘donor’ locus, *mat2P* or *mat3M*. The mechanism of donor choice ensuring that switching occurs primarily and productively to the opposite type, called directionality, is largely unknown. Here we identified the *mat1-Mc* gene, a mammalian sex-determination gene (*SRY*) homolog, as the primary gene that dictates directionality in M cells. A previously unrecognized, shorter *swi2* mRNA, a truncated form of the *swi2*, was identified, and its expression requires the *mat1-Mc* function. We also found that the *abp1* gene (human CENPB homolog) controls directionality through *swi2* regulation. In addition, we implicated a cis-acting DNA sequence in *mat2* utilization. Overall, we showed that switching directionality is controlled by judicious expression of two *swi2* transcripts through a cell-type-regulated dual-promoter. In this respect, this regulation mechanism resembles that of the *Drosophila* sex-determination *Slx* gene.

Keywords: Abp1/fission yeast/mating-type switching/recombination/directionality
Introduction

Fission yeast is primarily a haploid single-celled organism whose cells exist as one of two cell/mating types, called P (plus) or M (minus). Under nitrogen starvation growth conditions, haploid cells of opposite type mate and the resulting diploid zygotic cell undergoes meiosis to produce two mat1P and two mat1M haploid spore segregants. This 2:2 Mendelian segregation pattern observed in meiosis shows that cell type is controlled by two alleles of a single mat1 locus. However, the culture starting from a single cell of either type contains a roughly equal proportion of cells of both mating types. This cell-type change is due to the efficient mating-type switching phenomenon, called homothallism, which operates during mitotic growth of the culture (Arcangioli and Thon 2003; Egel 2005; Klar 2007). The mating-type switching process replaces the existing mat1 locus through gene conversion process with a copy derived from one of the two epigenetically silenced ‘donor loci’, mat2P and mat3M (Figure 1).

The switching process is tied to the DNA replication cycle so that only one in four ‘grandchildren’ of a non-switchable cell switches in ~ 90% of cell pedigrees (Egel and Eie 1987; Klar 1990a; Miyata and Miyata 1981). The switching process is initiated by a DNA strand-specific epigenetic entity, called an imprint (Klar 1987; Klar 1990b), found at the junction of the homology-box H1 and the mat1 allele-specific sequence (Figure 1). The imprint is either a strand- and sequence-specific nick and/or two ribonucleotides incorporated in DNA (Beach and Klar 1984; Kaykov and Arcangioli 2004; Nielsen and Egel 1989; Vengrova and Dalgaard 2004). Three genes (swi1, swi3 and swi7) are required for synthesis of the imprint (Dalgaard and Klar 2000; Egel et al. 1984; Singh and Klar 1993). The swi7, encoding DNA polymerase α, is an essential gene for cellular viability (Singh and Klar 1993). The swi1 and swi3 encode replication fork pause factors (Dalgaard and Klar 2000). DNA replication of the imprinted strand at mat1 is thought to create a transient double-strand break (DSB) in the resulting chromatid. The DSB is repaired by recombination by copying P or M information from one of the two donor loci through the synthesis-dependent strand-annealing (SDSA) mechanism (Arcangioli and de Lahondes 2000; Yamada-Inagawa et al. 2007). This strand-specific imprinting/segregation mechanism (Klar 1987; Klar 1990b) explains the generation of one-in-four-granddaughters switching pattern observed in cell pedigrees (Egel and Eie 1987; Klar 1990a; Miyata and Miyata 1981). Interestingly, a similar mechanism of asymmetric cell division, through epigenetic differentiation plus selective segregation of sister chromatids in mitosis, has been recently suggested for generating neuronal bilateral asymmetry in Caenorhabditis elegans (Nakano et al. 2011).

Interestingly, the donor locus selection is not random; mat1P prefers mat3M, and mat1M prefers the nearby mat2P in ~ 90% of switches (Figure 1). Consequently, switches to the opposite type occur predominantly in standard mat2P and mat3M-containing strains, called h90 strains (for homothallic, ~90% sporulation). This donor preference, called directionality of switching, is not because cells prefer the heterologous information-containing donor locus for switching, but it is because P cells prefer mat3 and M cells prefer mat2, regardless of
the donors’ genetic content. The directionality control was demonstrated by swapping the donor loci genetic content to mat2M and mat3P (called h09 genotype). Notably, h09 cells switched preferentially by futile homologous information replacement (Tthon and Klár 1993). Thus, the directionality control dictates mat1P to switch preferentially by choosing mat3, and mat1M by choosing mat2, regardless of the donors’ genetic content.

Several genes have been shown to effect directionality. Switching-recombination proteins Swi2 and Swi5 physically interact with each other and directly bind to the mat2/3 region to mediate switching (Akamatsu et al. 2003; Jia et al. 2004). The distribution pattern of the Swi2/Swi5 complex is cell-type regulated: in P cells, Swi2/Swi5 localizes only to the mat3 locus; in contrast, in M cells, it spreads to the mat2 locus (Jia et al. 2004). The precise mechanism of the cell-type control on the Swi2/Swi5 spread in the mat2/3 region is unknown, but the chromatin structure is thought to regulate the Swi2/Swi5 complex spreading because heterochromatin factors, such as Swi6, Clr4, Rik1, Sir2, Clr7 and Clr8, affect the mating-type switching efficiency (Aguilar-Arnal et al. 2008; Ivanova et al. 1998; Jia et al. 2004; Shankaranarayana et al. 2003; Tthon et al. 2005; Tuzon et al. 2004). The fission yeast Abp1 (encoding autonomously replicating sequence-binding protein 1), one of the human centromere protein B (CENPB) homologs, has been reported to regulate the activation of DNA replication, to maintain genome integrity by forming heterochromatin at retrotransposons and to protect replication forks during pausing (Cam et al. 2008; Halverson et al. 1997; Murakami et al. 1996; Okada et al. 2007; Zaratiegui et al. 2011). Interestingly, the Abp1 protein also controls switching directionality by regulating the Swi2/Swi5 spread on the mat2/3 region. The mechanism by which Abp1 controls Swi2/Swi5 spread at donor loci is also unknown. However, because Abp1 binds within the donor region, it has been postulated that such binding might regulate directionality (Aguilar-Arnal et al. 2008; Cam et al. 2008).

In this study, we discovered that mat1-Mc and abp1 genes are required for switching directionality. We showed that the swi2 gene produces two independent but overlapping transcripts by engaging two different promoters in M cells, but only the longer form is produced in P cells. The previously unrecognized short swi2 form (i.e., swi2S) is the one that controls directionality by promoting the mat2 donor locus selection in M cells. To some extent, such a two-promoter regulation mechanism resembles that of the sex determination gene (Sxl) of Drosophila. Furthermore, we identified a mat2P cis-acting site (named Swi2-dependent recombinational enhancer 2, SRE2), which is critical for donor locus utilization for switching.

Materials and methods

Strains, plasmids and media
The S. pombe strains used in this study and their genotypes are listed in Table II. The mat1 locus specific primers (TAAGTGAGATGAGCTTGGCTTTG and AGTTGAGATATATTAGGGAGTGCGGTAGCG) were used to amplify the mat1-mc mutants. The mating-type donor region is silenced and prohibited from
homologous recombination by an epigenetic mechanism (EGEL 1984). To introduce mutations in donor loci by DNA-mediated transformation, we used a clr1-5 genotype that permits homologous recombination in the mat2/3 region (THON and KLAR 1992). All subsequent studies were conducted with strains from which the clr1-5 mutation was removed by genetic crosses. All mutations were generated by standard PCR methods and confirmed by PCR or sequence analysis.

To construct plasmids pREP3-swi2L-HA, pREP3-swi2S-HA and pREP3-swi2S, PCR fragments amplified from genomic DNA of YA664 (AKAMATSU et al. 2003) or from a wild-type strain were inserted into the Ball- BamHI sites of the pREP3 vector (MAUNDRELL 1993).

Strains were cultured in yeast extract with supplementary adenine-containing medium (YEA). For switching, sporulation, Northern blot and Western blot experiments, Edinburgh minimal medium, supplemented with auxotrophic requirements of each strain (PMA), was used. Media and other standard conditions for growth and genetic analysis were employed as described previously (MORENO et al. 1991).

**Genomic DNA preparation and Southern blot analysis**
Genomic DNA preparation and Southern blot analyses with HindIII digested yeast genomic DNA were carried out as previously described (MORENO et al. 1991). The 10.6-kb mat1P-containing HindIII fragment was used as the probe.

**Total RNA preparation and Northern blot analysis**
Total RNA was extracted and purified using the TRIzol® Plus RNA Purification System (Invitrogen Ca. No12183-555). Each lane was loaded with 20 µg total RNA of each sample for Northern blot analysis using the kit provided by Ambion (Ca. No. AM1940). The Swi2P probe consisted of the PCR fragment copied from genomic DNA with primers (ATGCCCATTTGATGACCCACCA and AGGTGAAAGAAGACATATAGTTTG). The Swi2 5’P probe consisted of the PCR product copied from the yeast cDNA library with primers (TGATATTCACAAAGAAGGAGATTCGTACAGT and cDNA 5’-end adaptor primer AAGCAGTGTATCAACGCAGAGT).

**RACE analysis**
The mRNA was enriched from total yeast RNA with the Qiagen mRNA kit (Qiagen Ca. No.72022). A total of 200 ng mRNA was used for each RACE experiment. RACE analysis was performed according to instructions in the RACE cDNA Amplification Kit (Clonetaq Ca. No. 634923). The Swi2-specific primers used for 5’ and 3’ RACE comprised TTACACTCCCCCTAAGTCTGCTACCC and GGAGGTAGCAGACTTAGGGGGAGTGTAA, respectively.

**Analysis of mat1 locus M/P ratio**
To determine the genetic content at the mat1 locus, a quantitative multiplex PCR procedure was adapted from previous publications (AGUILAR-ARNAL et al. 2008; JIA et al. 2004). PCR amplified products were run on a 1.5% agarose-TBE gel stained with
0.5 g/ml ethidium bromide and captured using a Typhoon scanning machine. Imagequant software was used for quantitative analysis.

**Protein extraction and immunoblotting**
Strain SP976 cells harboring pREP3-swi2L-HA, pREP3-swi2S-HA or pREP3 plasmid was grown in PMA media lacking a Leucine supplement for one day at 30°C. The exponentially growing cells were collected by centrifugation, suspended in 5% trichloroacetic acid buffer and disrupted by vortexing with glass beads. The extracted protein was subjected to immunoblotting. Mouse anti-HA antibody (Sigma, Ca. No. H9658) was used as the first antibody, and peroxidase-conjugated anti-mouse IgG antibody was used as the second antibody.

**Results**

**Selection of mat1-mc mutations**
Ascospores synthesize starch during meiosis and sporulation, while non-sporulating cells do not. Therefore, efficiently switching colonies growing on sporulation medium (pombe minimal adenine [PMA] medium) stain black when exposed to iodine vapors because they contain ascospores, but colonies defective in switching do not stain (LEUPOLD 1955). Using the iodine-staining procedure to screen for mating-type switching-defective mutants from a strain containing the hypersporulation 1 (hsp1) mutation (MICHAEL et al. 1995), we found eight independent mutants with a reduced rate of switching. They were sterile for mating and also exhibited an unusual phenotype of repairing their mutations spontaneously. Both phenotypes might be explained should the inefficient mat1 switching process operate in them to repair/heal their mutations. We postulated that the mutations were located at the mat1 locus in regions that are replaced with wild-type donor information during the switching process. We also observed that in the hsp1 genetic background the switched and healed cells mate readily on PMA medium because of their hypersporulation property, and the resulting zygotic asci do not grow further. As a consequence, only the mat1 mutant cells multiply in PMA medium, making our mutation analyses possible despite the fact that mutations were subject to removal by the switching process (presented below). The DNA of the mat1 locus of the mutants was amplified with mat1-specific primers and sequenced. The eight mutations represented six different sites in the mat1-mc gene (Table I) (KELLY et al. 1988). Because the Mc gene is required for both mating and sporulation (KELLY et al. 1988), and therefore, both the sterility and mutations repair by switching are explained by their location within the mat1 cassette.

**The mat1 genes are not required for mat1 imprinting**
Although our procedure favored selection of mutant cells during growth on PMA solid medium, the persistence of mutations during growth of the culture suggested that the cells were defective in some aspect of the switching pathway. Specifically, we wanted to understand why the switching process had not readily repaired the mat1-mc mutations. Recombination required for mat1 switching is initiated with the synthesis of the mat1 imprint (Figure 1). The first possibility we considered was that the mat1-mc mutations might reduce the imprint level. That is, the Mc
transcription factor might be required for the imprinting process. It is not known whether mat1 genes influence the imprint level. The imprint forms a fragile DNA break site, resulting in a double-strand break (DSB) during the normal DNA extraction process, so the imprint level is usually evaluated by Southern blot analysis (Beach and Klar 1984). Since our mutations were subject to repair during mitotic growth, for this analysis we employed an mc mutant strain with deleted donor loci (Amat2/mat3) (Klar and Miglio 1986). Because of their donor locus deletion, such strains do not switch at all, and we could thus directly assess the role of Mc in imprinting without the complication of mutations disappearing due to switching-promoted repair during our analysis. The imprint level observed in the mc mutant was similar to that of the wild-type control (Figure 2A). The mat1P encodes mat1-Pc and mat1-Pi genes, and mat1-M encodes mat1-Mc and mat1-Mi genes (Kelly et al. 1988). The other three mating-type genes (pc, pi, mi) mutants were also not affected in the imprinting process. These results suggest that none of the four mating-type genes is required for the imprinting step of the switching pathway (Figure 2A).

**mat1-Mc gene controls the directionality of switching**

We next entertained an alternative model for explaining the persistence of our mutations in mat1-mc mutant stocks; the mat1-Mc gene might dictate mat2P donor preference for switching the mat1-M allele. In this hypothesis, our mat1-mc mutants might switch by a default mode, preferring the mat3M as a donor instead of mat2P; accordingly, homologous information would be transferred to the mat1 locus. We previously showed that only a portion of the cassette information can be replaced by switching during the homologous cassette replacement, rather than always replacing the entire cassette (Yamada-Inagawa et al. 2007). Accordingly, each switch event might not have repaired mat1-mc mutations. To determine whether the Mc gene dictates mat2 utilization, we engineered a mc mutation in the mat1 donor locus. Cells of this culture should alternate between mat1-P and mat1-mc alleles. The wild-type culture contains an equal proportion of mat1P and mat1M cells due to the efficient switching of both mat1 alleles, either one to the opposite mat1 allele. In contrast, according to our hypothesis, the mutant strain should predominate with mat1-mc cells. To test this possibility, we analyzed the mat1 locus M/P ratio by polymerase chain reaction (PCR) as described previously (Aguilar-Arnal et al. 2008; Jia et al. 2004). For wild-type h90 cells, the mat1P amount was observed to be nearly equal to the amount of mat1M, as expected (Figure 2B). By comparison, the mc mutant exhibited much more mat1M content. This result suggested that mat1-Mc controls directionality by promoting mat2 utilization in M cells.

We previously showed that donor preference depends on specific chromosomal location and not on the genetic content of donor loci (Thon and Klar 1993). To explore the directionality issue further, we checked the effect of a mc mutation on the mat1 content of an h90 strain (mat2-mc and mat3P) whose genetic contents have been swapped with respect to those of standard h90 cells. Here, the mat1P allele was greatly enriched in the mat3-mc mutant in comparison to the wild-type cells (Figure
This result confirmed that the mat2 donor locus is used inefficiently in mat1-mc h⁹⁰ cells, a result similar to that presented above with h⁹⁰ cells.

Similarly, we also determined whether the other three mating-type genes are involved in switching directionality. Our results showed that none of the three genes we tested (pc, pi and mi) was involved in the directionality of h⁹⁰ and h⁰⁹ cells (Figure 2B). We did not check the mat2-pc mutant in an h⁹⁰ cell background because a previous report indicated that the mat2-pc mutant does not affect the mat1 locus M/P ratio (RUUSALA 1991). Thus, the mat1-Mc gene is the only mat1 gene that controls specific donor-locus choice in both h⁹⁰ and h⁰⁹ genetic backgrounds.

Mc is a known transcriptional factor regulating many M cell-type specific genes in combination with another DNA-binding protein partner named Ste11 (KJÆRULFF et al. 1997). Is Ste11 also required for switching directionality? We found that the Δste11 mutation did not affect the mat1 M/P ratio in both h⁹⁰ and h⁰⁹ backgrounds (Figure 2C). This result is consistent with the notion that Mc uses a Ste11-independent pathway to control directionality. However, because the efficiency of switching cannot be determined directly in ste11 mutants owing to their sterile phenotype, in this case only knowing their mat1 M/P ratio is not sufficient to assess whether Ste11 controls directionality (see below).

A previously unknown, shorter swi2 (swi2S) transcript requires Mc and Abp1 factors for expression

Next, we addressed as to how Mc might control the switching direction. As described in the introduction, assembly of the Swi2/Swi5 complex spreads to both donor loci in M cells, while it is localized at the mat3 locus in P cells. The precise mechanism of this cell-type-specific spread of the Swi2/Swi5 complex is not known. Because mc mutants are defective in mat2 donor choice, we considered the possibility that such mutants may be defective in the spreading of the Swi2/Swi5 complex at the mat2/3 region. We therefore examined the swi2 and swi5 expression using Northern blot analysis of mRNA isolated from P and M cells, which lacked both donor loci and thus stably maintained their cell type (KLAR and MIGLIO 1986). Analysis of swi5 did not produce any clear signal, possibly due to its weak expression. swi2 showed one transcript that was 2.5 kb long (swi2L, L for longer) in P cultures, plus another, shorter one that was 2.0 kb long (swi2S, S for shorter) in M cultures (Figure 3A and 3B). Furthermore, the amount of swi2S transcript was about twice the amount of swi2L in M cultures. Most interestingly, the swi2S species was absent in the mc mutant. This result showed that expression of the swi2S transcript was Mc-dependent. Furthermore, these modes of expression were unaffected whether or not the cells contained donor loci (Figure 3B). In addition, since Mc is partially repressed in nitrogen-rich medium (KELLY et al. 1988), the swi2S transcript level was reduced in rich medium in comparison to the level found in nitrogen-starved conditions (Figure 3B). We concluded that Mc controls the swi2S mRNA expression.

It was previously known that Abp1 regulates switching directionality, but the molecular mechanism of its action is unknown (AGUILAR-ARNAL et al. 2008). We found that the swi2S transcript was absent in the Δabp1 mutant. Collectively, these
results led us to entertain a model in which both Mc and Abp1 factors affect directionality by regulating the swi2S gene expression.

Next we determined the ends of both transcripts. We conducted rapid amplification experiments of cDNA ends (RACE) and cloned the PCR products (Scotto-Lavino et al. 2006) (Figure 3C). Sequencing results showed that the common swi2L forms found in both P and M cells were identical. swi2L is a 2,476-nt-long transcript capable of encoding a 722-amino-acid-long open reading frame (ORF) (Figure 3A). The swi2S is a 1,962-nt-long transcript capable of encoding a predicted 518-amino-acid-long ORF. The 3’ ends of both forms were identical. Overall, the swi2S form is a 5’ end truncated species of the swi2L form, and these species share the same ORF in overlapping regions of the gene. Northern blot analysis with the 5’ end-specific probe from the swi2L form (Swi2 5’P) did not detect the swi2S signal (Figure 3B), confirming our RACE results.

**Swi2S and Swi2L proteins detected when expressed from a strong promoter**

We tested whether the Swi2S form is expressed as an ORF predicted protein in yeast cells. We could not detect hemagglutinin (HA)-tagged Swi2 protein when it was expressed at the genomic locus from its indigenous promoter (Figure 3D), a result in accord with a previous report suggesting that the level of expression is too low to be detected by Western blot analysis (Akamatsu et al. 2003). Then we attempted to express both swi2L and swi2S genes with a stronger nmt1 promoter of a pREP3 plasmid vector (Maundrell 1993). Two bands of about 80 kDa and 60 kDa, corresponding to the 722aa and 518aa predicted proteins, respectively, were detected (Figure 3D). These results suggested that the swi2S encodes an N-terminal truncated protein form of the Swi2L protein when these genes were expressed from the nmt1 promoter.

**swi2S gene plays a key role in regulating directionality**

Our molecular work predicted that swi2S is essential for directionality of switching. We genetically tested this possibility. We inserted the 1.8-kb DNA fragment, containing the ura4 gene in a transcription orientation reversed with that of the swi2 gene, into the swi2L translation initiation site in the genome (Figure 4A). The resulting strain was named swi2L:ura4. Northern blot analysis showed that the DNA insert did not alter the swi2S expression, but the swi2L form was missing (Figure 4B). This result demonstrated that the swi2S mRNA is independently transcribed from the swi2L form and that the swi2S promoter is probably located in the 5’ coding region of the swi2L gene. The iodine vapor dark-staining phenotype of the swi2L::ura4 strain was indistinguishable from that of the wild-type genotype, but the sporulation frequency of strains was somewhat reduced (50% versus 70%) (Figure 4C). Also, the mat1P content was increased relative to the mat1M content in the swi2L::ura4, in comparison to that of the wild-type cultures (Figure 4D). A colony of the swi2L::ura4 genotype stains dark (Figure 4C), and in comparison, the Δswi2 or Δabp1 colonies were reported previously to stain lightly (Aguilar-Arnal et al. 2008; Akamatsu et al. 2003). These results indicated that the swi2S form indeed
provides a mat1 switching function and that it facilitates mat2 donor utilization over mat3.

**Mc and abp1 genes regulate directionality specifically by inducing swi2S expression**

Do Mc and Abp1 control directionality specifically by promoting the expression of swi2S? We conducted complementation experiments to address this question. We expressed swi2S through the nmt1 promoter from an ectopic pREP3 vector (Maundrell, 1993). Ectopic expression of swi2S led to increased mat1P content over mat1M (Figure 5A), and consequently, otherwise wild-type colonies stained lighter and a reduced level of sporulation was observed in them (Figure 5B). swi2S expression in mc or Δabp1 mutants led to a dramatic mat1 M/P ratio reversal from > 4.0 to 0.6. These results indicated that increased swi2S expression greatly suppressed the mc and Δabp1 mutants’ switching defect. Specifically, the mat2P donor utilization defect of mc and Δabp1 mutants was suppressed through swi2S expression (Figure 5B). Expression from the nmt1 promoter can be inhibited by adding 0.05 uM thiamine to the medium (Javerzat et al., 1996). Moderating swi2S expression with the thiamine addition partially complemented the Δabp1-staining phenotype, and consequently, the mat1 M/P ratio changed from 4.4 to 2.3 (Figure 5A). These results strongly suggest that Mc and abp1 genes regulate directionality specifically, or predominantly, by inducing swi2S expression.

As shown above, Δste11 did not influence the P/M cell ratio in both h90 and h09 cells (Figure 2C). As we have implicated Swi2S in directionality, we tested whether Ste11 is required for swi2S expression. The Northern blot result showed that the Δste11 mutation reduced the swi2S mRNA level as compared to that of the swi2L form in both h90 and h09 background (Supplemental Figure 1). The reduction of swi2S is more likely due to reduction of the Mc gene expression, which is regulated by Ste11 (Kjaerulff et al. 1997; Xue-Franzen et al. 2006). Although swi2S expression is partially reduced, the P/M cell ratio is not affected by the ste11 mutation (Figure 2C). We think that there are two possible explanations for it: one is that the reduction of swi2S amount is not enough to affect switch directionality; the other is that Ste11 may control other target genes that can compensate for the swi2S reduction.

**A mat2 cis-acting SRE2 element implicated in donor preference**

A previous report identified an SRE (Swi2-dependent recombinational enhancer) element, located centromere-distal to the mat3 locus as the Swi2/Swi5 complex loading site (Jia et al. 2004). Here we refer to it as the SRE1 element. Similarly, we discovered a sequence located centromere-distal to the mat2 locus that promotes its utilization as a donor (Figure 6A). A 400 bp deletion mutation of mat2:H1 distal-region greatly reduced both colony staining and sporulation (Figure 6B). We named the region defined by the deletion as the SRE2 element. Analysis of the mat1 locus composition showed that mat1P predominates in ΔSRE1 and mat1M predominates in ΔSRE2 strains (Figure 6B). This work demonstrated that both mat2 and mat3 loci use flanking elements for their utilization as donors for switching, and they
probably help recruit the recombination Swi2/Swi5 complex in a cell-type specific fashion. This idea is supported by studies reporting the recruitment of Swi2/Swi5 at these two sites (Aguilar-Arnal et al. 2008; Jia et al. 2004).

Discussion
In this study, we found evidence that the mat1-Mc gene dictates directionality in M cells such that mat2 is preferred over mat3 as the donor for switching. Moreover, we discovered the mechanism of this preference. Specifically, Mc induces the transcription of a shorter form of swi2 mRNA by activating a previously uncharacterized promoter, which lies within the 5′-end side of the ORF of the longer form of the swi2 gene. We also found that Abp1 similarly dictates directionality by inducing swi2S mRNA synthesis. Furthermore, a new cis-acting element, SRE2, located next to mat2 was identified, and that also facilitates mat2 utilization. A diagram of the genetic pathway summarizing our results is presented in Figure 7A.

A model for Swi2L and Swi2S factors controlling directionality
A model for directionality controlled by two forms of the swi2 gene in P and M cells is proposed in Figure 7B. In mat1-M cells, the directionality is primarily governed by the mat1-Mc gene, which induces the M-cell-type-specific swi2S expression at the transcriptional level. It is not known whether the Mc factor regulates swi2S expression directly or indirectly through regulating other gene(s). Similarly, the abp1 gene is required for swi2S expression, also by acting either directly or indirectly. We postulate that, owing to their protein sequence differences, the Swi2L and Swi2S proteins possess different Swi2/Swi5 complex-spreading abilities at the donor-loci region. The Swi2L form contains a strong self-interaction domain at its N-terminal end, and it was postulated that this form determines the cell-type specific pattern of Swi2/Swi5 complex distribution (Akamatsu et al. 2003; Haruta et al. 2008). The Swi2L protein prefers to accumulate at the mat3 region, and it does not spread to other regions. The Swi2S protein lacks the strong self-interaction domain, and it might facilitate the spread to the entire donor loci heterochromatic region. It is also possible that the Swi2S helps bind the complex, preferably to the mat2 locus, by binding to the SRE2 element (Fig. 7B). The proposed model is in accord with our observations and those of others, including: (1) in Δabp1 background, the Swi2L is the only form expressed in P as well as in M cells, and the Swi2/Swi5 complex binds to the mat3 locus, as shown previously by chromatin immunoprecipitation experiments (Aguilar-Arnal et al. 2008; Jia et al. 2004); (2) the Swi2S is the predominant form in M cells, and the Swi2/Swi5 complex is assembled on the entire region to make mat2 as the preferred donor; (3) in the Δswi2L (swi2L::ura4) strain, where only the Swi2S form is expressed, the mat2 is a preferred donor when compared with wild-type cells; (4) in the Δswi2 strain, where both Swi2L and Swi2S forms are lacking, the mat3 donor is not preferred because Swi2L is absent; in this case, however, it is not possible to comment on the donor locus preference because of the very low rate of switching (Aguilar-Arnal et al. 2008; Jia et al. 2004). In Δswi2 cells, the mat1-P allele predominates, perhaps
because the nearby $mat2$ is at an advantage over the more distant $mat3$ locus for recombination with $mat1$.

**Mc and Abp1 may physically bind to a putative swi2S promoter to activate expression**

We learned here that the $swi2$ gene expresses two $swi2S$ and $swi2L$ forms. The $swi2S$ is more crucial than $swi2L$ for efficient mating-type switching. The $swi2S$ expression requires Mc and Abp1. Mc is a transcriptional factor and regulates M-cell-specific genes in conjunction with Ste11 (Kjaerulff et al. 1997). Our study showed that $\Delta ste11$ reduced $swi2S$ expression, perhaps indirectly through controlling $mat1$-$Mc$ expression, rather than by directly controlling $swi2S$ expression. We do not think the Ste11 directly regulate $swi2S$ expression because $\Delta ste11$ still show reduced level of $swi2S$ expression. We investigated the sequence upstream of the $swi2S$ transcription start site to look for a putative promoter element that might exist there. A short 12 bp motif (5′-ACAATGCCCATTGT) was found 45 bp upstream of the $swi2S$ transcript start site, and this motif contains two standard, inverted Mc-binding sites (ACAATG) (Kjaerulff et al. 1997). This finding reminds us of the mating-type switching directionality regulation of the distantly related budding yeast, *Saccharomyces cerevisiae*. Budding yeast mating-type $MAT\alpha2$ factor binds to two closely located recognition motifs at RE (recombinational enhancer) region as a dimer to repress the RE function for donor choice during mating-type switching (Coic et al. 2006; Houston et al. 2004). The Mc binding to this putative promoter might activate $swi2S$ transcription. A recent chromatin immunoprecipitation analysis showed that Mc physically interacts with the $swi2S$ promoter region, as predicted here (Matsuda et al. 2011). Whether Mc binding depends on the two inverted presumptive recognition sites needs further investigation.

The $abp1$ gene was originally identified as the ARS-binding protein (Murakami et al. 1996) and it is probably involved in DNA replication initiation. Abp1 and the other two CENPB homologs (Cbh1 and Cbh2) bind to the centromere, helping centromeric heterochromatin assembly to promote centromere function (Nakagawa et al. 2002). More recently, genome-wide Abp1 distribution mapping showed that Abp1 also binds to retrotransposons to help maintain genome stability by silencing transposons and by controlling replication fork pause release (Cam et al. 2008; Zaratiegui et al. 2011). The Abp1 was previously implicated in the directionality of switching by promoting Swi2/Swi5 distribution at donor loci (Aguilar-Arnal et al. 2008). Since there is a strong Abp1 binding signal near the CenH sequence in the $mat2/3$-region, it was postulated that Abp1 might control Swi2/Swi5 binding by directly binding to this site (Aguilar-Arnal et al. 2008). We deleted the region containing the putative Abp1-binding site, but no obvious colony-staining defect was observed (Supplementary Figure 2). This result indicated that the putative Abp1 binding site is not required for switching directionality. Instead, we found that Abp1 regulates directionality of switching primarily by regulating $swi2S$ expression. According to the high-resolution mapping data (S. pombe epigenome website: http://pombe.nci.nih.gov/index.html?org=S.+pombe&db=pombe&hgsid=59268), there is about a 2.5-fold Abp1 enrichment signal found at the putative $swi2S$
promoter region. Thus, it is possible that Abp1 induces the swi2S transcript by directly binding to our predicted swi2S gene promoter region. We speculate that Abp1 helps load Mc to the swi2S promoter to activate swi2S expression (Figure 7A).

**Regulation of the swi2 gene by two separate cell-type-activated promoters**

Different-sized transcripts and the encoded proteins are usually produced by alternative mRNA splicing. In the database, the swi2 gene (Genebank Access No. NM_001019690) is listed as encoding a 722 amino acid ORF, lacking introns reflecting the swi2L species. We found two swi2 species resulting from two promoters with overlapping transcripts that differ from each other only at the 5′-end. A precedent for one gene producing two overlapping transcripts, initiated from two different promoters, is found with the Drosophila sex-lethal (Sxl) gene. Although alternatively spliced products of Sxl eventually regulate sexual identity during development, the initial decision for sexual identity occurs through a choice between the maintenance promoter (Pm) and establishment promoter (Pe) (Keyes et al. 1992). The Sxl-Pm is transcribed in both sexes, but the Sxl-Pe, located in the region encoding the first intron of the Sxl-Pm transcript, shows female-specific expression. The protein product from Sxl-Pe regulates the Sxl-Pm splicing to produce the SXL protein in female embryos. The Sxl-Pm transcript found in males includes an extra exon, which renders the transcript non-functional (Salz and Erickson 2010). Similarly, we found that swi2L expresses in both yeast cell types, but the swi2S expresses only in M cells and at a level higher than that of swi2L. In this respect, swi2 regulation is analogous to that of the Sxl gene of Drosophila. However, both of the swi2L and swi2S species are functional and bind to the mating-type donor region with alternative distribution patterns to regulate directionality of switching. In sum, the two swi2 forms cooperate to regulate donor choice such that most switches to the opposite mating type occur in cells of either mating type.

We note that previously the swi2S form has escaped detection by microarray analysis or genome-wide sequence analysis possibly because it was regarded as a partial degradation product of the swi2L form. As a consequence, previous studies (Akamatsu et al. 2003; Jia et al. 2004) performed to define the function of swi2 in switching considered only the swi2L form and here we found that the swi2S form is a major determinant of directionality of switching.

While we were preparing this manuscript for publication, it was reported (Matsuda et al. 2011) that the Mc and Abp1 genes control the switching recombination directionality, that Mc protein directly binds to the Swi2 gene regulatory region and that binding requires Abp1. Results of that report are complementary to some parts of our study and support our conclusions. However, the discovery of the Swi2S form and its role in directionality is presented here only in our study.

**Acknowledgements:** We thank Sharon Moore, Ken Ishikawa, Stephan Sauer and Jeff Strathern’s laboratory members for discussions and advice. We also thank Drs. Fernando Azorin and Hiroshi Iwasaki for providing yeast strains. The Intramural
Research Program of the National Cancer Institute of the National Institutes of Health supported this work.

Author contributions: CY and AK designed and performed the experiments, analyzed the data and wrote the manuscript; MB provided technical support for this work.

Conflict of interest: The authors declare that they have no conflict of interest.
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Figure legends

Figure 1 The directionality phenomenon of mating-type switching in fission yeast. The diagram shows mat1, mat2P and mat3M genes located from left to right in chromosome 2. The mat1 locus is expressed and it confers cell type, while mat2P and mat3M, located in the silenced region, act only as donors for providing copies of genetic information for mat1 switching. The mat1 can be either P (black jagged line, representing 1104 bp long DNA sequence) or M (gray straight line, 1128 bp). Each mat cassette is flanked by the homology regions, boxes H2 (left empty box, 135 bp) and H1 (right solid box, 59 bp), which are used for switching-promoted recombination. The imprint site (solid triangle) is located at the junction of the mat1 allele-specific and the H1 box sequences. The donor preference is determined by the mat1 cell type; mat1-P cells choose mat3 and mat1-M cells choose mat2, both with ~ 90% preference (thicker arrow) over the other, less-preferred (~ 10%, thin arrow) donor locus. The HindIII restriction enzyme sites (H) flanking each cassette are shown. Digestion of yeast genomic DNA with the restriction enzyme generates three fragments of indicated sizes, each containing a specific mat locus. The figure is not drawn to scale.

Figure 2 Effect of mat1 mutations on mat1 imprint and on directionality of switching. (A) Imprint level determined by Southern blot analysis. The wild-type (wt, SP976, see Table II for complete genotype) was the homothallic h90 strain, which contains all three cassettes diagrammed in Figure 1. The stocks with mating-type gene mutations in pc (SP718), pi (SP717), mi (SP716), mc (SP715) had been deleted for both donor loci, thus bands reflecting mat2P and mat3M genes were lacking in them. Such strains lacking donor loci cannot switch, and therefore stably maintain their mat1 mutations. Genomic DNA was digested with HindIII and the blot was probed with mat1P gene-containing 10.6 kb long probe (Figure 1). Locations of the HindIII sites flanking each cassette are shown in Figure 1. The 5.6 kb and 5.0 kb bands result from the double stranded break (DSB) generated by shearing imprinted DNA during its preparation. The DSB reflects the level of mat1 imprint (BEACH and Klar 1984). (B) Effect of mat1 genes mutations on directionality of h90 (wt, SP976; mc, CY195; mi, CY200; pi, CY204) and h09 (wt, PG19; mc, CY196; mi, CY202; pi, CY207; pc, CY199) strains. Quantitative multiplex PCR analysis of cultures showed M/P ratio of the mat1 locus of strains in which mutations introduced in donor loci have been transposed to mat1 through switching. The PCR reaction included one primer from outside the mat1 allele-specific sequences and another one from the mat1-P- or mat1-M allele-specific sequences (See Methods and
The mat1 allele-specific PCR product bands are indicated. (C) Effect of Δste11 (h<sup>90</sup>, CY146; h<sup>90</sup>, CY148) on directionality. The mat1 M/P ratio of cultures was determined as described above.

**Figure 3** Characterization of the swi2L and swi2S products. (A) The swi2 gene, mRNAs, and predicted proteins. Upper drawing shows the swi2 gene’s DNA structure indicated along with its two translation initiation ATG codons. The Northern blot probes, Swi2 5’P, Swi2P, we used are also indicated. The two lower drawings indicate mRNA and ORF length of swi2L and swi2S forms of the swi2 gene. Both swi2L and swi2S predicted proteins share the same open reading frame (ORF) in regions where the genes overlap. The Swi2 protein domains, such as for other proteins interaction, self-interaction and two AT-hook motifs, are marked (AKAMATSU et al. 2003). The Swi2S lacks the self-interaction domain. The GenBank accession numbers for swi2L and swi2S are JQ308182 and JQ308183. (B) Northern blot of swi2 transcripts. The cultures with indicated genotype were grown in PMA media (Nitrogen-starvation condition) and YEA media (Nitrogen-rich condition). Strains: P, SP713; M, SP714; wt, SP976; mc in h<sup>90</sup>, CY195; mc in Δdonors, SP715; Δabp1, spaA160. (C) Rapid amplification of swi2 cDNA ends (RACE). Results show agarose gel electrophoresis of RACE products obtained by amplifying 5’- and 3’-end products of swi2 cDNA. (D) Western blot analysis of the Swi2L and Swi2S proteins. Analysis of the swi2HA tagged gene from the endogenous locus in donors-deleted P (CY347) and M (CY348) cells is presented in the left two lanes. The remaining lanes reflect analysis of swi2L-HA and swi2S-HA tagged genes that were expressed from a plasmid vector in h<sup>90</sup> cells (SP976). The anti-HA antibody was used for Swi2 detection. The h<sup>90</sup> wild type culture harboring empty pREP3 vector was used as a negative signal detection control.

**Figure 4** Effect of Δswi2L on mating-type switching. (A) Structure of the swi2L::ura4 allele is diagrammed. A 1.8 kb ura4 HindIII fragment was inserted in the translation initiation site (ATG) of the swi2L gene. (B) Northern blot analysis of wild-type (SP976) and the swi2L::ura4 (CY345) cultures. RNAs prepared from cultures grown in PMA medium were analyzed with the Swi2P probe (Figure 3A). (C) Iodine-staining colony phenotype. Numbers below each colony reflect their efficiency of sporulation. (D) The mat1 M/P ratio of cultures was determined with the method described in Figure 2B.

**Figure 5** Ectopically expressed swi2S suppresses mc (CY195) and Δabp1 (CY327) mutants’ switching defect. (A) The mat1 M/P ratio of cultures was determined with the method described in Figure 2B. Analysis of an empty pREP3 vector transformant was included for comparison, as a negative control (wt, SP976). (B) Iodine-staining colony phenotype. Moderate swi2S gene expression was achieved by 0.05 uM thiamine addition to the medium and thiamine was not added to achieve overexpression.

**Figure 6** Effect of ΔSRE (Switching Recombinational Enhancer) deletion on mating-type switching. (A) The diagram shows location of SRE1 and SRE2 elements with respect to donor loci. ΔSRE2 (CY212) comprises of a 400 bp deletion located distal to the H1 region of mat2P. The SRE1 is defined by a 454 bp deletion of sequences (ΔSRE1, CY294) located distal to H1 region of mat3M (JIA et al. 2004).
staining colony phenotype (B) and the mat1 M/P ratio (C) of indicated strains are presented.

**Figure 7** The mechanism directionality of switching. (A) Summary of genetic determinants controlling directionality of mat1 switching. In P cells, because Mc factor is absent, only swi2L form is expressed “ON”. In M cells, the Mc factor, in combination with the Abp1 factor, activates the swi2S gene. (B) Proposed role of Swi2L and Swi2S in directionality of switching. In P cells, the Swi2L protein promotes localization of the Swi2/Swi5 complex to the mat3 region to make it as the preferred donor. In M cells, perhaps Swi2S and Swi2L cooperate to promote assembly of the Swi2/Swi5 complex across the entire silenced region to make mat2 as the preferred donor. The two SRE elements might facilitate enrichment of Swi2/Swi5 complexes at donor loci in cell-type specific fashion.

**Supplementary Figure 1.** Δste11 reduced swi2S expression relative to swi2L form. Northern blot analysis of wild-type (h90, SP976; h09, PG19) and the Δste11 (h90, CY146; h09, CY148) cultures. RNAs prepared from cultures grown in PMA medium were analyzed with the Swi2P probe (Figure 3A).

**Supplementary Figure 2.** Deletion analysis of the putative Abp1 binding site located in the mat2/3 region. (A) Deleted sequence. The sequence deleted in Δ109 (CY303) mutation is underlined and that deleted in Δ121 (CY301) mutation is represented in gray color. A putative Abp1 binding site is represented in bold letters. To help create mutation constructs, both mutations contained the ura4 gene insertion at the indicated site. The iodine-staining phenotype of wild type (wt, CY308 only containing the ura4 insertion) and mutants (B) and their mat1 M/P ratios of indicated strains (C) are presented.
### Tables

**Table I. *mat1-mc* mutations**

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Table II Fission yeast stains

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