Euchromatin factors HULC and Set1C affect heterochromatin organization and mating-type switching in fission yeast *Schizosaccharomyces pombe*

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Mating-type (P or M) of fission yeast *Schizosaccharomyces pombe* is determined by the transcriptionally active mat1 cassette and is switched by gene conversion using a donor, either mat2 or mat3, located in an adjacent heterochromatin region (mating-type switching; MTS). In the switching process, heterochromatic donors of genetic information are selected based on the P or M cell type and on the action of two recombination enhancers, SRE2 promoting the use of mat2-P and SRE3 promoting the use of mat3-M, leading to replacement of the content of the expressed mat1 cassette. Recently, we found that the histone H3K4 methyltransferase complex Set1C participates in donor selection, raising the question of how a complex best known for its effects in euchromatin controls recombination in heterochromatin. Here, we report that the histone H2BK119 ubiquitin ligase complex HULC functions with Set1C in MTS, as mutants in the shf1, brl1, brl2 and rad6 genes showed defects similar to Set1C mutants and belonged to the same epistasis group as set1A. Moreover, using H3K4R and H2BK119R histone mutants and a Set1-Y897A catalytic mutant, we found that ubiquitylation of histone H2BK119 by HULC and methylation of histone H3K4 by Set1C are functionally coupled in MTS. Cell-type biases in MTS in these mutants suggested that HULC and Set1C inhibit the use of the SRE3 recombination enhancer in M cells, thus favoring SRE2 and mat2-P. Consistent with this, imbalanced switching in the mutants was traced to compromised association of the directionality factor Swi6 with the recombination enhancers in M cells. Based on their known effects at other chromosomal locations, we speculate that HULC and Set1C control nucleosome mobility and strand invasion near the SRE elements. In addition, we uncovered distinct effects of HULC and Set1C on histone H3K9 methylation and gene silencing, consistent with additional functions in the heterochromatic domain.

Key words: gene conversion, chromatin structure, histone modifications, mating-type switching, *Schizosaccharomyces pombe*

INTRODUCTION

Homothallic strains (h<sup>0</sup>) of the fission yeast *Schizosaccharomyces pombe* switch between two mating types, P and M. This process is known as mating-type switching (MTS) and takes place at the *mat* locus on chromosome 2. The *mat* locus comprises three cassettes, *mat1*, *mat2* and *mat3* (Fig. 1A). The active *mat1* cassette expresses either P or M mating-type-specific genes and determines the mating type of a haploid cell (Kelly et al., 1988). The genes in the *mat2* and *mat3* cassettes, P (mat2-P) and M (mat3-M), respectively, are invariant, and are silenced by heterochromatin. Each cassette is flanked by short homology boxes called *H1* and *H2*. MTS is a result of
gene conversion repair of a programmed double-strand break at the mat1 cassette, using the homology boxes at either the mat2-P or mat3-M donor cassette (Klar et al., 2014).

A cell-type-specific regulation takes place at the donor selection step, where cells usually select the mating-type donor cassette opposite to the allele present at mat1; thus, P cells (mat1-P) preferentially choose mat3-M as a donor, while M cells (mat1-M) preferentially choose mat2-P (Fig. 1B) (Thon et al., 2018). This donor selection, also known as directionality of MTS, requires the mating-type switching factors Swi2 and Swi5, the heterochromatin protein 1 (HP1) homolog Swi6, and two cis-acting DNA elements (Egel et al., 1984; Jia et al., 2004b; Jakociunas et al., 2013). The cis-acting DNA elements are called Swi2-dependent recombination enhancers, SRE2 and SRE3, and are located next to the H1 box of mat2-P and mat3-M, respectively (Jia et al., 2004b; Jakociunas et al., 2013). The Swi2 protein localizes to these elements according to cell type: in P cells, Swi2 localizes only to SRE3; in M cells, it localizes to both SRE2 and SRE3 (Jia et al., 2004b; Jakociunas et al., 2013). From yeast two-hybrid assays, Swi2 has been shown to interact with Swi5 and with the recombination factor Rad51; therefore, it has been suggested that the Swi2–Swi5 complex promotes homologous recombination at the SRE2- or SRE3-adjacent cassette (Akamatsu et al., 2003). Furthermore, the Swi6 protein spreads over the mat2 and mat3 region between the flanking boundary elements IR-L and IR-R (Fig. 1A) and it contributes to the formation of heterochromatin over this entire region (Noma et al., 2001; Thon et al., 2002). The association of Swi6 with the region differs between P and M cells, with greater enrichment of Swi6 in M cells than in P cells, although overall cellular levels of Swi6 are comparable between P and M cells (Noma et al., 2001). As Swi2 interacts with Swi6
Swi6 localization at the mat locus is controlled by several histone modifications (Allshire and Madhani, 2018). An essential histone modification for Swi6 localization is di- or trimethylation of histone H3 at lysine 9 (H3K9me2 or -me3, respectively) catalyzed by the methyltransferase Clr4, the homolog of human SUV39H1 and SUV39H2 (Ekwall and Ruusala, 1994; Rea et al., 2000; Nakayama et al., 2001). H3K9me2 and -me3 are detected in constitutive heterochromatin including mat, centromeres and telomeres, and in facultative heterochromatin such as meiotic genes (Zofall et al., 2012; Allshire and Ekwall, 2015). Constitutive heterochromatic regions contain repetitive DNA sequences that nucleate H3K9 methylation through RNA interference (RNAi) (Allshire and Ekwall, 2015). At the mat locus, RNAi is triggered by the transcription of cenH, homologous to centromeric repeats (Hall et al., 2002; Volpe et al., 2002). dsRNAs originating from cenH are cleaved by the ribonuclease Dcr1, and the resulting siRNA (small interfering RNA) products are bound by the RNAi-induced transcriptional silencing complex (RITS) (Irvine et al., 2006; Buker et al., 2007). RITS loaded with siRNAs recruits the Clr4–Rik1–Cul4 complex (CLRC) to methylate H3K9 (Zhang et al., 2008). At the mat locus, there is an additional silencing pathway involving the CREB-like transcription factor Atf1–Pcr1 (Jia et al., 2004a). The Atf1–Pcr1 dimer binds to consensus sequences within the mat locus that exist at the REIII silencer and ~1.4 kbp away from REIII, close to cenH (Fig. 1A) (Thon et al., 1999). Other histone-modifying enzymes such as histone deacetylases (HDACs) are also required for donor selection and heterochromatin establishment. These include the NAD+–dependent histone deacetylase Sir2 (Shankaranarayana et al., 2003) and the Snf7/Hdac-containing repressor complex (SHREC), Clr1, Clr2 and Clr3 (Thon and Klar, 1992; Ekwall and Ruusala, 1994). Clr4, Clr3 and another HDAC, Clr6, interact with Atf1; therefore, Atf1–Pcr1 has been suggested to recruit these histone-modifying enzymes to the mat locus (Jia et al., 2004a; Kim et al., 2004; Yamada et al., 2005).

We recently conducted a genetic screen for factors that affect the directionality of mating-type switching. We identified the six genes (set1, swd1, swd2, swd3, ash2 and spf1) encoding components of the H3K4 mono-, di- and trimethyltransferase complex Set1C/COMPASS (Roguev et al., 2003). We also identified the brl2 gene encoding a component of the H2BK119 monooubiquitin ligase HULC complex that is composed of four subunits, Brl1, Brl2, Rad6 and Shf1 (Maki et al., 2018; Thon et al., 2018). In S. pombe, no other enzyme catalyzes these reactions. Ubiquitylation of H2B (H2Bub at K119 in fission yeast, at K123 in budding yeast, and at K120 in human) is required for the trimethylation of H3K4 by the Set1C/COMPASS family from yeast to human (Shilatifard, 2012). Therefore, we hypothesized that H2Bub and H3K4me work together to regulate MTS. Interestingly, the histone modifications by HULC and Set1C are generally observed at active genes, in euchromatin, and have been proposed to antagonize heterochromatin formation at the mat locus (Noma et al., 2001; Zofall and Grewal, 2007; Greenstein et al., 2020). However, HULC and Set1C have also been reported to have a positive effect on gene silencing in heterochromatin (Kanoh et al., 2003; Chen et al., 2008; Mikheyeva et al., 2014). HULC was proposed to promote loading of heterochromatin factors in centromeric repeats by facilitating a wave of transcription that fuels the RNAi machinery during S-phase (Chen et al., 2008). In the case of Set1C, deletion of the catalytic subunit Set1 causes a slight derepression of centromeric and telomeric reporter genes, and of transcription of the cenH element in the mat region (Kanoh et al., 2003; Mikheyeva et al., 2014). In addition, Set1C also functions in the repression of the stress-response gene ste11 (Materne et al., 2015, 2016) and T2 retrotransposons (Mikheyeva et al., 2014). Interestingly, Set1 localizes to Atf1 binding sites at centromeres, where it contributes to heterochromatin assembly with the HDAC Clr3 (Lorenz et al., 2014).

In this study, we performed genetic and molecular analyses of HULC, Set1C and histone mutants to better understand the role of the HULC and Set1C complexes in mating-type switching. Our results support the view that, like Set1C (Maki et al., 2018), HULC affects donor choice by reducing the effectiveness of the SRE3 enhancer in M cells and that H2B ubiquitylation by HULC and H3K4 methylation by Set1C operate in the same pathway for this function. Both modifying enzymes regulate Swi6 enrichment positively at the SRE2 and SRE3 elements. On the other hand, the two enzymes differentially affect heterochromatin formation and heterochromatic silencing at the mat locus. When combined with dcr1Δ, the set1Δ mutation caused derepression of a reporter gene at SRE3 while shf1Δ, a HULC mutant, did not. Thus, beyond increasing our understanding of donor selection through HULC and Set1C, our findings also shed light on gene silencing mechanisms by these complexes.

**RESULTS**

The histone H2B ubiquitylation complex HULC is involved in MTS. Recently, we determined that a subunit of HULC, the brl2+ gene product, is required for efficient MTS by screening the gene deletion library Bioneer version 5 (Maki et al., 2018). In an independent screen for MTS defects with version 2 of the same library, we identified another subunit of HULC, Shf1 (Small Histone Ubiquitination Factor 1). Therefore, we systematically
investigated the switching phenotypes of mutants in each of the four HULC subunits: the shf1Δ, rad6Δ, brl1Δ and brl2Δ mutants. MTS defects can be detected by measuring mating-type ratios in saturated liquid cell cultures, under conditions where the cells do not conjugate, because efficient switching results in an equal proportion of each cell type. Mutants in a few factors, such as Swi2 mutants, display variable mating-type ratios in independent cultures, while other mutations uniformly bias cell populations toward P or M (Jakočiūnas et al., 2013; Maki et al., 2018). Thus, for each HULC mutant, four independent clones were constructed and analyzed to evaluate clonal variation by multiplex PCR. The wild-type h90 strain, PG4045, had nearly equal proportions of P and M cells, as expected, but all HULC mutants displayed a biased mating-type ratio which was around 33% P cells.

Fig. 2. HULC is involved in MTS. (A) Multiplex PCR was used to measure the content of mat1 using primers that bind specifically to either mat1-P or mat1-M together with a mat1-specific primer. The P and M band intensities were measured. The graph shows the mean value ± standard deviation (SD) for four independent colonies of a WT (h90) or single deletion mutants of each of the subunits of HULC and rad18. (B) Iodine staining of mutants lacking individual HULC subunits and a rad18Δ strain. WT (h90) colonies capable of forming spores stain darkly upon exposure to iodine vapor due to the high content of starch within the spore wall. Colonies deficient in MTS stain lightly. (C) Quantification of mat1 content estimated by multiplex PCR. Mean values ± SD of the % of P band intensity are displayed for four independent colonies of the single shf1Δ mutant (same samples as in A) and for double deletion mutants combining shf1Δ with deletions affecting other HULC subunits. Two-tailed paired Student’s t test was used to compare the mean of each sample to the control (the single shf1Δ mutant); n.s., not statistically significant. (D) Spot test using single deletion mutants of the HULC subunits and the rad18Δ mutant on a rich medium (YES) where a plate was subjected to UV radiation (100 J/m²). Five-fold dilution series are shown.
in all cultures examined (Fig. 2A and Supplementary Fig. S1A). The deletion mutants were also examined by iodine staining of plate-grown colonies, a classical assay for MTS efficiency based on the conjugation and subsequent sporulation of diploids. Colonies of the control PG4045 strain grown on MSA plates stained darkly with iodine, indicative of efficient MTS. The HULC mutants were less stained (Fig. 2B and Supplementary Fig. S1B), consistent with the observed cell-type bias. Double deletion of \( \text{shf1} \) and either \( \text{rad6, brl1} \) or \( \text{brl2} \) did not show any additive effect (Fig. 2C and Supplementary Fig. S1C). We concluded that HULC is involved in MTS.

The Rad18/Rad6 pathway does not influence MTS
In addition to its interaction with the E3 ubiquitin ligase Brl, the E2 Rad6 enzyme also operates with E3 Rad18 in a conserved DNA damage tolerance pathway by mono-ubiquitylating PCNA (Hedglin and Benkovic, 2015). We tested whether the Rad6/Rad18 pathway is required for MTS. As expected, both the \( \text{rad6} \) and \( \text{rad18} \) deletion mutant strains were sensitive to UV exposure, similar to a \( \text{rad51} \) deletion mutant strain (Fig. 2D). On the other hand, the HULC mutants \( \text{shf1Δ, brl1Δ and brl2Δ} \) did not show UV damage sensitivity (Fig. 2D). Conversely, multiplex PCR and iodine staining assays did not detect any switching defect in the \( \text{rad18Δ} \) mutant, unlike for the \( \text{shf1Δ, brl1Δ and brl2Δ} \) mutants (Fig. 2A and 2B, Supplementary Fig. S1A and S1B). These results indicate that Rad6 functions in different complexes for MTS and DNA damage tolerance.

HULC regulates donor selection at SRE3
Depending on which step is affected, mutants deficient in MTS can be separated into three classes, Class Ia, Class Ib and Class II, by Southern blotting (Egel et al., 1984). Class Ia mutants lack an imprint at \( \text{mat1} \); the imprint is required for MTS as it is converted to a single-ended double-strand break just past the \( \text{H1} \) homology box during DNA replication, allowing invasion of \( \text{mat2-} \text{P} \) or \( \text{mat3-} \text{M} \) by the \( \text{H1} \) sequence (Fig. 1) (Klar et al., 2014; Thon et al., 2018). The imprint also creates a fragile site that undergoes breakage during DNA preparation and it is thus visible on Southern blots as a DNA double-strand break. Class Ib mutants have the imprint but fail to use it properly. Class II mutants are deficient at a later step, in the resolution of the gene conversion, which causes characteristic \( \text{mat2-} \text{mat3} \) cassette duplications inserted at \( \text{mat1} \) that are not found in Class I mutants. The \( \text{shf1Δ} \) deletion was assigned to Class Ib in which a functional imprint is detected at the \( \text{mat1} \) cassette (Supplementary Fig. S2A) similar to the previously examined \( \text{brl2Δ} \) deletion (Maki et al., 2018). The Class Ib group includes muta-

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Fig. 3. Analysis of donor preference in HULC mutants. (A) Quantification of \( \text{mat1} \) content in the \( h^{0} \) strain, in which the donor loci are swapped from \( \text{mat2-} \text{P mat3-} \text{M} \) to \( \text{mat2-} \text{M mat3-} \text{P} \), by multiplex PCR. Mean values ± SD of the % of P band intensity of four independent colonies for single deletion mutants in each subunit of HULC in the \( h^{0} \) background. (B) Quantification of \( \text{mat1} \) content of mutants of \( \text{SRE} \) elements, \( \text{SRE3Δ} \) and \( \text{SRE2Δ} \), by multiplex PCR. Mean values ± SD of % of P band intensity of four or three independent colonies of strains with each of the mutated \( \text{SRE} \) elements. Two-tailed paired Student’s \( t \) test was used to compare the mean of each sample to control; n.s., not significant.
tions that impair donor selection. These can be identified and further classified according to their effects in $h^{90}$ cells that have swapped mat2-M mat3-P donor cassettes (Thon and Klar, 1993). At least four groups of $h^{90}$ Class Ib mutants can be distinguished: Group 1a, displaying a strong bias toward P cells; Group 1b, with a mild bias toward P cells; Group 2, with clonal variations; and Group 3, with a bias toward M cells (Maki et al., 2018). Deletions removing HULC subunits each caused a mild bias towards P cells in the $h^{90}$ background (~48% P cells instead of ~19% in the presence of functional HULC), placing them in Group 1b (Fig. 3A and Supplementary Fig. S2B).

The SRE elements are necessary for donor selection (Jia et al., 2004b; Jakociūnas et al., 2013). The changes in cell-type ratios caused by HULC mutations in $h^{90}$ (Fig. 2A; increased proportion of M cells) and $h^{90}$ populations (Fig. 3A and Supplementary Fig. S2B; increased proportion of P cells) suggested in both cases an increased use of SRE3 over SRE2. We further investigated the importance of SRE2 and SRE3 in the presence or absence of the $shf1$ gene using SRE deletion mutants (Fig. 3B). In an SRE3Δ strain, ~84% of the population were P cells (i.e., the use of SRE2 is favored). This ratio was unaffected in $shf1$ SRE3Δ cells (~83%) (Fig. 3B and Supplementary Fig. S2C). In an SRE2Δ strain, the $shf1$ deletion slightly shifted the mating-type ratio toward M cells (~12% P cells in $shf1$ Δ instead of ~19% in $shf1^+$ cells) (Fig. 3B and Supplementary Fig. S2C). The slight shift in $shf1$ SRE2Δ cells does not seem decisive, but it is statistically significant. These results suggest that, in M cells, HULC normally prevents the use of SRE3. They also suggest that HULC does not facilitate the use of SRE2, at least not when SRE3 is absent. It remains possible, however, that HULC enhances the use of SRE2 when SRE3 is present and this effect would be missed in the SRE3Δ mutant. We do not exclude such a scenario as it would be consistent with a proposed model where SRE2 and SRE3 compete in the process of donor selection (Jakociūnas et al., 2013).

**Histone residues modified by HULC and Set1C are required for MTS** We noticed that the genetic analyses in Fig. 3 showed similar trends for the MTS defects detected in HULC mutants and the defects previously reported for set1 deletion mutants (Maki et al., 2018). The ubiquitylation of H2BK119 by HULC is essential for H3K4me3 by Set1C in *S. pombe* (Roguev et al., 2003); therefore, we performed an epistasis analysis to test the relationship between HULC and Set1C in MTS. The switching defect of the $h^{90}$ shf1Δ set1Δ double mutant was similar to that of the $h^{90}$ set1Δ strain (~42% P cells in shf1Δ set1Δ mutant versus ~43% P cells in set1Δ mutant in the multiplex PCR assay), with set1Δ slightly suppressing the cell-type bias of the shf1Δ strain (~33% P cells in shf1Δ mutant) (Fig. 4A and Supplementary Fig. S3A). This placed the two mutations in the same epistasis group, suggesting coordinated action of HULC and Set1C.

Next, we investigated the importance of the histone H2BK119 and H3K4 residues that are modified by HULC and Set1C, respectively, in MTS. The proportion of P cells in the H2BK119R mutant (~34% P cells) was similar not only to the shf1Δ deletion mutant, but also to the double mutant shf1Δ H2BK119R (Fig. 4B and Supplementary Fig. S3B). On the other hand, the proportion of P cells in the H3K4R mutant was ~27% P cells, less than in the set1Δ deletion mutant (~44% P cells) but similar to the H3K4R set1Δ double mutant (~30% P cells) (Fig. 4C and Supplementary Fig. S3C). We also investigated the requirement for the catalytic activity of Set1 in MTS. The SET domain has a highly conserved tyrosine residue that is suggested to be a catalytic residue from the crystal structure (Supplementary Fig. S3D) (Trievel et al., 2002), and replacement of this tyrosine (Y1054) with alanine in *S. cerevisiae* Set1 causes loss of H3K4 methylation (Williamson et al., 2013). We created a Set1-Y897A mutant in *S. pombe*, corresponding to Set1-Y1054A in *S. cerevisiae* (Supplementary Fig. S3D). The mating-type ratios in the set1-Y897A mutant analyzed by multiplex PCR were similar to those in the set1Δ mutant (Fig. 4D and Supplementary Fig. S3E). We verified that the Set1-Y897A protein tagged with 9×V5 epitope (9×V5-Set1-Y897A) was present in the cells (Supplementary Fig. S3F). The *svd1* gene encodes a subunit of the Set1C complex (Roguev et al., 2003). An *svd1*Δ single mutant and the *svd1*Δ Set1-Y897A double mutant also showed similar ratios to the set1Δ mutant (Fig. 4D and Supplementary Fig. S3E). These observations point to both H2BK119 ubiquitylation by HULC and H3K4 methylation by Set1C playing an important role in the directionality of MTS.

Several lines of evidence have indicated that the RNA polymerase II-associated factor 1 complex Paf1C (Krogan et al., 2003; Ng et al., 2003; Wood et al., 2003), which is functionally conserved from yeast to mammals (Kim et al., 2009; Mbogning et al., 2013), can recruit HULC and Set1C (Sadeghi et al., 2015). In fission yeast, Paf1C prevents heterochromatin propagation across the IR-L boundary of the mat locus, among other effects (Sadeghi et al., 2015). To test the requirement for Paf1C in MTS, we constructed strains lacking the Paf1C components Leo1 and Paf1. The *leo1Δ* and *paf1Δ* mutants did not show a switching defect in the multiplex PCR assay (Fig. 4E and Supplementary Fig. S3G). Thus, the effects of HULC and Set1C in MTS occur independently of Paf1C.
Set1 and Shf1 are involved in the mating-type-specific localization of Swi6 at SRE2 and SRE3, but they have different effects on histone H3K9 methylation. The results presented so far allow us to update a previous model (Maki et al., 2018) by now proposing that both HULC and Set1C reduce use of the SRE3 recombination enhancer in M cells by modifying histone H2BK119 and H3K4. Given that differential Swi6 enrichment in the mating-type regions of M and P cells is a determinant of donor choice (Thon and Klar, 1993; Jia et al., 2004b; Jakociunas et al., 2013), we next investigated the effects of HULC and Set1C on Swi6 occupancy at SRE2, SRE3 and the K region located between SRE2 and cenH (Fig. 5A). To this end, we performed ChIP-qPCR with Flag-tag antibody for 3×Flag-Swi6 using heterothallic strains with a fixed mating type, P (mat1-PΔ17) or M (mat1-Msmt-0). In a wild-type background, Swi6 showed an approximately three-fold higher enrichment at both SRE2 and SRE3 in M cells compared to P cells (Fig. 5B). No significant difference between P and M cells was observed within the K region, specifically at a location between mat2-P and cenH analyzed in this study.
In both the shf1Δ and set1Δ backgrounds, the high, M-specific, Swi6 occupancy at SRE2 and SRE3 was decreased (Fig. 5B). It thus appears likely that Shf1 and Set1, and by extension HULC and Set1C, control donor choice at least in part by ensuring high Swi6 occupancy at recombination enhancers in M cells. We note, however, that the double shf1Δ swi6Δ deletion mutant showed a slightly but significantly lower (in h90 cells) or higher (in h09 cells that have swapped mat2-M mat3-P donor cassettes) proportion of P cells compared with the single swi6Δ mutant (Supplementary Fig. S4), suggesting that some effects of HULC in MTS are not through Swi6.

Swi6 recognizes di- and trimethylation of histone H3K9 (Nakayama et al., 2001; Jih et al., 2017). We thus examined the di- and trimethylation levels of H3K9 in shf1Δ and set1Δ strains using the same chromatin-fixed samples as in Fig. 5B and antibodies specific for di- and trimethylation of H3K9 (H3K9me2 and H3K9me3, respectively). (Fig. 5B). In both the shf1Δ and set1Δ backgrounds, the high, M-specific, Swi6 occupancy at SRE2 and SRE3 was decreased (Fig. 5B). It thus appears likely that Shf1 and Set1, and by extension HULC and Set1C, control donor choice at least in part by ensuring high Swi6 occupancy at recombination enhancers in M cells. We note, however, that the double shf1Δ swi6Δ deletion mutant showed a slightly but significantly lower (in h90 cells) or higher (in h09 cells that have swapped mat2-M mat3-P donor cassens. Error bars indicate SD (n = 3 or 4). Two-tailed paired Student's t test was used to compare the mean obtained for each gene deletion to WT of the same mating type; *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant.

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Comparing P and M cells, we observed that H3K9me2 enrichments in wild-type strains were comparable at the three locations examined and did not vary with cell type, with the possible exception of SRE2 for which H3K9me2 was slightly lower in M cells (Fig. 5C). In shf1Δ strains, H3K9me2 was significantly reduced in both cell types with the similar tendency of slightly lower enrichments in M cells (Fig. 5C).

For H3K9me3, M cells showed somewhat higher enrichments than P cells in the wild-type background (Fig. 5D). This difference between P and M cells was attenuated in the shf1Δ background, but globally the levels of H3K9me3 enrichments remained unchanged in shf1Δ cells (Fig. 5D). As observed for H3K9me2 (Fig. 5C), H3K9me3 levels were increased by set1Δ, at all locations and in both cell types (Fig. 5D). These ChIP-qPCR analyses suggest that the enrichment levels of H3K9me2 and H3K9me3 are regulated by Set1; however, these regulations work in different manners and correlations between H3K9 methylation and Swi6 enrichment levels were not systematically observed.

Shf1 and Set1 have differential effects on silencing and MTS

Two main pathways of heterochromatin formation have been well documented at the mat locus: one is RNAi nucleating heterochromatin at the cenH region (Hall et al., 2002; Volpe et al., 2002; Allshire and Ekwall, 2015), and the other is an RNAi-independent pathway involving the Atf1–Pcr1 complex (Jia et al., 2004a; Kim et al., 2004; Wang and Moazed, 2017; Greenstein et al., 2018). Both H2Bub and H3K4me are believed to control transcription by regulating RNA polymerase II activity in S. pombe (Tanny et al., 2007; Zofall and Grewal, 2007; Mikheyeva et al., 2014). This suggests that HULC and Set1C participate in silencing the mat locus by facilitating RNAi that uses transcription products from cenH to nucleate heterochromatin. Alternatively, the facts that H2Bub and H3K4me mediate gene silencing at the ste11 gene locus (Materne et al., 2015, 2016), and that Set1 localizes to Atf1 binding sites at centromeres and at the ste11 gene locus (Lorenz et al., 2014), suggest that

Fig. 6. Set1 works in a different pathway from Dcr1 for gene silencing and MTS. (A) Schematic view of the mating-type region of an h90 strain containing a ura4 reporter gene (PG1899). Red arrows indicate binding sites for Atf1–Pcr1 dimers. (B) Silencing in PG1899 was assayed by plating ten-fold dilution series of cells with the indicated gene deletions on YES medium or medium containing 5-FOA. (C) Quantification of mat1 content by multiplex PCR, showing the % of P band intensity for the strains analyzed in the silencing assay in (B). Error bars indicate SD (n = 3).
HULC and Set1C cooperate with Atf1–Pcr1 also in the mating-type region. To investigate whether Shf1 or Set1 participates in the RNAi or Atf1–Pcr1 pathway of heterochromatin formation, we created double mutants, namely shf1Δ dcr1Δ, shf1Δ pcr1Δ, set1Δ dcr1Δ and set1Δ pcr1Δ, in a strain with a urad4 reporter gene inserted in the SRE3 region (PG1899 with (EcoRV)::urad4') (Fig. 6A). As previously reported (Jia et al., 2004a), dcr1Δ and pcr1Δ single deletion mutants with the (EcoRV)::urad4' reporter were resistant to 5-FOA, which is toxic to cells that express urad4', while the dcr1Δ pcr1Δ double deletion caused sensitivity, at the same level as the swi6Δ strain (Fig. 6B). The shf1Δ single mutant was resistant to 5-FOA, and the shf1Δ dcr1Δ and shf1Δ pcr1Δ double mutants remained resistant. In contrast, the set1Δ and set1Δ pcr1Δ strains were resistant to 5-FOA, but the set1Δ dcr1Δ strain was clearly sensitive: its growth on 5-FOA was as severely affected as that of the dcr1Δ pcr1Δ strain (Fig. 6B). This places set1Δ, but not shf1Δ, in the same epistasis group as pcr1Δ for the effects of these mutations on silencing in the mating-type region.

We examined the switching phenotype of the strains shown in Fig. 6B by multiplex PCR (Fig. 6C and Supplementary Fig. S5). The PG1899 strain carrying (EcoRV)::urad4' at SRE3 was slightly biased toward P cells (~59% P cells) in comparison with the normal h90 configuration (~50% P cells). This switching phenotype probably comes from the insertion of the urad4' gene slightly affecting SRE3 due to its proximity (see Fig. 6A). However, populations of a swi6Δ derivative of PG1899 showed mating-type biases similar to the h90 swi6Δ mutant; hence, we analyzed all mutant strains derived from PG1899 for MTS. The single dcr1Δ and pcr1Δ mutants showed mating-type ratios similar to PG1899. The double dcr1Δ pcr1Δ mutant was biased towards M cells (~45% P cells). The double shf1Δ dcr1Δ and shf1Δ pcr1Δ mutants had cell-type ratios similar to the shf1Δ single deletion mutant. In the case of set1Δ, the set1Δ pcr1Δ deletion strain had a cell-type ratio similar to the set1Δ strain, but the set1Δ dcr1Δ double deletion strain showed a larger bias toward M cells than the set1Δ strain, the largest bias of all strains examined. Thus, both gene silencing and multiplex PCR assays converge to show that Set1 functions in parallel to Dcr1 at the silent mat locus.

**DISCUSSION**

Mating-type switching is governed by chromatin conformation. Thus, the H3K9 methyltransferase CLRC and several HDACs have well-documented roles in the formation of heterochromatin required for the directionality of switching. Our genetic screens identified euchromatic factors, the H2B ubiquitin ligase HULC and the H3K4 methyltransferase Set1C, as additional MTS factors (Fig. 2) (Maki et al., 2018). H3K4me3 has been put forward as a regulator of DSB formation, promoting meiotic recombination in some eukaryotes (Tock and Henderson, 2018; Serrano-Quilez et al., 2020). In the MTS of *S. pombe*, the lysine-specific demethylases Led1/Led2 are recruited to the matl locus and involved in imprint formation (Holmes et al., 2012; Raimondi et al., 2018). On the other hand, we found that mutants in HULC or Set1C do not show an imprinting defect (Supplementary Fig. S2) (see also Maki et al., 2018). This places the action of HULC and Set1C at the donor choice step. It indicates that euchromatic histone modifications function not only in DSB events, but also in the selection of donor templates by regulation of chromatin conformation. A potentially important feature is that these complexes engage in cross-talk whereby H2B ubiquitylation upregulates H3K4 methylation by Set1C (Tanny et al., 2007). Here, we obtained evidence that HULC and Set1C function in a common pathway for MTS to inhibit the preferential use of the SRE3 enhancer in M cells, plausibly through this cross-talk. Notably, the effects of the two complexes as determined by mutational analyses were not identical; there are distinct contributions to gene silencing detected for Set1 and Shf1 that likely reflect some divergent functions in heterochromatin formation.

Strong biases toward the M cell-type were observed in both HULC and Set1C mutants, denoting increased use of the SRE3 enhancer in these mutants (Fig. 2 and 3) (Maki et al., 2018). An epistasis analysis assigned the two complexes to the same pathway (Fig. 4), even though a slightly more pronounced bias towards M cells was observed in shf1Δ cells than in set1Δ or shf1Δ set1Δ cells (Fig. 4A). It has been reported that Set1 is still expressed in cells that lack HULC, and low levels of ubiquitylation of H2BK119 in vivo (Tanny et al., 2007; Zofall and Grewal, 2007; Mikhayeva et al., 2014) and H3K4 dimethylation are still detected in an H2BK119R strain (Tanny et al., 2007). In *S. cerevisiae*, H3K4 monomethylation is still detected in rad6 deletion cells (Schneider et al., 2005). Therefore, we speculate that the residual mono- and/or dimethylation of H3K4 in cells lacking H2Bub increases the bias toward M cells, either directly through effects on recombination or indirectly. Our study also revealed that while the H2BK119R, shf1Δ and combined H2BK119R shf1Δ mutations had the same effect on MTS (Fig. 4B), the switching defects in the H3K4R and H3K4R set1Δ mutants were much stronger than in the single set1Δ strain (Fig. 4C). Transient acetylation of H3K4 has been proposed to facilitate the association of Swi6 with histone H3K9me2 through a chromodomain switch where Swi6 replaces H3K9me2-bound Chp1 or Clr4 at centromeres (Xhemalce and Kouzarides, 2010). The same mechanism facilitating Swi6 association at SRE elements at the mat locus may account for the pronounced effects of H3K4R on MTS where Swi6 association is paramount.
The differential association of Swi6 with the mating-type region is a distinguishing factor between P and M cells: Swi6 is present at a low level over the region in P cells, coinciding with Swi2 specifically at SRE3, but at a high level in M cells, coinciding with Swi2 at both SRE2 and SRE3 (Jia et al., 2004b). These associations are believed to favor the use of SRE2 over SRE3 in M cells (Yu et al., 2012; Jakociūnas et al., 2013). We found here that Shf1 (and to a lesser extent Set1) is required for the high Swi6 occupancy at SRE2 and SRE3 in M cells. In the shf1Δ and set1Δ mutants, Swi6 occupancy remained abnormally low at both enhancers in M cells, similar to what is normally seen in P cells (Fig. 5). This profile could account for SRE3 being preferred over SRE2 in both cell types in the mutants, where all cells would in essence behave as P cells. The association of Swi6 with the enhancers was most strongly reduced in the shf1Δ mutant (Fig. 5), where donor choice was also most impaired (Fig. 4). These effects would place HULC and Set1C upstream of the enhanced Swi6 association with recombination enhancers, SRE2 and SRE3, in M cells (Fig. 7), without excluding other points of action. We propose that the resultant chromatin organization is at least in part responsible for the altered donor choices in HULC and Set1C mutants (Fig. 7).

Our experiments are consistent with and support an active repression by HULC and Set1C at SRE3, relevant to the central question of how SRE2 outcompetes SRE3 in M cells when Swi2 is present at both enhancers. SRE2 may be inherently more efficient than SRE3 under these conditions, or recombination may be actively repressed at SRE3. Here, shf1Δ and set1Δ mutants showed a bias toward M cells in h30 cells (Fig. 2 and 4) and toward P cells in h30 cells (Fig. 3A), consistent with the two factors repressing the use of SRE3. Moreover, the ~80% bias towards P in SRE3Δ cells remained unchanged in the shf1Δ mutant, indicating that SRE2 is functional in the absence of Shf1. In contrast, in the SRE2Δ strain, SRE3 was increasingly used in shf1Δ cells, indicating that Shf1 inhibits the use of SRE3 (Fig. 3B). Thus, our genetic analyses suggest that HULC inhibits selection of SRE3 – and thereby mat3-M donor selection – in h30 cells.

Fig. 7. Proposed working model for the regulation of MTS involving HULC and Set1C. In M cells, HULC and Set1C fine-tune the organization of Swi6-containing chromatin. The Swi2–Swi5 complex, a mediator of strand exchange, associates with both SRE2 and SRE3 but its action at SRE3 is counteracted by the established chromatin structure, resulting in preferential use of the competing SRE2 enhancer. In P cells, the Swi2–Swi5 complex localizes at SRE3 specifically and promotes mat3-M donor choice, as previously suggested by Thon et al. (2018).
similar to Set1C (Fig. 7) (Maki et al., 2018). Furthermore, the result that neither HULC nor Set1C promoted mat2-P choice at SRE2 raises an additional possibility: Swi6 could inhibit the utilization of mat3-M, and HULC and Set1C might inhibit the utilization of SRE3 by promoting Swi6 binding there. Interestingly, the majority of Swi2 binding to SRE3 is independent of Swi6 (Jia et al., 2004b). Swi6 may be dispensable for the gene conversion reaction per se during MTS. If so, instead, Swi6 binding at SRE3 promoted by HULC/Set1C may inhibit mat3-M choice. In support of this possibility, experiments where silent cassette contents or enhancers were swapped or deleted revealed that the donor cassette controlled by SRE3 always becomes predominantly used in the absence of Swi6 (Jakociūnas et al., 2013). Thus, the model will be worth exploring further.

How might an inhibition of recombination by HULC/ Set1C take place at SRE3? Together with increasing Swi6 association, a relevant effect of HULC/Set1C could be by controlling nucleosome occupancy or positioning. In S. cerevisiae, nucleosome occupancy is decreased genome-wide in H2BK123R, rad6A and lge1Δ mutants (Chandrasekharan et al., 2009; Battà et al., 2011). In S. pombe, H2Bub decreases chromatin remodeling by RSC at the ste11 promoter to repress transcription (Materner et al., 2015, 2016). In this case, the effect of H2Bub is through H3K4me and histone deacetylation, supporting the idea that HULC, Set1C and HDACs might work in concert to position nucleosomes at the mat locus as well. Nucleosome positioning at SRE3 may mask the enhancer or prevent strand invasion. An intriguing alternative that our results do not exclude is that nucleosome positioning or modification by HULC/Set1C might facilitate recombination near SRE2 in M cells sufficiently for SRE2 to outcompete SRE3 when both recombination enhancers are present. Positive effects on recombination repair and on recombination-dependent bypass of DNA lesions have been reported for the RNF20/RNF40 mammalian homolog of Bre1 (Moyal et al., 2011; Nakamura et al., 2011) and for the S. cerevisiae counterpart of HULC (Hung et al., 2017). Nucleosome depletion by associated remodelers, rather than nucleosome stabilization, often appears instrumental during repair, highlighting the context dependency of the effects of the modifying and remodeling complexes (Nakamura et al., 2011; Challa et al., 2021). It will be important in the future to understand how remodeling complexes might contribute to MTS, taking into account the fact that Swi6 also interacts with many remodelers (Motamed et al., 2008).

Finally, we uncovered effects of HULC and Set1C on the heterochromatic structure of the mating-type region that illuminate how these complexes might affect Swi6 association and MTS. In the case of shf1Δ, we observed reduced H3K9me2 at the three locations tested (Fig. 5C). HULC has been suggested to associate with the RNAi machinery in centromeric regions when repetitive sequences are transcribed during S-phase (Chen et al., 2008); by analogy, cenH in the mating-type region may be an entry point for HULC. In the case of set1Δ, we observed a synthetic silencing defect when combining the set1Δ and dcr1Δ mutations (Fig. 6B), showing that Set1 and Dcr1 participate in parallel pathways of heterochromatin formation. The effect is clearly relevant to MTS as the double mutant showed a strong bias toward M cells (Fig. 6C). Similarly, Dcr1 and the Clr3 HDAC operate in parallel to recruit Clr4 to the mat locus, Dcr1 through RNAi at cenH and Clr3 through the Atf1–Pcr1 binding sites (Yamada et al., 2005). Here, we placed set1Δ in the same epistasis group as per1Δ for the effects of these mutations on silencing (Fig. 6B), suggesting that the Atf1–Pcr1 binding sites at the mat locus constitute entry points not just for Clr3 but also for Set1C. It has been suggested that H3K9me2 is associated with RNAi machinery and that the transition from H3K9me2 to -me3 is associated with an RNAi-independent mechanism in centromeric regions (Jih et al., 2017). Distinct effects of shf1Δ and set1Δ on modifications of H3K9 and silencing of the ura4 reporter gene inserted at SRE3 may be due to the differential functions of HULC and Set1C in relation to RNAi machinery in the cenH region and RNAi-independent machinery using the Atf1–Pcr1 complex. Independently, a genome-wide study showed that Set1 cooperates with Clr3 to repress transcription at other Atf1–Pcr1 binding sites (Lorenz et al., 2014) and an important effect of Clr3 in heterochromatin is to suppress histone turnover (Aygun et al., 2013; Wang and Moazed, 2017; Greenstein et al., 2018). Thus, taking these observations altogether, HULC and Set1C may be recruited in several ways to the mat locus where they would cooperate with Clr3 to regulate aspects of heterochromatin formation and nucleosome occupancy that are important for donor selection. To further analyze this mechanism, we will need to understand how the regulation would be exerted in a cell-type-specific manner, as well as spatially, and whether the regulation has to occur locally at the enhancers, or whether global effects on nucleosome mobility that would differ in P and M cells might lead to the observed biases in enhancer use.

**MATERIALS AND METHODS**

**Yeast strains, strain construction and strain manipulation** Schizosaccharomyces 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 (Ekwall and Thon, 2017). Strains were generated by transformations or genetic crosses. The H3K4R mutant strain (TM504: h⁰ hht1-H3K4R hht2-H3K4R leu1-32 his3-D1) was generated from EM20 (h⁰ leu1-32 his3-D1 ade6-M375) by CRISPR/
Cas9-mediated gene editing. EM20 was transformed by a gRNA expression plasmid (pEM59), a Cas9 expression plasmid and HR donor templates. To mutate the hht1 and hht2 genes, the common target sequence 5′-TCTACCGGGTGTTAAGGCACC-3′ was inserted into the BbsI site in the gRNA scaffold sequence in pEM59. To select cells in which Cas9 was active, the ade6-M375 mutation was edited in the same transformation. The gRNA targeting ade6-M375, 5′-CTCGGCAACAAATTGATTG-3′, was also expressed from pEM59. The HR donor templates for mutagenesis, purchased from Integrated DNA Technologies, were amplified using three primer sets, hht1-H3K4R (5′ and 5′-GGACGATAACGATGAGGCTTC-3′) and hht2-H3K4R (5′-GGGAACCGAAAAATCGCAATC-3′ and 5′-CCAGGACGATAACGATGAGGCTTC-3′) and ade6- (5′-GTGGTCAATTGGGCGCTAT-3′ and 5′-CGTGCACTTCTTAGACAGTTCA-3′) by PCR. Cells were grown on low-adenine plates and white colonies (ade6′) were selected. TM863 and TM896 were derived from TM504. TM501 was also generated by Cas9-mediated gene editing, with the gRNA target sequence 5′-TACTTATGATTACAAGTTTC-3′ and the HR donor template amplified by primer set 5′-GGGAATATCGCGCGTTTCTC-3′ and 5′-CTAGTTTAAATAGGCCAGCATGT-3′. All mutants selected for further analysis were confirmed by PCR and sequence analysis. The plasmid pEM59, allowing expression of an inserted gRNA sequence, was constructed by Emil Damgaard Jensen in the Thon lab. The sequence data are provided in Supplementary Fig. S6. The plasmid is available from G.T. upon request. The sequences of the PCR-amplified HR donor templates are shown in Supplementary Fig. S7.

Iodine staining The efficiency of MTS was estimated by iodine staining as described previously (Thon and Klar, 1993). Briefly, cells were streaked on an MSA plate and grown at 26 °C for 3–4 days. The plate was inverted over a cylindrical glass beaker (diameter 90 mm) containing a few crystals of iodine in a fume hood. The iodine vapor stained the colonies in about 10 min at room temperature.

Multiplex PCR Multiplex PCR was performed as previously described (Maki et al., 2018). Schizosaccharomyces pombe cells were propagated in 2 ml YE5S culture at 30 °C to saturation. A 500-μl aliquot was transferred to a 1.5-ml microcentrifuge tube, followed by DNA extraction with a Dr. GenTLE (from Yeast) High Recovery kit (Takara Bio). The genomic DNA concentration was measured using the Quantifluor ONE dsDNA Dye System (Promega) and 5–20 ng was added to a PCR reaction mixture (total 20 μl). The primers used were FAM-MT1 (5′-AAATAGTGGGTAGCCTGGAAGG-3′) at 400 nM, MP1 (5′-ATCTATCCAGAGATTGGCAG-GTG-3′) at 200 nM and MM1 (5′-GGGAACCGCTGATTAATCTTGG-3′) at 200 nM. The 5′ end of FAM-MT1 and FAM-MT3 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, 2.5 mM MgCl2) (Shigemori et al., 2005). 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 5-min extension at 72 °C. PCR fragments corresponding to mat1-P and mat1-M alleles were resolved on 5% polyacrylamide gels. Fluorescence was detected and quantified using Typhoon FLA9500 (GE Healthcare) and ImageQuant (GE Healthcare).

UV damage sensitivity Serial dilutions of exponentially growing cell cultures were plated on complete medium (YES) and subjected to UV irradiation by exposure to a germicidal lamp (254 nm; 100 J/m²). UV intensities were measured with a UV Radiometer (TOPCON UVR-2). Plates were incubated at 30 °C for 3–4 days.

Chromatin immunoprecipitation (ChIP) ChIP was performed as in Kimura et al. (2008), with several modifications. 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 propagated to 1.0 × 10⁷ cells/ml at 30 °C, and then shifted to 18 °C for 2 h. Cells (5.0 × 10⁸) 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 by a multi-beads shocker (Yasui Kikai) at 4 °C, with 15 cycles of 1 min on and 1 min off, and sonicated using a Bioruptor UCD-200 (Diagenode) at 2 cycles of 10 min each with alternating pulses of 40 s on and 30 s off at high level. The sheared samples were centrifuged at 20,000 g for 10 min at 4 °C. The supernatants were incubated with 30 μl Dynabeads Protein A (Thermo Fisher Scientific) preloaded with 1.2 μl anti-FLAG M2 antibody (Sigma-Aldrich) for 6 h at 4 °C. The beads were washed sequentially with wash buffer 1 (50 mM HEPES-KOH [pH 7.5], 1 mM EDTA, 0.1% sodium deoxycholate, 0.5 M NaCl), wash buffer 2 (10 mM Tris-HCl [pH 8.0], 0.25 M LiCl, 1 mM EDTA, 0.5% NP40, 0.5% SDS) and TE (twice), and 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 at 65 °C overnight to reverse cross-links and were 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 (GL Sciences). Quantitative PCR was performed with SYBR Premix Ex Taq II (Takara Bio) or TB Green Premix DimerEraser (Takara Bio) on an
Mx3000P qPCR system (Agilent). Primer sequences are in Supplementary Table S2. For ChIP of H3K9me2 and -me3, 20 μg of H3K9me2 antibody or H3K9me3 antibody (Hayashi-Takanaka et al., 2011) was preloaded onto 40 μl Dynabeads M-280 Sheep anti-Mouse IgG (Thermo Fisher Scientific).

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AUTHOR CONTRIBUTIONS

A. E.-C. and T. M. conducted experiments. T. M. and H. I. were responsible for conceptualization and project design. T. M., H. T., H. K., G. T. and H. I. supervised the study. T. M., G. T. and H. I. were responsible for data analysis and funding acquisition.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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